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Biosynthesis of D-series resolvins in skin provides insights into their role in tissue repair

Jason Hellmann[1], **Brian E. Sansbury**[1], **Blenda Wong**[1], **Xiaofeng Li**[1], **Mansher Singh**[2], **Kristo Nuutila**[2], **Nan Chiang**[1], **Elof Eriksson**[2], **Charles N. Serhan**[1], and **Matthew Spite**[1,*]

[1]Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

[2]Division of Plastic Surgery, Department of Surgery, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

Abstract

Cutaneous injury causes underlying tissue damage that must be quickly repaired to minimize exposure to pathogens and to restore barrier function. While the role of growth factors in tissue repair is established, the role of lipid mediators in skin repair has not been extensively investigated. Using a mass spectrometry-based lipid mediator metabolomics approach, we identified D-series resolvins and related pro-resolving lipid mediators during skin injury in mice and pigs. Differentiation of human epidermal keratinocytes increased expression of 15-lipoxygenase and stereospecific production of 17*S*-hydroxydocosahexaenoic acid, the common upstream biosynthetic marker and precursor of D-series resolvins. In human and pig skin, specific receptors for D-series resolvins were expressed in the epidermal layer and mice deficient in RvD1 receptor *Alx/Fpr2* showed an endogenous defect in re-epithelialization. Topical application of Dseries resolvins expedited re-epithelialization during skin injury and they enhanced migration of human epidermal keratinocytes in a receptor-dependent manner. The enhancement of re-epithelialization by RvD2 was lost in mice genetically-deficient in its receptor and migration of keratinocytes stimulated with RvD2 was associated with activation of the PI3K-AKT-mTOR-S6 pathway, blockade of which prevented its pro-migratory actions. Collectively, these results demonstrate that resolvins have direct roles in the tissue repair program.

INTRODUCTION

Upon cutaneous injury (e.g., penetrating force, burn, pressure ulcers, surgery), a dynamic wound healing response is enacted to rapidly restore barrier function and tissue homeostasis

*Corresponding author: Matthew Spite, Ph.D., Brigham and Women's Hospital, Building for Transformative Medicine, Room 3016K, 60 Fenwood Rd., Boston, MA 02115; Ph: 617-525-5133; mspite@bwh.harvard.edu.

Conflict of interest

The authors state no conflict of interest.

and to protect the host against pathogen invasion (Robbins *et al.*, 2010). Several co-morbidities such as metabolic disease, smoking, cardiovascular disease and advanced age, can perturb skin tissue repair (Demidova-Rice *et al.*, 2012; Diegelmann and Evans, 2004). Clinical management of cutaneous wounds exceeds $6 billion annually in the U.S. despite the current standard of care, which includes debridement, dressings, and antibiotics (Demidova-Rice *et al.*, 2012; Junker *et al.*, 2013; Singer and Dagum, 2008; Zielins *et al.*, 2014). Given this broad clinical problem, a further understanding of the mechanisms regulating normal tissue repair is critical for the development of novel therapeutics.

Following primary hemostasis, a highly coordinated tissue repair program consisting of temporally distinct but overlapping phases of inflammation, proliferation and remodeling is enacted (Gurtner *et al.*, 2008; Robbins *et al.*, 2010). The inflammatory phase serves to eradicate pathogens and its subsequent resolution is characterized by the clearance of apoptotic cells and debris from injured tissues. Resolution is regulated in part by specialized pro-resolving lipid mediators (SPM), such as resolvins, which are biosynthesized in exudates by immune cells and/or via transcellular biosynthesis involving endothelial or epithelial cells (Serhan, 2014). Their complete structures and stereochemical assignments have been systematically elucidated (Hong *et al.*, 2003; Serhan, 2014; Serhan *et al.*, 2002; Spite *et al.*, 2009; Winkler *et al.*, 2016). Resolvins play well-defined roles in the inflammation-resolution program; they halt excessive infiltration of neutrophils, diminish production of pro-inflammatory lipid mediators and chemokines, stimulate macrophage efferocytosis, and enhance host-defense (Serhan, 2014). Their distinct roles in the transition in tissue repair are less defined. Other SPM, such as the maresins and their sulfido-conjugates, promote tissue regeneration in *Planaria* and we have previously found that resolvins rescue defective resolution of inflammation in diabetes and that this translates to improved tissue repair (Dalli *et al.*, 2014; Serhan *et al.*, 2012; Tang *et al.*, 2013). This led us to question whether SPM are produced in wounds and have a direct receptor-mediated role in the normal tissue repair program.

RESULTS

Identification of SPM in skin wounds from mice and pigs

We performed targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of SPM and their biosynthetic pathway markers in skin and wounds. This consisted of multiple reaction monitoring (MRM) of individual products comprising metabolomes from arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). We identified AA-derived lipoxins (e.g., LXA_4, LXB_4), EPA-derived resolvins (e.g., RvE1, RvE2) and DHA-derived resolvins, protectins and maresins in biopsies of mouse skin (see Table S1 and *Supplementary Abbreviations and Full Chemical Names*). Using interaction network pathway analysis, we observed enrichment of the D-series resolvin biosynthetic pathway in wounds, with statistically significant increases in RvD1 (7*S*, 8*R*, 17*S*-trihydroxy-docosa-4*Z*, 9*E*, 11*E*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid), RvD2 (7*S*, 16*R*, 17*S*-trihydroxy-docosa-4*Z*, 8*E*, 10*Z*, 12*E*, 14*E*, 19*Z*-hexaenoic acid), and RvD4 (4*S*, 5*R*, 17*S*-trihydroxy-docosa-6*E*, 8*E*, 10*Z*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid) (Fig. 1a and Table S1). We also identified the 17*R*-epimer of RvD3, which has previously been shown to promote

proliferation of lung epithelial cells and wound healing (Colby *et al.*, 2016). Of note, AA-derived LXB_4 and 5*S*, 15*S*-dihydroxyeicosatetraenoic acid, a marker of 5- and 15-lipoxygenase (LOX) activity, were also increased in wounds (Fig. S1 and Table S1). Representative MRM chromatograms of D-series resolvins are shown in Figure 1b, with MS/MS spectra and diagnostic fragmentation ions shown in Figure S2. Individual levels of D-series resolvins in mouse wounds are shown in Figure 1c. To confirm the identification of SPM in a pre-clinical model of wound healing, we collected skin biopsies and wounds from Yorkshire pigs (Daly *et al.*, 2016). Similar to mice, we identified SPM across all three metabolomes, including, RvD1, RvD2 and RvD4 (Table S2). Representative MRM chromatograms of D-series resolvins identified in pig wounds are shown in Figure 1d, with MS/MS spectra and diagnostic fragmentation ion assignments shown in Figure S3.

Expression and regulation of human 15-LOX in human epidermal keratinocytes

The biosynthesis of most SPM we identified in skin and wounds is dependent upon 15-LOX mediated conversion of polyunsaturated fatty acids (Hong *et al.*, 2003; Spite *et al.*, 2009). In human skin biopsies, we identified that 15-LOX-2 is predominantly expressed in the epidermis in both the keratin 14 (K14)-positive basal layer and suprabasal layers (Fig. 2a). Normal human epidermal keratinocytes (NHEK) expressed both human isoforms of 15-LOX, namely *ALOX15* and *ALOX15b* (Fig. 2b). Expression of *ALOX15b* increased significantly in keratinocytes undergoing differentiation induced by elevating extracellular calcium (Elsholz *et al.*, 2014). While 15-LOX-1 protein (corresponding to *ALOX15*) was present and increased with differentiation, a statistically significant increase in 15-LOX-2 protein was observed with differentiation (Fig. 2c). To determine whether NHEK utilize DHA to initiate biosynthesis of resolvins, we measured levels of their common biosynthetic precursor and pathway marker, 17-hydroxydocosahexaenoic acid (17-HDHA), by LC-MS/MS. We found that while it was undetectable in undifferentiated keratinocytes, 17-HDHA was biosynthesized endogenously at day 7 post-differentiation (Fig. 2d, **left panel**). As an indicator of biosynthetic capacity, addition of DHA substrate led to a substantial increase in 17-HDHA formation (Fig. 2d, **right panel**). Using chiral chromatography and synthetic standards for 17*S* and 17*R* forms of 17-HDHA, we determined that the majority of 17-HDHA was the 17*S* stereoisomer, indicative of stereo-specific enzymatic biosynthesis (Fig. 2e). A representative MS/MS spectrum of 17*S*-HDHA biosynthesized by NHEK is also shown in Fig. 2e, with diagnostic fragmentation ions indicated. These results demonstrate that 15-LOX is expressed in differentiated epidermal keratinocytes and could potentially initiate the D-series resolvin biosynthetic pathway upon injury.

D-series resolvins enhance wound re-epithelialization

We next questioned the role of D-series resolvins in re-epithelialization. For this, we topically applied RvD1, RvD2 or RvD4 to wounds of male mice immediately after injury and monitored the initial phases of re-epithelialization. Wounds were splinted with silicone rings to prevent contraction. All three resolvins significantly enhanced the rate of re-epithelialization, decreasing the time to 50% wound closure (T_{50}) by ~1 day (Fig. 3a). Representative images of wounds treated with RvD2 are shown, with the leading edge of the neo-epidermal layer indicated. Histological analysis of hematoxylin and eosin (H & E) stained cross sections of wounds collected from saline or RvD2 treated mice (day 5)

confirmed the identification of the neo-epidermis (Fig. 3b). The original wound edge is indicated by arrows, with the neo-epidermis demarcated; granulation tissue (GT) is apparent in the wound bed. We note that, in addition to male mice (Fig. 3), topical RvD2 also enhanced wound re-epithelialization in female mice (Fig. S4). Using deuterium-labeled RvD1 and RvD2 to distinguish them from endogenously produced resolvins, we also found that both d5-RvD1 and d5-RvD2 are rapidly (within 2h) cleared from wounds after topical administration (Fig. S5). They were both distributed in the plasma following topical wound delivery, although plasma levels of d5-RvD1 were greater than d5-RvD2 (Fig. S5).

We next asked whether the promotion of re-epithelialization by RvD2 was secondary to growth factors; no changes in *Ctgf, Fgf2, Fgf7* (also known as keratinocyte growth factor) or *Tgfb1* at day 5 post-wounding were found in RvD2-treated wounds (Fig. 3c). We also measured protein levels of these growth factors in wound treated with RvD2 for 5 days. Consistent with mRNA levels, no changes in growth factors were observed in wounds upon RvD2 treatment (Fig. S6). Additionally, pro-inflammatory cytokine, *Il1β*, was significantly decreased in RvD2-treated wounds, while levels of pro-inflammatory chemokines and cyclooxygenase-2 (*Ptgs2*) were not significantly altered at this time point (Fig. 3c).

Role of pro-resolving receptors in skin wounds—The biological actions of resolvins are mediated by specific receptors (Chiang and Serhan, 2017). RvD1 binds and activates ALX/FPR2 and RvD2 was recently shown to be a potent ligand for GPR18, now denoted DRV2 (Chiang *et al.*, 2015; Chiang *et al.*, 2017; Krishnamoorthy *et al.*, 2010). Given the potent actions of these mediators in promoting wound re-epithelialization, we questioned whether their specific receptors are expressed in the epidermis. Immunofluorescence imaging of human skin biopsies demonstrated that DRV2 is expressed in the epidermal layer (Fig. 3d). The basal epidermal border is indicated with K14 staining and is demarcated with the white line. Consistent with our previous report in which we identified ALX/FPR2 in mouse skin (Tang *et al.*, 2013), ALX/FPR2 was also expressed in human skin (Fig. S7). Additional human skin biopsies revealed heterogeneity in each receptor (Fig. S7). Because we identified that resolvins are produced in pig wounds, we also assessed expression of these receptors in skin biopsies of Yorkshire pigs. Epidermal staining of both DRV2 and ALX/FPR2 was also observed in skin biopsies from pigs (Fig. S8). Similar results were obtained with mouse skin, where DRV2 was expressed in the epidermal layer (Fig. S9). The specificity of the anti-human antibody for mouse DRV2 was confirmed using both positive (murine testis) and negative (skin and testis from *Gpr18*-deficient mice) controls (Fig. S9).

We next questioned whether genetic deficiency of *Alx/Fpr2* or *Gpr18* modulates re-epithelialization. To this end, we assessed the time course of wound re-epithelialization in *Alx/Fpr2*-deficient or *Gpr18*-deficient mice. Mice deficient in *Alx/Fpr2* showed an endogenous defect in wound re-epithelialization, while *Gpr18*-deficient mice had no impairment (Fig. 3e, f). Topical application of RvD2 significantly enhanced re-epithelialization in WT mice as compared with saline, reducing the time to re-epithelialization (T_{50}) by nearly 2 days (Fig. 3f). This enhancement in re-epithelialization by RvD2 was completely lost in *Gpr18*-deficient mice, indicating that the effects of RvD2 are receptor dependent.

Resolvins promote keratinocyte migration in a receptor-dependent manner

To elucidate the role of RvD2 on epidermal keratinocytes, we investigated whether expression of DRV2 is modulated during differentiation. For this, we used a 3-D air-liquid interface culture of primary human epidermal keratinocytes that allows for complete stratification into basal, spinous, granular and cornified layers and closely mimics the structure of human skin, but is devoid of other cell types. A representative H & E stained cross-section is shown in Figure 4a. Using immunofluorescence microscopy, we determined that DRV2 is expressed throughout all suprabasal layers, with relatively weaker staining observed in the K14-positive basal layer. We extended this analysis to ALX/FPR2 and found a similar expression profile (Fig. S10a). We validated expression of DRV2 in primary keratinocytes by Western blot and further determined that both DRV2 and ALX/FPR2 are expressed on the cell surface using flow cytometry (Fig. 4b and Fig. S10b). Because DRV2 was most prominently localized to differentiated epidermal layers, we asked how its expression changes during differentiation. Calcium-induced differentiation increased mRNA expression of *GPR18* in both human and mouse keratinocytes (Fig. 4c). Expression of keratinocyte differentiation marker, involucrin (*IVL*) was used as a positive control. These results demonstrate that DRV2 is expressed on primary keratinocytes from humans and mice and increases with differentiation.

Migration of keratinocytes is fundamental for re-epithelialization (Seeger and Paller, 2015). As RvD2 promoted wound re-epithelialization *in vivo* and because its specific receptor was expressed in epidermal keratinocytes, we asked whether RvD2 promotes migration in these cells. Using an electric cell-substrate impedance sensing system (ECIS), we found that RvD2 enhanced the rate of keratinocyte migration (Fig. 4d). Pre-incubation with an antagonist to DRV2 (i.e., O-1918) abolished this effect (Fig. 4e) (McHugh *et al.*, 2010). Under the same conditions, RvD2 did not affect proliferation, as determined by 5-ethynyl-2'-deoxyuridine incorporation (Fig. 4f). Similar to RvD2, RvD1 enhanced keratinocyte migration in a manner that was abolished by ALX/FPR2 receptor antagonist, WRW4 (Fig. S10c). Neither RvD1 nor RvD2 affected the viability of keratinocytes at concentrations ranging from 1-100nM (Fig. S10d).

The PI3K-AKT pathway plays a causal role in the enhancement of keratinocyte migration by RvD2

We next sought to determine the mechanisms whereby RvD2 promotes keratinocyte migration. Recent studies utilizing genome-wide shRNA libraries identified the PI3K-AKT pathway as a central hub regulating cellular migration (Seo *et al.*, 2014) and we found that RvD2 increased phosphorylation of PI3K p85 subunit (Fig. 5a, b). This was associated with phosphorylation of AKT^{S473}, which is a downstream target of PI3K. Among the downstream effectors of AKT, we focused on the mammalian target of rapamycin (mTOR) and one of its targets, ribosomal protein S6, because this pathway plays a causal role in epidermal repair and cytoskeletal remodeling (Kakanj *et al.*, 2016; Qian *et al.*, 2004; Squarize *et al.*, 2010). We found a significant increase in the phosphorylation of mTOR and a relatively more robust activation of S6 by RvD2, as determined by phosphorylation at serine 235/236 (Fig. 5a, b). To test whether activation of the PI3K-AKT pathway plays a causal role in the enhancement of migration by RvD2, we assessed migration in the presence

of inhibitors of PI3K and AKT activation. As shown in Figure 5c, inhibition of either PI3K (Wortmannin) or AKT (MK2206) slightly reduced basal migration of keratinocytes. Migration of keratinocytes was enhanced by RvD2 and this effect was completely and significantly abolished in cells pretreated with PI3K or AKT inhibitors. Taken together, these results demonstrate that RvD2 activates its receptor on human keratinocytes to enhance migration via the PI3K-AKT pathway.

DISCUSSION

Results of the present study demonstrate that SPM are biosynthesized in cutaneous wounds of both mice and pigs and that they enhance normal re-epithelialization upon injury. These protective actions are mediated in part through direct receptor-mediated actions of resolvins on epidermal keratinocytes. Thus, in addition to their well-documented roles in regulating immunity, the present results uncover alternate cellular mechanisms underlying the tissue reparative roles of resolvins and expand our understanding of the diverse roles of lipid mediators in the wound-healing program.

D-series resolvins were originally identified during self-limited inflammation and their specific structures and biosynthetic pathways have been elucidated. Specifically, RvD1 and RvD2 are produced via the sequential action of 15-LOX and 5-LOX via a common 7,8-epoxide intermediate (Hong *et al.*, 2003; Serhan, 2014; Spite *et al.*, 2009). In contrast, RvD4 biosynthesis proceeds through a 4,5-epoxide, but shares the common upstream 17-hydroperoxide intermediate with RvD1 and RvD2 (see Fig. 1) (Hong *et al.*, 2003; Winkler *et al.*, 2016). Reduction of this biosynthetic intermediate generates 17-HDHA which serves as a marker for D-series resolvin biosynthesis and 15-LOX activity. Here, we found that human epidermal keratinocytes express both human 15-LOX isoforms and readily convert DHA to 17-HDHA. It is notable that a secreted phospholipase with substrate preference for DHA-containing phospholipids, denoted PLA2G2F, is selectively expressed in the skin and regulates epidermal homeostasis (Yamamoto *et al.*, 2015). Whether this or related enzymes play a role in regulating the availability of DHA for conversion to 17-HDHA during wound healing warrants further study. The chirality of the hydroxyl group in 17-HDHA was found to be predominantly in the *S* configuration, which is characteristic of mammalian lipoxygenases and consistent with the original identification of D-series resolvins (Hong *et al.*, 2003). These results suggest that keratinocytes are a potential source of 17-HDHA, and that during tissue injury, infiltrated leukocytes that express 5-LOX (e.g., neutrophils, macrophages) could readily form resolvins through transcellular biosynthesis. Indeed, we have previously shown that 17-HDHA is converted to D-series resolvins in human leukocytes (Hong *et al.*, 2003; Spite *et al.*, 2009). In support of an important role for this biosynthetic pathway in epithelial repair, mice deficient in the murine isoform of *Alox15* (denoted 12/15-LOX) have defective re-epithelialization in corneal and cutaneous wounds (Gronert *et al.*, 2005; Hong *et al.*, 2014). Murine 12/15-LOX is expressed in both epidermis and other cells in the dermal layer in wounds (Hong *et al.*, 2014). Moreover, genetic deficiency of *Alox15* in mouse wounds decreases 17-HDHA and we have previously demonstrated that 17-HDHA is lower in wounds of diabetic animals that show defective re-epithelialization (Hong *et al.*, 2014; Tang *et al.*, 2013). These results collectively lend support to an important role of D-series resolvins produced via this pathway in wound

healing. It is notable that we identified several other SPM in skin biopsies, as well as in wounds. Some of these SPM, such as the lipoxins and maresins, promote tissue repair and regeneration in other models and organisms (e.g., *Planaria*); their individual roles in tissue repair in the skin remains to be established (Biteman *et al.*, 2007; Serhan *et al.*, 2012).

We present several lines of evidence demonstrating that resolvins have direct actions on epidermal keratinocytes. We identified that receptors for both RvD1 and RvD2 are expressed in the epidermal layer of human keratinocytes using both skin biopsies as well as 3-D air-liquid interface cultures of primary cells. Surface expression of both ALX/FPR2 and DRV2/GPR18 was validated using flow cytometry. With intact epidermis, receptor expression was also apparent within the cytoplasm. This is consistent with a recent report demonstrating that GPR18 undergoes a high degree of constitutive trafficking and that the receptor is largely localized to intracellular pools (Finlay *et al.*, 2016). Using mice deficient in *Gpr18*, we established that the enhancement of re-epithelialization by RvD2 was completely abolished. However, no endogenous defect in re-epithelialization was observed. We note that prior studies have demonstrated that mice deficient in *Gpr18* have an endogenous defect in ischemic-revascularization and in resolution in bacterial peritonitis, while reperfusion injury in the lung is not affected by *Gpr18*-deficiency (Chiang *et al.*, 2015; Zhang *et al.*, 2016). These results indicate that its endogenous role may be masked in a site-specific manner when other pro-resolving receptors are expressed. Indeed, our results demonstrate that mice deficient in *Alx/Fpr2* had an endogenous defect in wound re-epithelialization. This more prominent role may be because several pro-resolving mediators (e.g., RvD1, LXA_4, RvD3) activate signaling through ALX/FPR2 (Chiang and Serhan, 2017). Nonetheless, both RvD1 and RvD2 promoted migration but not proliferation of human keratinocytes *in vitro* and these responses were blocked with receptor antagonists to ALX/FPR2 or DRV2. This enhancement of keratinocyte migration explains in part the effects of RvD1 and RvD2 on re-epithelialization in skin wounds, as migration of keratinocytes is required for re-epithelialization and occurs independently of proliferation (Seeger and Paller, 2015; Usui *et al.*, 2008; Usui *et al.*, 2005). Interestingly, keratinocytes from diabetics that have defective wound repair display reduced migration while being highly proliferative (Usui *et al.*, 2008). These results also extend our prior findings that microparticles released from activated immune cells during resolution are enriched with resolvins promote keratinocyte migration *in vitro* (Norling *et al.*, 2011). We note that, because resolvins have well-defined actions on leukocytes (e.g., neutrophils, macrophages), it is likely that their roles in wound healing are multi-factorial. In fact, these multiple cellular targets could be potentially advantageous for both promoting tissue repair as well as host-defense in chronic wounds. Future studies will be required to interrogate fully their receptor-mediated roles on distinct cell types during wound healing.

Given that resolvins promoted keratinocyte migration, it was of interest to determine the downstream signaling pathways involved in this response. Recent studies utilizing genome-wide shRNA libraries identified the PI3K-AKT pathway as a central hub regulating cellular migration (Seo *et al.*, 2014). Our results show that RvD2 rapidly activates phosphorylation of PI3K and its downstream target, AKT. Receptor-dependent activation of AKT by resolvins has also been demonstrated in macrophages and endothelial cells, suggesting that this may be a common downstream target of resolvin receptor-dependent signaling in

multiple cell types involved in immunity and tissue repair (Chiang *et al.*, 2017; Maekawa *et al.*, 2015; Ohira *et al.*, 2010). Further analysis identified activation of mTOR and ribosomal S6 protein by RvD2, a signaling pathway previously identified to regulate macrophage phagocytosis by another SPM, resolvin E1 (Ohira *et al.*, 2010). Constitutively active forms of PI3K, AKT and S6 kinase (an upstream activator of S6) are sufficient to induce migration in fibroblasts by regulating actin filament remodeling, and levels of phosphorylated AKT and S6 are increased in the epidermis during cutaneous wound re-epithelialization (Qian *et al.*, 2004; Squarize *et al.*, 2010). Accordingly, epidermal keratinocyte-specific loss of *Tsc1*, a negative regulator of the mTOR complex 1, impairs wound closure in mice (Squarize *et al.*, 2010). This pathway is also sufficient to promote epidermal migration in *Drosophila melanogaster*, indicating that it is an evolutionarily conserved module for epidermal repair (Kakanj *et al.*, 2016). In our studies, inhibition of upstream modulators of this pathway (i.e., PI3K and AKT) was sufficient to reverse the enhancement of keratinocyte migration by RvD2 indicating that SPM are tightly coupled to tissue repair programs in epithelial cells.

In summary, the results presented here identify epidermal keratinocytes as cellular targets for SPM that enhance wound re-epithelialization. Using targeted LC-MS/MS, we identified that D-series resolvins are increased in cutaneous wounds of mice and pigs and that receptors for RvD1 and RvD2 are expressed in the epidermis. These results indicate that resolvins and their receptors may be targets for tissue repair, particularly in conditions that impair resolution of inflammation and the normal wound healing response such as diabetes. Because these SPM potently resolve inflammation and enhance host-defense, targeting these pathways for tissue repair may offer distinct advantages over traditional anti-inflammatory therapies that perturb tissue repair and are immunosuppressive.

MATERIALS AND METHODS

Detailed methods are discussed in the Supplementary Materials and Methods.

Animals and human samples

Male and female C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Male *Alx/Fpr2*-deficient mice (C57BL/6 background) were provided by Actelion pharmaceuticals (see Acknowledgements). Male *Gpr18*-deficient mice (129/SvEv-C57B/6) and their wild-type (WT) littermates were generated as recently described (Chiang *et al.*, 2015). All mice were maintained on normal laboratory chow diet, housed in a temperature controlled and 12 h light-dark cycling environment and were randomized to treatment groups. The only pre-established exclusion criterion for mouse studies was unintentional removal of wound splints, which causes rapid contraction and impairs the assessment of re-epithelialization. Female Yorkshire pigs (6 months of age; 50–60 kg) were purchased from Parson's Farm (Hadley, MA). All murine and porcine procedures were approved by the Harvard Medical Area Standing Committee on Animals (murine: #05125, porcine; #693). De-identified human skin biopsies were collected under a protocol approved by Brigham and Women's Hospital Institutional Review Board (#2010P002947/BWH) after obtaining written informed consent.

Solid phase extraction and LC-MS/MS lipid mediator and SPM profiling

Murine wounds (day 1 post-wounding) or skin were collected from euthanized animals using a 12 mm skin punch. Porcine wounds (day 6 post-wounding) and skin were collected using a sterile scalpel. The tissue was weighed and immediately snap frozen in liquid nitrogen and stored at −80°C. The day before extraction, tissue was placed into 1 mL of ice-cold methanol containing deuterium-labeled internal standards (d_5-RvD2, d_5-LXA_4, d_4-LTB_4, d_4-PGE_2, d_8-5-HETE) and finely minced. Solid phase extraction and LC-MS/MS analysis were carried out with the NIH-funded Center for Experimental Therapeutics and Reperfusion Injury Metabololipidomics Core as in (Colas *et al.*, 2014). Briefly, lipid mediators were extracted using C18 solid phase extraction cartridges with an automated extraction system (RapidTrace, Biotage). Methyl formate fractions were collected and subjected to evaporation using a slow and steady stream of N_2 gas, followed by re-suspension in methanol:water (50:50). Samples were then analyzed by LC-MS/MS using a Poroshell reverse-phase C18 column (100 mm × 4.6 mm × 2.7 μm; Agilent Technologies) equipped high performance liquid chromatography system (HPLC; Shimadzu) coupled to a QTrap 5500 mass spectrometer (AB Sciex). The mobile phase consisted of methanol:water:acetic acid (50:50:0.01 vol/vol/vol) and was ramped to 85:15:0.01 (vol/vol/vol) over 10 min, followed by ramping to 98:2:0.01 (vol/vol/vol) over the next 8 min and held for additional 2 min. The entire sample elution was performed using a flow rate of 400 μL/min at a constant temperature of 50°C. The QTrap was operated in negative ionization mode using scheduled multiple reaction monitoring (MRM) and coupled with information-dependent acquisition (IDA) and enhanced product ion-scanning (EPI) (Colas *et al.*, 2014). Mediators were identified using retention time and six diagnostic MS/MS ions, as compared with synthetic standards (Cayman Chemical). Concentration was determined using standard curves generated for each mediator with standards after normalization of extraction recovery based on deuterium-labeled internal standards. In order to visualize the changes in lipid mediator abundance between control skin and wounds, network analyses for each parent polyunsaturated fatty acid was then performed using the Cytoscape open source software platform (version 3.5.1). Once lipid mediator profiles for each sample were obtained, the data underwent a missing value imputation that replaced non-detected values with half the minimum value for each mediator, followed by log transformation.

In some experiments, the stereochemistry of the alcohol group in 17-HDHA generated by differentiated keratinocytes was determined by chiral LC-MS/MS. For this, a chiral column (Chiracel AD-RH, 150 mm × 2.1 mm) was used to separate 17*S*-HDHA and 17*R*-HDHA (Cayman Chemical). The column was coupled to an HPLC (Agilent HP 1100 Chemstation with DAD) and isocratic methanol:water:acetic acid (95:5:0.01) was run for 10 minutes at a flow rate of 350 μL/min while being monitored by MS/MS as above.

Statistics

Data are presented as mean ± standard error of the mean (SEM). For small sample sizes, nonparametric statistical analysis was performed. Direct comparisons were made using nonparametric two-tailed Mann-Whitney tests. Nonparametric Kruskal-Wallis tests were used for multiple comparisons, followed by Dunn’s post-tests. In cases with higher sample numbers, we first tested for normality using D’Augostino and Pearson omnibus tests,

followed by unpaired two-tailed Student's *t*-tests for direct comparisons. Parametric one-way or two-way ANOVA, followed by Bonferroni, Tukey's or Dunnett's post-tests, were used for multiple group comparisons as appropriate. We assumed equal variance among groups. The investigators were not blinded during group assignment or data analysis. In all cases, a $P<0.05$ was considered significant. All statistical analysis was performed with GraphPad Prism 6.0.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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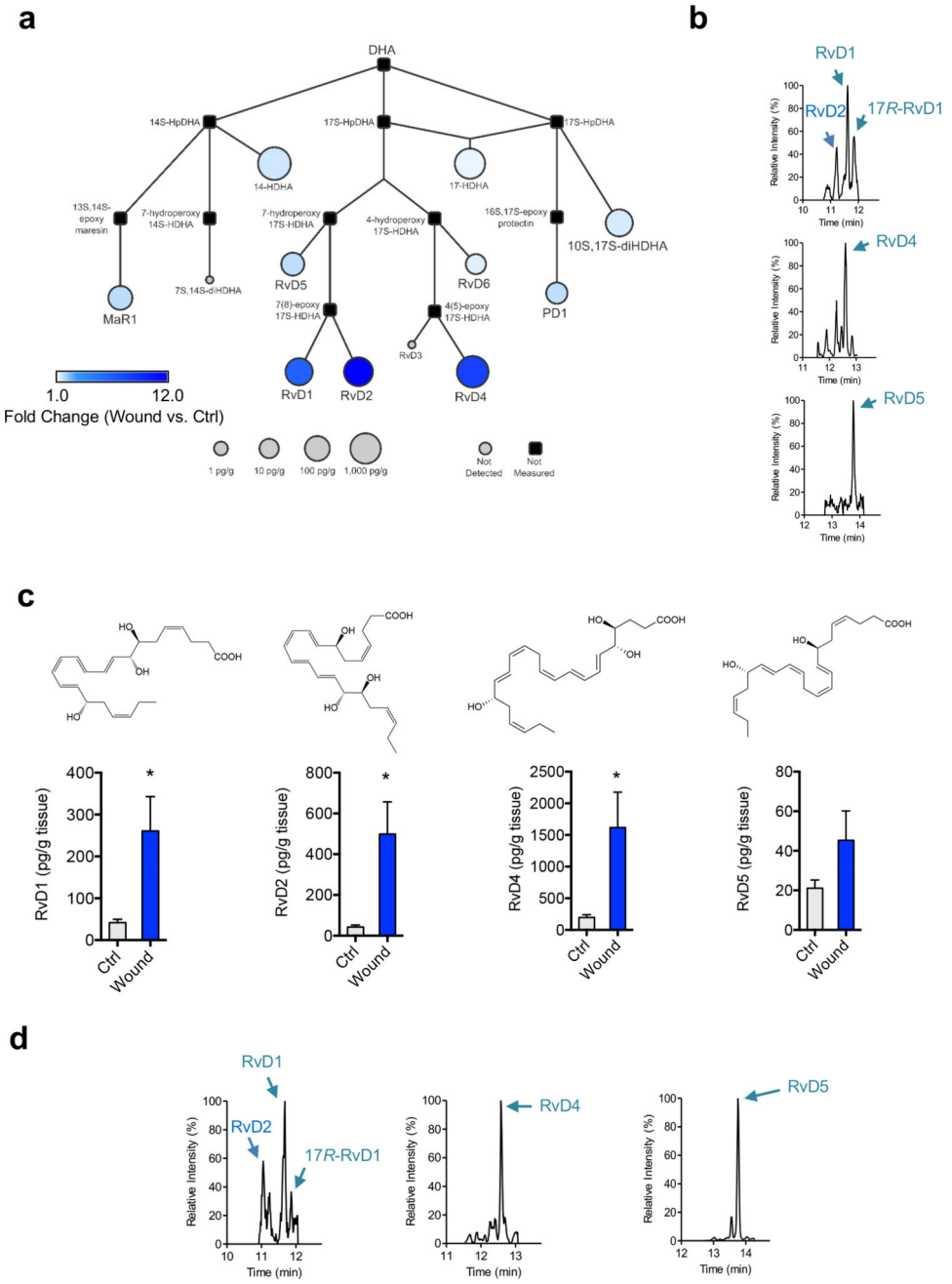

Figure 1. D-series resolvins are biosynthesized in murine and porcine cutaneous wounds

Cutaneous wounds were collected from C57BL/6J mice or Yorkshire pigs and the levels of D-series resolvins were determined by targeted LC-MS/MS. (**a**) Interaction network pathway analysis of the docosahexaenoic acid (DHA) metabolome depicting relative levels of specialized pro-resolving mediators and their biosynthetic pathway markers in murine cutaneous wounds 1 day after wounding. (**b**) Multiple reaction monitoring (MRM) chromatograms of D-series resolvins in murine cutaneous wounds. (**c**) Levels of individual D-series resolvins in murine control skin (Ctrl; n=10) or cutaneous wounds (day 1 postwounding; n=7), with structures shown as inset. (**d**) MRM chromatograms of D-series

resolvins in porcine cutaneous wounds collected at day 6 post-wounding (n=2). Data are mean ± SEM; *P<0.05 by a two-tailed Mann-Whitney test (**c**).

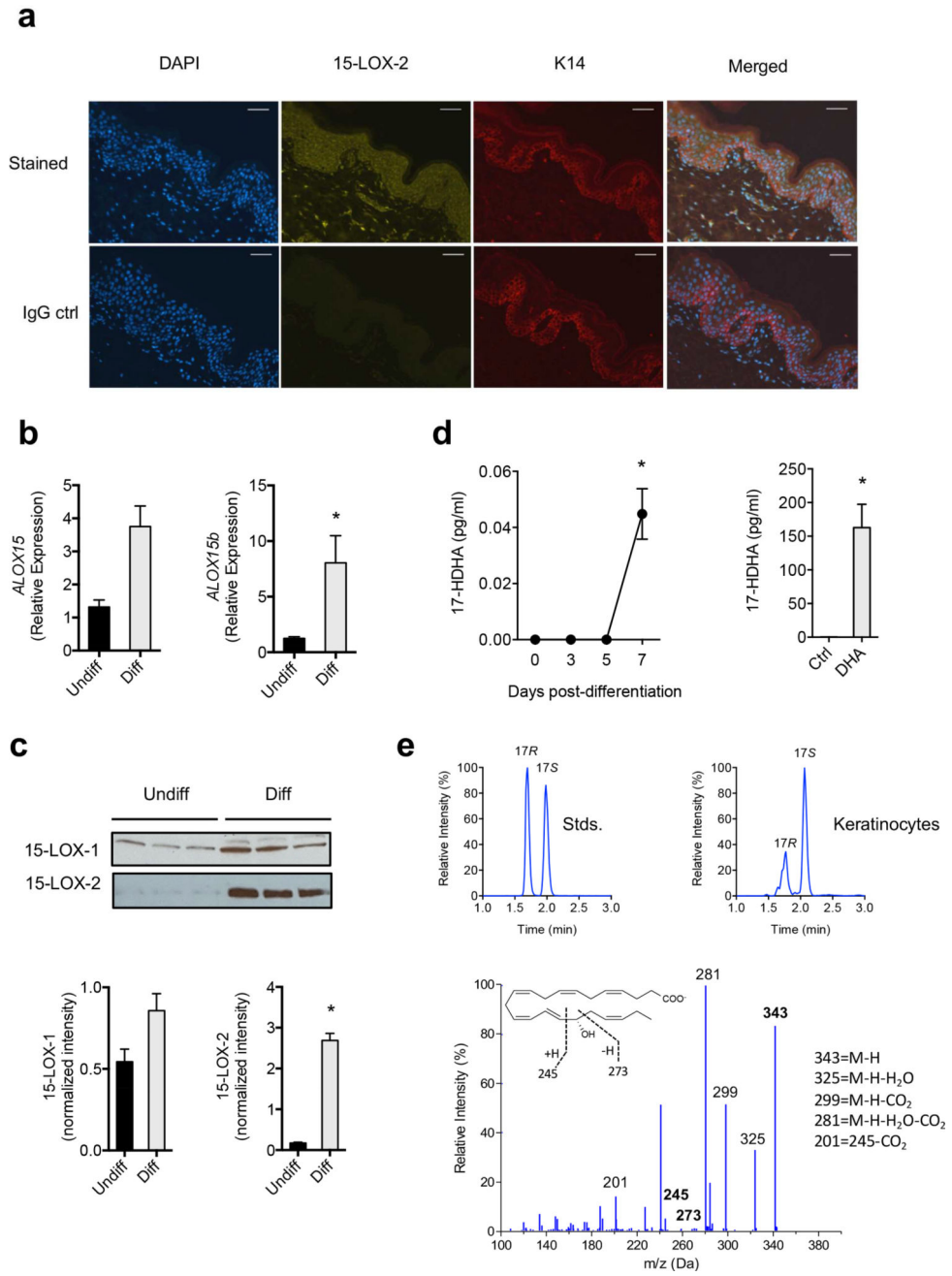

Figure 2. Differentiation of human epidermal keratinocytes increases 15-lipoxygenase expression and production of D-series resolvin biosynthetic precursor and pathway marker, 17-HDHA

(**a**) Immunofluorescence images of 15-LOX-2 in human skin, with basal cell marker keratin 14 (K14) and nuclei staining with DAPI indicated. Results are representative of 5 separate human donors. (**b**) qRT-PCR analysis of *ALOX15* and *ALOX15b* expression in undifferentiated (Undiff) or differentiated (Diff) primary normal human epidermal keratinocytes (NHEK) (n=4 per group). Expression is relative to *HPRT*. (**c**) Western blots and quantitative analysis of human 15-LOX-1 and 15-LOX-2 in NHEK undergoing differentiation (normalized to total protein by amido black staining). Data are representative

of 5 (undifferentiated) or 6 replicates (differentiated), from two independent experiments. (**d**) Left panel, *de novo* biosynthesis of 17-HDHA in NHEK during differentiation, as determined by LC-MS/MS (n=3 replicates per time point). Right panel, production of 17-HDHA in differentiated NHEK incubated in the absence (n=7) or presence (n=8) of DHA (10μM, 30 min). (**e**) Upper panels: MRM chromatograms of 17*R*-HDHA and 17*S*-HDHA synthetic standards and solid-phase extracts of differentiated keratinocytes incubated with DHA. Lower panel: Representative MS/MS spectrum of 17*S*-HDHA from differentiated keratinocytes. Data are mean ± SEM; *P<0.05 by a two-tailed Mann-Whitney test (**b**, **c**, **d**; right panel) or nonparametric one-way ANOVA (Kruskal-Wallis), followed by Dunn's post-test (**d**; left panel).

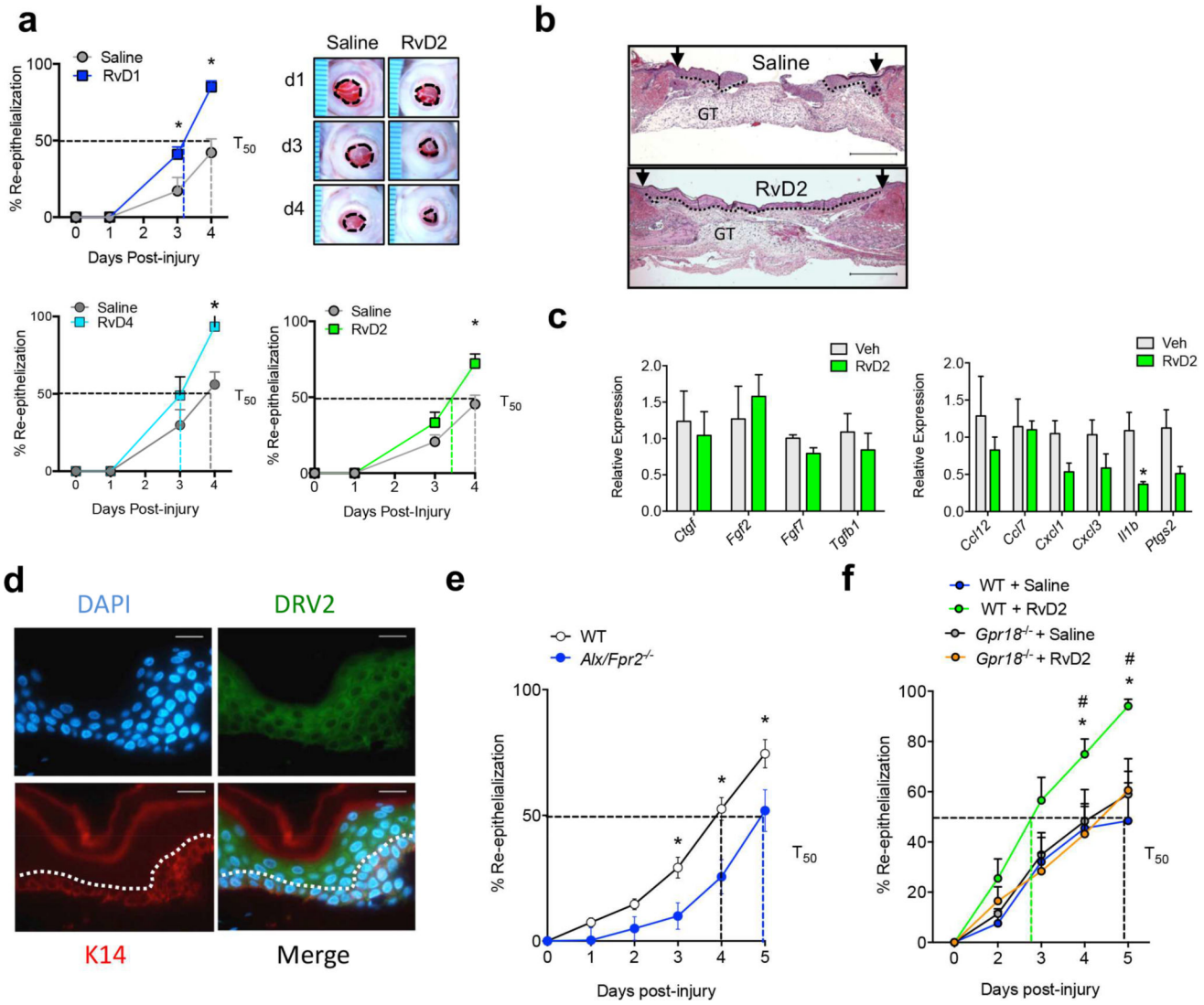

Figure 3. D-series resolvins promote cutaneous wound re-epithelialization

(**a**) Quantitative time-dependent analysis of re-epithelialization in splinted cutaneous wounds of mice treated topically with saline, RvD1, RvD2 or RvD4 for 4 days (n=4–6 per group). T_{50} indicates the time point at which the wounds were re-epithelialized by 50%. Representative images of splinted cutaneous wounds treated with saline or RvD2, with the neo-epidermis indicated with a black dotted line, are shown. (**b**) Images of hematoxylin and eosin stained sections of cutaneous wounds treated with saline or RvD2 for 5 days, with the original wound border indicated by arrows and the neo-epidermis demarcated with a black dotted line. GT: granulation tissue. Scale bars: 500 μm. (**c**) qRT-PCR analysis of growth factors, cytokines/chemokines and COX-2 *(Ptgs2)* in murine cutaneous wounds treated with saline vehicle (Veh) or RvD2 for 5 days (n=4 per group), with gene expression normalized to *Hsp90ab1*. (**d**) Immunofluorescence images of DRV2 expression in human skin, with nuclei stained with DAPI and the basal epidermal layer identified by keratin 14 (K14) staining (white dotted line indicates the suprabasal cell border). Scale bars: 50 μm. (**e**) Wound re-epithelialization in WT and *Alx/Fpr2*-deficient mice (n=8 per group). (**f**) Time-dependent re-

epithelialization of cutaneous wounds of WT and *Gpr18*-deficient mice treated with saline or RvD2 for 5 days (n=5–6 per group). Data are mean ± SEM; *P<0.05 by two-way ANOVA, followed by Bonferroni post-test (**a, e**), or *P<0.05 (WT saline vs. WT + RvD2); #P<0.05 (WT + RvD2 vs. $Gpr18^{-/-}$ + RvD2) by two-way ANOVA, followed by Tukey's multiple comparisons post-test (**f**).

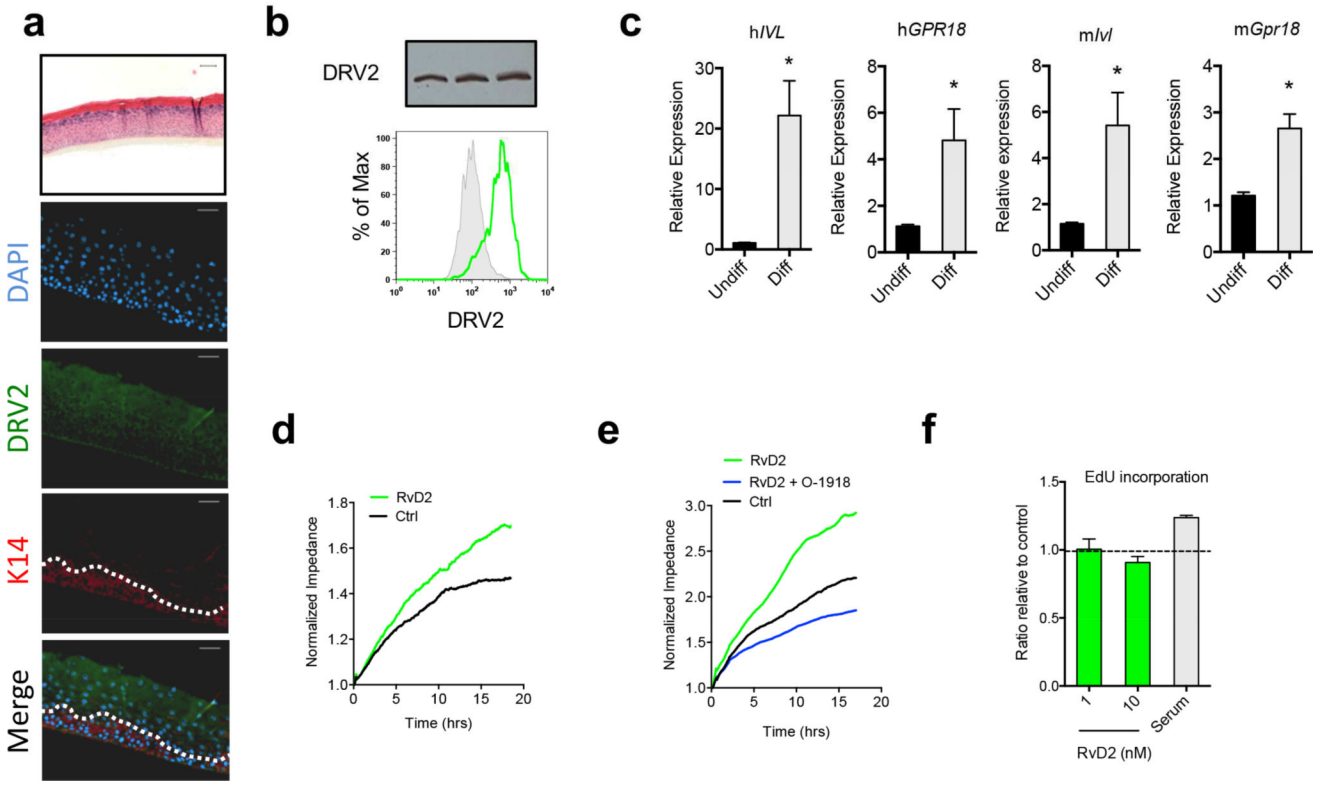

Figure 4. RvD2 promotes epidermal keratinocyte migration in a receptordependent manner

(**a**) Images of hematoxylin and eosin stained primary human keratinocytes during stratification in a 3-D air-liquid interface culture system (scale bar: 100 μm), with immunofluorescence imaging of RvD2 receptor, DRV2 shown in the lower panels (white dotted line demarcates the basal and suprabasal layers); scale bars: 20 μm. (**b**) Western blot (top panel) and representative flow cytometry histogram (bottom panel; n=3) of DRV2 in undifferentiated primary normal human epidermal keratinocytes (NHEK). (Grey: unstained secondary Ab only; Green, anti-DRV2 Ab stained). (**c**) mRNA expression of *GPR18* in undifferentiated (Undiff) or differentiated (Diff) human (h) primary NHEK (left panels) or mouse (m) keratinocytes (right panels), with involucrin (*IVL*) expression shown as a positive control for differentiation (n=3–4 per group). Expression is relative to *HPRT*. (**d, e**) Real-time analysis of epidermal keratinocyte migration in response to wounding (see Methods), as determined using an electric cell substrate impedance sensing (ECIS) system. Cells were either left untreated (control; Ctrl) or incubated with RvD2 (10nM) in the presence or absence of the RvD2 receptor antagonist, O-1918 (20μM). Results are representative of three independent experiments. (**f**) Assessment of proliferation in NHEK treated with RvD2 at indicated concentrations and under identical conditions to those presented in panels **d** and **e**, as assessed with 5-ethynyl-2'-deoxyuridine (EdU) incorporation. Full serum was used as a positive control. Data are relative fluorescence units (RFU) and expressed as mean ± SEM of ratios relative to the untreated group from three independent experiments. Data are mean ± SEM; *P<0.05 by a two-tailed Mann-Whitney test (**c**).

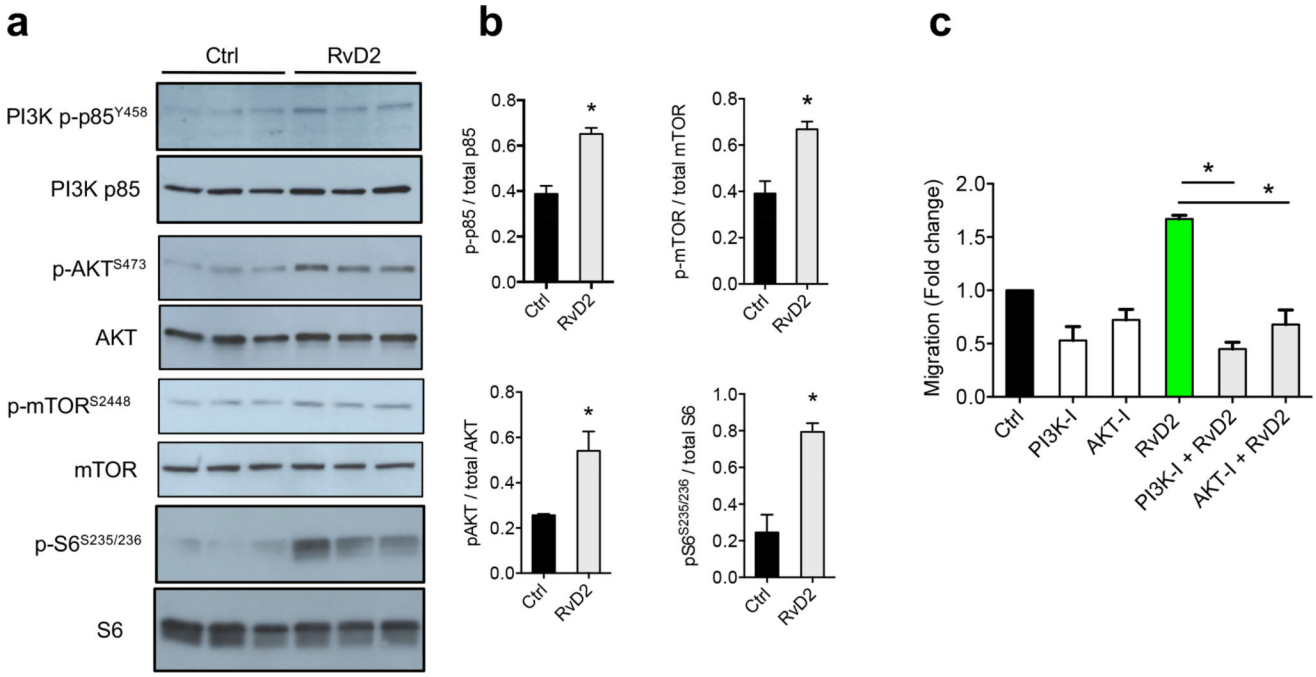

Figure 5. RvD2 promotes epidermal keratinocyte migration by activating the PI3K-AKT-mTOR-S6 pathway

(**a, b**) Western blots and quantification of the phosphorylation of PI3K ($p85^{Y458}$), AKT^{S473}, $mTOR^{S2448}$ and $S6^{S235/236}$ in epidermal keratinocytes left untreated (Ctrl) or stimulated with RvD2 (10nM; 15 min.) (n=3 per group). (**c**) Migration (scratch assay) of epidermal keratinocytes stimulated with RvD2 (10nM) in the presence or absence of phosphatidylinositol 3 kinase inhibitor (PI3K-I) Wortmannin (0.5μM), or AKT inhibitor (AKT-I) MK2206 (5μM). Data are mean ± SEM; *P<0.05 by an unpaired Student's *t*-test (**b**) or by one-way ANOVA, followed by Dunnett's multiple comparisons post-test (**c**).