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Re-epithelialization of pathological cutaneous wounds is improved by local mineralocorticoid receptor antagonism

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Short title: A novel approach for improving wound healing

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ABSTRACT

Impaired cutaneous wound healing is a social burden. It occurs as a consequence of glucocorticoid treatment and in several pathologies. Glucocorticoids (GC) bind not only to the glucocorticoid receptor (GR), but also to the mineralocorticoid receptor (MR), both expressed by keratinocytes. In addition to its beneficial effects through the GR, GC exposure may lead to inappropriate MR occupancy. We hypothesized that dermatological use of MR antagonists (MRA) may be beneficial by overcoming the negative impact of GC treatment on pathological wounds.

The potent GC clobetasol, applied as an ointment to mouse skin, or added to cultured human skin explants, induced delayed wound closure and outgrowth of epidermis with reduced proliferation of keratinocytes. Delayed wound re-epithelialization was rescued by local MRA application. Normal skin was unaffected by MRA. The benefit of MR blockade is explained by the increased expression of MR in clobetasol-treated mouse skin. Blockade of the epithelial sodium channel by phenamil also rescued cultured human skin explants from GC-impaired growth of the epidermis.

MRA application over post-biopsy wounds of clobetasol-treated skin zones of healthy volunteers (from the SPIREPI clinical trial) also accelerated wound closure.

In conclusion, we propose repositioning MRA for cutaneous application to improve delayed wound closure occurring in pathology.
Key words: Skin, Wound healing, Re-epithelialization, Mineralocorticoid receptor, Blockade, Glucocorticoid.

INTRODUCTION
Impaired cutaneous wound healing is a major clinical problem that occurs in various situations including in patients treated with corticosteroids/dermocorticoids, in the elderly, and in pathological conditions such as diabetes, sickle cell disease, or Cushing syndrome. Efforts to improve wound healing, and hence the quality of life, represent a major challenge. Wound healing is a complex biological phenomenon that comprises successive stages including initial inflammation and activation of the coagulation cascade, followed by new tissue formation and tissue remodeling (Gurtner et al., 2008). Full healing occurs over a long period (about one year in humans). Re-epithelialization of injured skin is an early step in the formation of new tissue, involving keratinocyte migration and proliferation from the edges of the wound. This process is activated by signaling pathways originating from epithelial and non-epithelial cells at the wound edges, which release cytokines and growth factors. Nuclear receptors such as peroxisome-proliferator-activated receptors, androgen, estrogen, and glucocorticoid receptors also mediate wound re-epithelialization (Gurtner et al, 2008).

Glucocorticoids (GC) are given systemically or topically to treat a wide number of inflammatory diseases. One of the major adverse reactions to glucocorticoid treatment is skin atrophy and delayed wound healing, causing great concern to patients and limiting the use of GC. GC classically act through binding to the glucocorticoid receptor (GR); they can also bind with high affinity to another closely related member of the nuclear receptor superfamily, the mineralocorticoid receptor (MR) (Farman and Rafestin-Oblin, 2001; Jaisser and Farman, 2016). The MR and its mineralocorticoid ligand aldosterone are regulators of renal sodium reabsorption, fluid homeostasis, and blood pressure, due to their action in the kidney (Farman and Rafestin-Oblin, 2001; Jaisser and Farman, 2016). In mineralocorticoid-sensitive tissues, GC are inactivated by the enzyme 11 beta hydroxysteroid dehydrogenase type II (HSD2), preventing their binding to MRs, which are therefore selectively activated by aldosterone (Farman and Rafestin-Oblin, 2001; Odermatt and Kratschmar, 2012). In some tissues/cells in which little or no enzyme is present, GC (which largely prevail in the plasma) may activate MRs, for example, in the heart or brain (Jaisser and Farman, 2016).

Both MRs and GRs are expressed by epidermal keratinocytes (Farman et al., 2010). GC delay wound healing through binding to GRs (Youm et al., 2013; Tiganescu et al., 2014). The role of epidermal MRs is however mostly unknown (Farman et al, 2010). We hypothesize that some cutaneous side effects of GC could depend on inappropriate MR activation by GC, because there is no (or little) HSD2 in the epidermis (Kenouch et al., 1994). We propose that local (cutaneous) MR antagonism may bring some benefit to patients treated with GC. We have previously shown that the epidermal atrophy induced by clobetasol, a potent dermocorticoid, could be limited by co-administration of MR blockers (Maubec et al., 2015).

Several reports have also shown the involvement of the renin-angiotensin-aldosterone system (RAAS) in diabetes and insulin resistance, leading to the proposal that administration of MR antagonists could reduce deleterious events in diabetes; beneficial effects of treatment with MR antagonists on cardiovascular and renal functions have been reported (Jaisser and Farman, 2016). We reasoned that delayed skin wound healing in diabetic mice might also be improved by MR blockade (Guo et al., 2006; Guo et al., 2008; Hirata et al., 2009; Sato, 2015).

In this study, we examined whether locally applied MR blockers could improve the early phase of cutaneous wound closure in mouse models of delayed wound healing (glucocorticoid treatment, type I diabetes) and in glucocorticoid-treated cultured human skin explants. Acceleration of wound closure would limit the risk of infection, thus favoring skin healing. We searched for mechanisms
involved in the observed beneficial effects such as regulation of MR expression, keratinocyte proliferation, and the involvement of the epithelial sodium channel (ENaC), a classical target of MR in epithelia. We also monitored wound closure after skin biopsy in healthy volunteers treated for one month with the dermocorticoid clobetasol, to evaluate the effect of spironolactone applied to the skin, as a post-hoc study of the SPIREPI clinical trial (ClinicalTrials.gov: NCT01407471).

RESULTS

MR blockade rescues glucocorticoid-induced impairment of wound re-epithelialization in mice

Five days after wounding, we found that the cutaneous expression of MR mRNA was four-fold higher in wounded skin and the surrounding normal skin of clobetasol-pre-treated mice than in control mice (Figure 1a), providing clues for MR occupancy by locally-applied glucocorticoids. In contrast, cutaneous GR mRNA levels were marginally affected or not at all by clobetasol treatment (Figure 1b). MR activation in the wound was suggested by clobetasol-induced enhanced expression of the alpha subunit of the epithelial sodium channel (αENaC), that was blunted by the topical MR antagonist canrenoate (Canre) (Table 1). We observed a low level of HSD2 mRNA expression in the wounds of control mice, that was not modified in the clobetasol-treated mice (Figure 1c). We thus questioned whether MR blockade could improve GC-induced delayed wound closure in mouse skin. GC pre-treatment delayed wound closure, as expected; MR blockade by the topical application of Canre on the wounds rescued the impaired wound closure: three and five days after wounding, the wound surface, expressed as a percent of the wound surface at day 0, was not different from those of control mice (Figure 1d and e). Topical application of Canre did not modify wound closure in control mice (Figure 1d and e).

Macroscopic evidence for a reduction in wound surface could be due to wound contraction and/or re-epithelialization. Histological analysis of wound sections five days after wounding showed that topical MR blockade induces re-epithelialization of wounds in clobetasol-pre-treated mice, as illustrated by the longer neo-epidermis at the edges of the wound sections labeled with keratin-14 (K14) (Figure 1f and g); Canre-treated mice had shorter residual wound length (the distance between two edges) than PBS-treated control mice (Figure 1f and h). Moreover, this improved re-epithelialization was accompanied by a higher number of Ki67-positive proliferating keratinocytes in the neo-epidermis of the wounds, revealing that cellular proliferation of the neo-epidermis increased in response to topical treatment with Canre (Figure 1i and j). Thus, MR blockade stimulated the proliferation of epidermal keratinocytes and restored the delayed re-epithelialization of the wounds of glucocorticoid-treated mouse skin in vivo.

MR antagonism rescues the impairment of keratinocyte outgrowth induced by GC in cultured human skin explants

To overcome the contribution of in vivo wound contraction to wound closure, we also examined the effects of MR antagonists on an ex vivo model of re-epithelialization in human skin explants. In this situation, contraction of the wound edges does not occur, because keratinocytes grow at the edges of the explants, as illustrated by the presence of epidermal tongues. Human skin explants were maintained in organotypic culture; the specificity of the MR blockade was assessed by incubating the explants with three different MR antagonists: canrenoate or spironolactone (that block the MR and can also interfere with other steroid hormone receptors), or the highly MR-specific antagonist eplerenone. The formation of epithelial tongues at the edges of cultured human skin explants exhibit several aspects of wound healing including re-epithelialization (Meier et al., 2013). In the absence of clobetasol in the incubation medium, the length and surface of epithelial tongues were not influenced by addition of any of the three MR blockers (Figure 2a-c). In the presence of 10 µM clobetasol, epidermal growth from the edges of explants was significantly less than that of controls.
The addition of the MR blockers restored the impaired epidermal outgrowth (Figure 2a-c). The beneficial effect observed with canrenoate or spironolactone was similar to that obtained with eplerenone, arguing for MR specificity. We analyzed explant sections stained for Ki67 and showed that fewer ki67-positive proliferating cells were present in the epidermis of explants treated with clobetasol (8.0%) than in those of controls (12.1%). This shows that GC inhibits keratinocyte proliferation of human skin explants in culture (Figure 2d and e). This decreased keratinocyte proliferation was rescued by each of the three MR blockers, whereas these drugs did not affect the epidermal proliferation in explants cultured without clobetasol (Figure 2d and e). Thus, addition of the MR antagonists restored re-epithelialization that was impaired due to GC treatment.

We also performed ex vivo culture of skin explants from newborn mice with MR overexpression in keratinocytes (K5-MR mice, (Sainte Marie et al., 2007)). Overexpression of MR during development led to impaired epidermal outgrowth of explants relative to control littersmates, and the addition of canrenoate during explant culture partially (but significantly) restored this delay in K5-MR explants (Supplementary Figure S1 online).

**Blockade of the epithelial sodium channel rescues the defect of epidermal outgrowth induced by GC in human skin explants**

MR regulates the expression and activity of the amiloride-sensitive ENaC in kidney, and MR overexpression in mouse epidermis leads to an increase in ENaC transcript levels (Maubec et al., 2015). We monitored the effect of the ENaC blocker phenamil on the formation of epithelial tongues in human skin explants to evaluate whether ENaC activity is involved in the GC-dependent impairment of wound re-epithelialization. Neo-epithelialization, reduced by clobetasol treatment, was rescued by phenamil, suggesting that the activity of the ENaC participates in the GC-induced defect of epithelial growth (Figure 3a-c). The improvement of epithelialization by phenamil treatment was accompanied by a higher number of proliferating epidermal keratinocytes (Figure 3d and e).

**MR blockade improves wound closure in diabetic mice**

Wound healing is impaired in diabetic patients and in a mouse model of Type I diabetes induced by streptozotocin injections (STZ mice) (Luo et al., 2004; Gurtner et al., 2008). We confirmed that the skin heals more slowly in STZ mice than in control mice (Figure 4d and e). We also found that STZ mice had a 6-fold higher level of MR mRNA in intact skin, as well as over the wound, than control mice (Figure 4a), whereas GR mRNA levels were only moderately (1.5-fold) higher (Figure 4b). MR activation in the wound of diabetic mice was suggested by enhanced eENaC mRNA expression, that was reversed upon local treatment with canrenoate (Table 1). HSD2 mRNA expression in the wounds from control and STZ mice was low and did not differ (Figure 4c). Application of canrenoate to the wounds of STZ mice reduced the wound surface relative to the application of PBS (Figure 4d and e). Improved wound closure in STZ mice treated with canrenoate was associated with increased re-epithelialization (Figure 4f and g), and was accompanied by increased keratinocyte proliferation (Figure 4h and i). Thus, in this model of type I diabetes, local MR blockade accelerated wound closure.

**MR blockade and wound closure in healthy volunteers**

The SPIREPI clinical trial was designed to evaluate the efficiency of MR blockade to limit clobetasol-induced epidermal atrophy in 23 healthy subjects (Maubec et al., 2015). After 4 weeks of clobetasol application over the skin (with/without spironolactone), a wound was created by biopsy. We compared the surface of wounds three days and seven days following skin biopsy, i.e. at the early stage of wound healing before crust formation, as a post-hoc analysis of the SPIREPI study. Application of clobetasol gel for one month resulted in a larger wound area relative to placebo, whereas application of clobetasol gel with spironolactone significantly decreased wound size at day 3 post-biopsy (Figure 5a). The size of the wound was similar to that of placebo gel administration.
in the zone where spironolactone was applied in the absence of clobetasol. The benefit of spironolactone addition to clobetasol-treated zones was still visible seven days after biopsy, despite the withdrawal of clobetasol application following biopsy (as it delays wound healing), whereas spironolactone was continued (Figure 5b). Thus, the improvement of the early phase of wound healing by spironolactone is also observed in vivo for glucocorticoid-treated skin of healthy subjects.

DISCUSSION

Several reports have highlighted the involvement of steroids and their nuclear receptors in cutaneous wound healing. Estrogens accelerate epidermal healing of acute wounds in elderly women, an effect shown to be mediated by the beta (not alpha) epidermal estrogen receptor in mice (Campbell et al., 2010). Androgens have the opposite effect, i.e. delayed wound healing (Ashcroft and Mills, 2002). Overexpression of the GR in keratinocytes or glucocorticoid administration delay wound healing (Sanchis et al., 2012). Here, we add another nuclear receptor to the list of transcription factors that modulate wound healing, and show that MR activity can be targeted by the local application of antagonists. The activation of MRs in the wound was suggested by the ability of the MR blocker canrenonate to reverse the clobetasol-induced (or diabetes-induced) enhanced expression of the αENaC (a target gene downstream of MR activation). We found that MR blockade favors re-epithelialization of the wound in pathological wound models (GC treatment, diabetes), while it was ineffective in normal (healthy) skin. The ability of spironolactone or canrenonate to promote epidermal growth over wounds on the back of the mouse or at the edges of cultured human skin explants suggests that the effect is MR-dependent. However, these drugs are not fully specific for MR; indeed they can antagonize other nuclear receptors, particularly the androgen receptor (Kolkhof and Borden, 2012). The equal ability of eplerenone, a highly specific MR antagonist, to promote epidermal growth over wounds shows that the restoration of keratinocyte proliferation and epidermal growth are MR-dependent. Thus, MR blockade improves re-epithelialization of GC-induced delayed wound closure. This effect may be complementary to that mediated by the GR. Indeed, local application of the GR blocker RU38486 alleviates the healing defect observed in GC-treated skin or stressed mice (Youm et al, 2013). Both GR and MR are effectors of wound healing; use of MR antagonists (rather than GR blockers) may be preferred to counter GC-induced delayed wound closure, while retaining the beneficial effects of GC.

GC, rather than aldosterone, may preferentially bind to cutaneous MRs because of the very low epidermal expression of HSD2, at least in normal skin (Kenouch et al, 1994). In addition to exogenous (therapeutical) glucocorticoids or adrenal hypersecretion (Cushing disease), there is evidence of local corticosteroidogenesis in the skin (Vukelic et al., 2010; Slominski et al., 2013). Whether local steroidogenesis is subjected to regulation in pathological situations (and in wound healing) remains unknown. The regulation of HSD2 in disease is largely unknown, even in the most typical MR target cells such as the renal collecting duct. The extent of HSD2 activation may depend on the status of the skin. The basal enzyme levels may be very low in in vivo and may increase as a result of tissue injury or glucocorticoid exposure. For example, HSD2 mRNA or protein levels were reported to be low under control conditions, but enhanced following UVA exposure or wounding (Vukelic et al., 2011; Skobowiat et al., 2013; Tiganescu et al, 2014). Tiganescu et al. reported that HSD2 mRNA and activity were undetectable in wounds of mouse skin (Tiganescu et al, 2014). We found very low HSD2 mRNA levels in the wounds of control mice that were not modified by glucocorticoid treatment or diabetes. Active GC may be locally generated by the enzyme HSD1 (acting as reductase, opposite to the action of HSD2) present in epidermis (Kenouch et al, 1994), thus modulating local activation of MRs. The role of HSD1 has been highlighted in aging skin, where wound healing is also impaired, despite no major change in the plasma cortisol concentration; one mechanism is the age-dependent increase in the enzyme HSD1, that enhances regeneration of glucocorticoids (Tiganescu et al., 2013). Topical treatment with an HSD1 inhibitor accelerates the healing of dorsal wounds in wild-type mice, and aged HSD1 KO mice show
improved cutaneous healing (Tiganescu et al, 2013). This mechanism has been proposed to be one of the major pathways for atrophy and delayed healing in aging human skin (Tiganescu et al, 2013). In this study, we found that MR mRNA expression was enhanced in the skin of a diabetic mouse model, as well as following local application of the potent dermocorticoid clobetasol. There is very limited information on cutaneous MR levels in skin pathologies. An increase in cutaneous MR has been reported in a mouse model of aging skin that associates UV irradiation and metabolic syndrome (Nagase et al., 2013). The ability of cutaneous MR blockade to improve wound closure as reported here in pathological situations may be explained by enhanced MR expression. The notion of excessive/inappropriate MR activity in disease originates from the observed benefits of the treatment of several diseases with MR blockers (Jaissler and Farman, 2016). The ability of MR antagonists to improve cardio-vascular pathologies has been demonstrated in experimental and clinical situations. Pioneer clinical trials showed that the addition of spironolactone or eplerenone to standard care limits morbidity and mortality in patients with cardiac insufficiency or following myocardial infarction (Pitt et al., 1999; Pitt et al., 2003). MR blockade improves vascular dysfunction (Leopold et al., 2007), limits progression towards renal failure, (Shavit et al., 2012) and restores vision in patients with chronic serous chorioretinopathy, a vision-threatening eye disease (Zhao et al., 2012). In diabetes and metabolic syndrome, MR antagonism has also been shown to improve glucose tolerance, and to decrease insulin resistance and plasma levels of triglycerides and pro-inflammatory cytokines (Guo et al, 2008; Hirata et al, 2009).

In murine skin, wound healing may be influenced by the hair cycle stage. Our experiments in adult mice were performed in mice with pink skin (see Methods) i.e. most likely when hair follicles are at telogen stage. Cultured mouse skin explants were from neo-natal mice, where hair follicles are synchronized. Thus it is anticipated that no major difference in hair cycle stage between our experimental conditions should interfere with our results. Contraction of subcutaneous muscles occurs in rodent skin, but re-epithelialization is also required following wounding. The proliferation rate of keratinocytes is lowered by glucocorticoids; we found that MR antagonism restores the epidermal proliferation rate in mouse skin, as well as in cultured human explants (where contraction of the wound do not occur), thus providing a mechanism for this beneficial effect. Reports of an effect of MRs on proliferation are limited; experimental evidence concerns essentially endothelial cells in the context of cardiac or vascular ischemic diseases (Gravez et al., 2015).

The mechanisms of the beneficial effect of MR antagonists on the GC-induced impairment of wound closure may include the reduction of ENaC activity in keratinocytes. ENaC is a classical aldosterone-MR target in the renal distal nephron, that regulates sodium reabsorption. Changes in chemical gradients and electrical fields at the wound site are important, although the nature of the transporters or channels are not fully defined (Zhao et al., 2006). Whether excessive ENaC activity at the edges of the wounds participate in GC-dependent pathological healing through alterations of the wound electrical fields is unknown. MR blockade may limit this phenomenon through the reduction of ENaC expression and activity, thus improving cutaneous wound closure in some pathological situations.

The dermis may also be affected by MR blockade, perhaps during later stages of wound healing, to influence inflammation and dermal remodeling. It has been reported that aldosterone and MR antagonists modulate the elastin and collagen content of human skin (Mitts et al., 2010). MR antagonists may reduce dermal inflammation, oxidative stress, and fibrosis, as suggested by the effects of MR blockers in diseases (myocardial infarction, vascular and renal remodeling), thus providing additional benefits to improve wound healing (Palatinus et al., 2010).

We have treated human skin explants and mice with topical MR antagonists. We believe that this is feasible in a clinical setting as they are small steroid molecules that can cross the epidermal barrier and reach the basal epidermal layers, particularly when the skin barrier is altered. This treatment
route not only targets the lesion site, but also reduces the risk of systemic adverse effects: risk of hyperkaliemia in subjects with impaired renal function (for spironolactone and eplerenone); gynecomastia and sexual dysfunctions, due to blockade of the androgen receptor (for spironolactone). Toxicity assays are required, and novel formulations designed to improve epidermal delivery of MR antagonists must be developed, as skin is highly impermeable. Dedicated clinical trials should be designed to ensure the safety and efficacy of MR antagonists reformulated for skin application for selected pathologies, such as hypercorticism and diabetes.

In conclusion, we suggest that local application of MR antagonists may bring significant benefit to patients to improve impaired cutaneous wound closure, as it does to limit glucocorticoid-induced epidermal atrophy (Maubec et al, 2015). It may increase the tolerance of prolonged topical glucocorticoid use by preventing some of its major cutaneous side effects. It may also be an important tool for the management of diabetic wounds.
MATERIALS AND METHODS

Detailed informations are provided in the Supplementary Data. Two models of impaired wound healing were studied in vivo in mice: pre-treatment with topical clobetasol of the back skin for 10 days, and streptozotocin-induced type I diabetes, with their appropriate controls. Wound healing was generated with 6 mm biopsy punch; MR blockade over the wound was achieved by local application of the MR antagonist potassium canrenoate (Canre) or PBS and wounds were photographed to evaluate wound closure. At day 5 mice were euthanized and wound and skin specimens were collected for RNA extraction and immunolabelling.

Ex vivo model of wound healing was achieved by organotypic culture of skin explants from newborn mice with epidermal overexpression of MR (and their controls) (Sainte Marie et al, 2007) and from human skin explants cultured in the presence of clobetasol with/without MR antagonists.

Real-time PCR analysis of gene expression and immunofluorescence analyses of skin and wound specimens are detailed in supplementary data.

A post hoc analysis of wound closure in subjects from the SPIREPI clinical trial (ClinicalTrials.gov Identifier: NCT01407471) is reported.
CONFLICT OF INTEREST
The authors state no conflict of interest

AUTHOR CONTRIBUTIONS

V.T.N. designed research studies, conducted experiments, data analysis and interpretation, and participated in writing the manuscript;

N.F. participated in the conception and design of the study, data analysis and interpretation and in writing the manuscript;

E.M. was the PI of the SPIREPI clinical trial and followed the wound closure;

D.N. collected the initial data;

D.D. and L.W. participated in the experiments on the STZ mice;

S.A. participated in conception and design of the study, data analysis and interpretation and in writing the manuscript;

F.J. participated in the conception and design of the study, data analysis and interpretation and in writing the manuscript;

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Figure 4

(a) Relative MR mRNA expression

(b) Relative GR mRNA expression

(c) Relative HSD2 mRNA expression

(d) Images of wound healing:
   - Day 0
   - Day 3
   - Day 5

(e) Wound area (% of day 0)

(f) Immunofluorescence images:
   - K14/Dapi
   - STZ+PBS
   - STZ+Canre

(g) Neoeipidermis (µm)

(h) Ki67/Dapi images:
   - Epi
   - Der
   - STZ+PBS
   - STZ+Canre

(i) Ki67 positive cells (%)

Legend:
- STZ (n=4)
- Clo (n=8)
- CT (n=7)
- STZ+PBS (n=4)
- STZ+Canre (n=4)

Notes:
- * Significant difference
- ** Very significant difference
- ns Not significant
**Figure 5**

(a) Day 3

- Placebo
- Spiro
- Clo
- Clo + Spiro

(b) Wound area at D3 (A.U.)

(c) Day 7

- Placebo
- Spiro
- Clo
- Clo + Spiro

(d) Wound area at D7 (A.U.)
SUPPLEMENTARY MATERIAL AND METHODS

Reagents

Reagents and drugs were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) unless specified. Eplerenone was obtained from Tocris Bioscience (R&D System Europe, Lille, France). For GC treatment, clobetasol administration (rather than dexamethasone) was chosen because this potent dermocorticoid is commonly used in dermatology. Culture medium was purchased from Gibco® (Life Technologies, Saint-Aubin, France).

Skin explants from newborn mice overexpressing the MR

Mice with overexpression of the MR in basal keratinocytes (K5-MR) have been previously characterized by Sainte Marie et al. Skin explants from newborn (they die shortly after birth) K5-MR mice and their control littermates were cultured as described previously (Mazzalupo et al. 2002). After decapitation, patches from 0.5-1 day-old pups were obtained from the ventral skin, using a 4 mm biopsy punch (Kai Europe GmbH, Solingen, Germany); each patch was placed directly on a well of 24-wells tissue culture plates with the dermis facing the plastic for 30 min to favor adhesion. Then 0.5 ml of the keratinocyte culture medium warmed at 37°C (cFAD: DMEM/HamF12 (3/1, v/v), insulin 5 µg/ml, T3 2nM, transferrin 5 µg/ml, epidermal growth factor 10 ng/ml, fungizone 0.5 µg/ml, 5% decomplemented charcoal-stripped fetal bovine serum (Life Technologies, Saint-Aubin, France) and cholera toxine 0.1 nM (Tebu-Bio, Le Perray-en-Yvelines, France) were gently added. Medium was changed daily. After 7 days of culture, explants were harvested and fixed for 10 min with 4% paraformaldehyde, rinsed in PBS; antibody against cytokeratine 17 (K17, 1:5000, kindly provided by Coulombe PA, Maryland, USA) was used for immunolabelling to visualize the outgrowth of keratinocytes. The outgrowth of keratinocytes was measured using the ImageJ software (NIH); the surface is expressed in mm² (after substraction of the surface of the initial punch). Of note, hair follicles are synchronized in neonatal skin.
In vivo wound healing assay in mice

The wound healing assay was performed either in normal C57BL/6 mice, mice pre-treated once a day with topical clobetasol on the skin of the back (Dermoval gel: 0.05% clobetasol propionate, GlaxoSmithKline, Marly le Roi, France) for 10 days before wounding, or diabetic mice. Type I diabetes was induced by streptozotocin (4 weeks after 5 daily i.p. injections of 50 mg/kg streptozotocin). All mice were 10-12 week-old females. We selected mice with hair follicles presumably at the telogen stage, indicated by pink dorsal skin observed after hair clipping. Histological examination of the lateral skin surrounding the healing wounds at day 5 showed an absence of anagen hair follicles.

Mice were anesthetized by inhalation of isofluorane (Aerane, Baxter, Deerfield, IL). After depilation, four full-thickness wounds were generated on the back of the mice with 6 mm diameter disposable biopsy devices (Kai Europe GmbH, Solingen, Germany). All tissues above the panniculus carnosus were excised. Wounds were then left uncovered and 0.5 mM of the MR antagonist potassium canrenoate (Canre) or PBS (50 µl for each wound), were locally applied twice daily until sacrifice (day 5). Pictures of the wounds were taken using a Sony Cybershot H 10.1 megapixels DSC-W180 digital camera. The wound surface was expressed as the percentage of the initial surface of each wound at day 0. For tissue collection, mice were euthanized in a CO₂ chamber. Tissues were harvested, snap-frozen in liquid nitrogen, and stored at −80 °C for RNA extraction or immunolabeling. Complete wound specimens were harvested; for morphological studies, we selected the median sections of the wounds to ensure adequate comparison between sections; molecular analyses were performed on the full thickness of the wounds.

Human skin explant culture

Human skin explant culture was performed for six days as described (Maubec et al. 2015). Explants were cultured in the presence of clobetasol (10 µM in 0.2% ethanol) with/without MR antagonists.
Three MR antagonists were used: spironolactone (10 µM in 0.2% ethanol) or potassium canrenoate (10 µM in PBS) or eplerenone (10 µM in 0.2% DMSO). Control explants were incubated with the drugs alone, or the solvents alone. The effect of phenamil (100 nM), a blocker of the epithelial sodium channel ENaC, was also evaluated. After six days in culture, explants were frozen and processed for measurement of the length and surface of the epidermal tongues (Meier et al. 2013). Keratinocyte proliferation was measured as described (Maubec et al. 2015).

**Wound healing in healthy subjects: a post-hoc analysis of the SPIREPI clinical trial**

The SPIREPI clinical trial (ClinicalTrials.gov Identifier: NTC01407471) was designed to evaluate the interest of topical spironolactone administration to prevent corticoid-induced epidermal atrophy in healthy volunteers as primary outcome. The detailed protocol has been previously provided (Maubec et al. 2015). Four zones on the forearm of each participant (2 zones on each arm) were treated with hydro-alcoholic gels containing placebo, 5% spironolactone, 0.05% clobetasol propionate, or 0.05% clobetasol + 5% spironolactone, administered six days a week during 29 days. Skin biopsies (3 mm biopsy punch) were performed on day 29. Subsequent treatments (up to day 48) were either placebo (over zones previously treated with placebo or clobetasol) or spironolactone (over zones previously treated with spironolactone alone or spironolactone + clobetasol); clobetasol applications were not continued for ethical reasons, as it is known to delay wound healing. After biopsy, clinical examination and photography of the treated zones was performed by dermatologists to monitor eventual local reactions. The surface of the wounds three days and seven days after biopsy was measured (using Image J software) by two investigators blind to the treatments.

**Immunofluorescence experiments**

Immunolabeling was performed on 6 µm cryosections. Sections were fixed in ice-cold acetone and blocked with 2% BSA/PBS. Samples were then incubated with primary antibodies for 2 h at room temperature. Isotype IgG served as a negative control. The following antibodies were used for
immunofluorescence staining: rabbit polyclonal anti-keratin 14 (1:1000, Covance, Denver, PA), rabbit polyclonal anti-Ki67 (1:200) (Abcam, Paris, France), and rabbit anti polyclonal ki67 (1:100) (Novocastra, Newcastle, UK). The secondary antibodies used were Alexa Fluor 488 and Alexa Fluor 555 conjugated antibodies (1:500) (Molecular Probes, Saint-Aubin, France). Samples were examined and photographed using a Nikon Eclipse 90i fluorescent microscope equipped with a Nikon DS-Fi1C digital camera (Nikon, Tokyo, Japan). Labeled cells were counted without knowledge of the corresponding experimental condition in three different fields and reported as a percentage of total DAPI stained nuclei. Wound re-epithelialization was assessed in a blind manner.

**Quantitative real time RT-PCR**

Procedures for RNA extraction and real-time PCR were as described (Maubec et al. 2015). Samples were assayed in triplicate. The expression of genes was normalized to the relative levels of beta-actin (values in controls were set as 1 and values in treated zones are expressed as fold changes). Primers were purchased from Eurogentec (Angers, France); forward and reverse primers for beta Actin (CAT CCG TAA AGA CCT CTA TGCCAA C and ATG GAG CCA CGG ATC CAC A), MR (CCA GAA GAG GGG ACC ACA TA and GGA ATT GTC GTA GCC TGC AT), GR (TTC GCA GGC CGC TCA GTG TT and TTG GGA GGT GGT CCC GTT GCT), alpha ENaC (CGG AGT TGC CAA CAT and TGG AAG CCA GTA CCG GC T); HSD2 (ACC-CCT-GCT-TGG-CAG-CCT-ACG-GCA and TCA-CAT-TAG-TCA-CTG-CCT-CTG-TCT-TG).

**Statistical analysis**

Results are expressed as the means ± SEM. Differences between the means of two groups were assessed using the Mann Whitney test. Differences between multiple groups were analyzed by 1-way ANOVA followed by the Newman-Keuls Multiple Comparison test. The wound size of each treated zone of the arms of healthy volunteers (SPIREPI study) was compared using 1-way
ANOVA for repeated measures followed by the Newman-Keuls Multiple Comparison test. $P < 0.05$ was considered to be statistically significant.

**Study approval**

The use of mice was in accordance with the guidelines of the European Community and approved by our Institutional Animal Care and Use Committee.

Human skin was collected from breast reduction surgery (obtained after informed consent of the subjects and ethical approval of the Comité de Protection des Personnes Ile de France V).

The members of the SPIREPI group provided agreement to use the post-biopsy wound closure data. The healthy volunteers of the SPIREPI study provided written informed consent for the use of photographs for investigation and publication purposes.
SUPPLEMENTARY REFERENCES


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Figure S1. MR blockade improves outgrowth of keratinocytes in neonatal skin explants issued from mice with epidermal MR overexpression (K5-MR)

A: Explants of dorsal skin from newborn control (CT) and transgenic mice overexpressing the MR in keratinocytes (K5-MR) cultured for 7 days with/without Canrenoate (Canre); K14 staining (orange) shows keratinocyte outgrowth around the explants.

B: Quantification of outgrowth of keratinocytes; explants issued from pups of the same litter are joined by line (mean values per litter in each condition, n = 18 CT and 17-18 K5-MR pups from 7 litters). MR overexpression during gestation (K5-MR explants) was associated with reduced keratinocyte outgrowth, and incubation of explants with Canrenoate partially improved the impaired outgrowth.

Comparison between groups was made by 1-way ANOVA followed by Newman-Keuls Multiple Comparison test. *P<0.05.