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Early Induction of NRF2 Antioxidant Pathway by RHBDF2 Mediates Rapid Cutaneous Wound Healing

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Abstract

Rhomboid family protein RHBDF2, an upstream regulator of the epidermal growth factor (EGF) receptor signaling, has been implicated in cutaneous wound healing. However, the underlying molecular mechanisms are still emerging. In humans, a gain-of-function mutation in the *RHBDF2* gene accelerates cutaneous wound healing in an EGFR-dependent manner. Likewise, a gain-of-function mutation in the mouse *Rhbdf2* gene (*Rhbdf2^{cub/cub}*) shows a regenerative phenotype (rapid ear-hole closure) resulting from constitutive activation of the EGFR pathway. Because the RHBDF2-regulated EGFR pathway is relevant to cutaneous wound healing in humans, we used *Rhbdf2^{cub/cub}* mice to investigate the biological networks and pathways leading to accelerated ear-hole closure, with the goal of identifying therapeutic targets potentially effective in promoting wound healing in humans. Comparative transcriptome analysis of ear pinna tissue from *Rhbdf2^{cub/cub}* and *Rhbdf2^{+/+}* mice at 0h, 15 min, 2h, and 24h post-wounding revealed an early induction of the nuclear factor E2-related factor 2 (NRF2)-mediated anti-oxidative pathway (0h and 15 min), followed by the integrin-receptor aggregation pathway (2h) as early-stage events immediately and shortly after wounding in *Rhbdf2^{cub/cub}* mice. Additionally, we observed genes enriched for the Fc fragment of the IgG receptor IIIa (FCGR3A)-mediated phagocytosis pathway 24h post-wounding. Although cutaneous wound repair in healthy individuals is generally non-problematic, it can be severely impaired due to aging, diabetes, and chronic inflammation. This study suggests that activation of the NRF2-antioxidant pathway by rhomboid protein RHBDF2 might be beneficial in treating chronic non-healing wounds.

Introduction

Chronic non-healing wounds are debilitating and contribute significantly to the growing healthcare costs (Fife and Carter, 2012; Rice et al., 2014; Sen et al., 2009). Normal wound healing is a complex process involving several overlapping phases, including inflammation, proliferation, migration, and tissue remodeling (Eming et al., 2014; Gurtner et al., 2008;

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Martin, 1997). Several factors contribute to chronic non-healing wounds, including hypoxia, excessive inflammation, incomplete re-epithelialization, delayed keratinocyte proliferation and migration, ineffective resolution of cellular debris/foreign bodies, and wound infection (Broughton et al., 2006; Frykberg and Banks, 2015). A better understanding of the biological pathways underlying these overlapping phases of wound healing is required to develop effective treatment strategies to not only suppress the hypoxic and inflammatory environment but also facilitate efficient progression of tissue repair.

Although the ability to regenerate lost organs and appendages is a feature of lower vertebrates (e.g., zebrafish, newts, and salamanders), adult mammals fail to regenerate lost or damaged tissues, with the exception of the MRL/MpJ inbred mouse strain (*Mus musculus*) and wild African spiny mice (*Acomys kempfi* and *Acomys percivali*) (Gawriluk et al., 2016; Heydemann, 2012; Seifert et al., 2012). Both MRL/MpJ and spiny mice are useful tools for identifying the molecular mechanisms underlying scarless regeneration, the goal of which is to recapitulate such capabilities in humans. However, substantial evidence in the MRL/MpJ strain suggests that wound healing has polygenic inheritance (Li et al., 2001; Masinde et al., 2001), and inheritance is also very likely to be polygenic in spiny mice. Thus, it can be difficult to identify individual genes linked to a regenerative phenotype in these mice, hindering efforts to identify the underlying molecular mechanisms. Importantly, we recently identified a spontaneous mouse mutation, curly bare (*Rhbdf2^{cub/cub}*), on the C57BL/6J inbred background, in which the wound healing phenotype is caused by a single gene mutation (Hosur et al., 2014) in the *Rhbdf2* gene.

The curly bare (*Rhbdf2^{cub/cub}*) mouse mutation results from a spontaneous deletion of exons 2 through 6 in the *Rhbdf2* gene (Hosur et al., 2014; Johnson et al., 2003). We found that through-and-through (2-mm) ear holes heal rapidly in *Rhbdf2^{cub/cub}* mice. Molecular, cell biological, and *in vivo* genetic approaches revealed that RHBDF2 mediates rapid wound healing (ear-hole closure) in *Rhbdf2^{cub/cub}* mice through enhanced secretion of the epidermal growth factor (EGF) ligand amphiregulin (AREG). Consistently, loss of AREG impairs the rapid wound-healing phenotype in *Rhbdf2^{cub/cub}* mice (Hosur et al., 2014). Thus, *Rhbdf2^{cub/cub}* mice, which carry a single gene mutation on the C57BL/6J inbred strain, a non-healer strain, provide a powerful *in vivo* system with which to elucidate the effects of RHBDF2 on accelerated cutaneous wound healing. Notably, *Rhbdf2^{cub}* is a mouse model of human skin disease tylosis, which is caused by mutations in the human *RHBDF2* gene. Tylosis patients present with a wound healing phenotype—keratinocytes isolated from skin biopsies of tylosis patients show significantly increased migration in an EGFR-dependent manner (Blaydon et al., 2012).

Here, we performed transcriptome analyses to identify early-stage pathways and biological processes that function post-wound healing in *Rhbdf2^{cub/cub}* and control *Rhbdf2^{+/+}* mice. Two-mm through-and-through holes were punched into the ears of 4-wk-old *Rhbdf2^{+/+}* and *Rhbdf2^{cub/cub}* female mice and ear pinna tissue was collected at 0h, 15 min, 2h, and 24h post-wounding (three mice per time point). We identified several differentially expressed genes acting within the nuclear factor E2-related factor 2 (NRF2)-mediated oxidative stress pathway immediately and shortly after wounding (0h and 15 min). Additionally, at 2h and 24h post-wounding we observed genes enriched for the integrin receptor aggregation

pathway and the Fc γ receptor-mediated phagocytosis pathway, respectively. This study identifies an essential role for the NRF2-mediated oxidative stress pathway in the early stages of cutaneous wound healing, and provides insight into specific pathways that are potentially valuable not only for testing novel hypotheses but also for identifying therapeutic interventions to facilitate cutaneous healing in chronic non-healing wounds.

Results and Discussion

Transcriptome analysis of ear-hole closure in *Rhbdf2*^{cub/cub} mice

To identify downstream targets of the AREG-EGFR pathway, and to identify the gene-expression pattern that explains the accelerated healing response in *Rhbdf2*^{cub/cub} mice, we performed transcriptome analysis over time post-wounding. Four pairwise comparisons of sample groups (*Rhbdf2*^{cub/cub} versus *Rhbdf2*^{+/+} mice at 0h, 15 min, 2h, and 24h time points post-wounding) were used to determine how genotype affects the prediction of gene expression values using the R/maanova analysis package (Wu et al. 2003). Differentially expressed probe sets were declared at an FDR q-value threshold of 0.05. We identified 846, 1,492, 696, and 750 probe sets as significantly differentially expressed between genotypes at 0h, 15 min, 2h, and 24h, respectively. Each set of significantly differentially expressed probe sets was used as input in Venny, a software package that creates Venn diagrams (Oliveros, 2007), and results showed 33 probe sets to be common among all four contrasts (Fig. 1A). A heatmap of gene expression for 32 (of the 33) significantly differentially expressed probe sets across all time points was generated using R/pheatmap (Fig. 1B). One probe set was excluded because it was assigned to a putative gene; the other 32 probe sets map to known genes. Via the heatmap, we identified several groups of genes differently expressed between *Rhbdf2*^{+/+} and *Rhbdf2*^{cub/cub} mice, including genes involved in re-epithelialization and angiogenesis, as detailed below. We also validated the gene-expression changes of a subset of these genes—the keratin *Krt77*, the kallikrein proteases *Klk7* and *Klk8*, and the chemokine ligand-16 *Cxcl16*—using qRT-PCR 24h post-wounding (Fig. 1C).

Epithelialization involves replacement of an injured or damaged epithelial surface (Pastar et al., 2014). Keratinocytes play a central role in forming a protective layer over denuded epithelial surfaces (Savagner et al., 2005). Keratins are essential proteins for maintenance of keratinocyte cell structure and of the integrity of skin (Pastar et al., 2014; Werner and Grose, 2003). Upregulation of several keratin genes correlates with keratinocyte activation, proliferation, and migration (Mazzalupo et al., 2003; Tomic-Canic et al., 1998; Werner and Grose, 2003). Induction of these keratins is essential for enhancing the synthesis of other proteins, including collagens, for successful re-epithelialization (Tomic-Canic et al., 1998). Substantial literature suggests that expression of keratin 6 and 16 is significantly upregulated in epithelial cells at the wound edge, and significant downregulation of these keratins is observed in non-healing wounds (Cheng et al., 2013; Mazzalupo et al., 2003). Additionally, induction of keratin 17 in injured skin has been shown to initiate keratinocyte proliferation and migration through the AKT/mTOR signaling pathway (Kim et al., 2006). In MRL healer mice, significant upregulation of keratin genes, including keratin 6, 16, and keratin 17, has been observed during regeneration in both the ear and the digits compared with non-healer C567BL/6J mice (Cheng et al., 2013; Podolak-Popinigis et al., 2015). In line with these

findings, keratinocyte stimulation using exogenous keratins has shown promise in the treatment of cutaneous wounds by facilitating rapid re-epithelialization (Davidson et al., 2013; Than et al., 2012). In our present study, while there were no significant alterations in the expression of *Krt6*, *Krt16*, or *Krt17* (not shown) between *Rhbdf2^{cub/cub}* and *Rhbdf2^{+/+}* mice at any of the four time points, expression of *Krt77* was significantly upregulated immediately (0 h and 15 min) and at 2h and 24h post-wounding (Fig. 1C, D). Additionally, we observed a fourfold increase in *Krt77* expression and a twofold reduction in *Krt24* expression over the 24h period after injury at all stages tested in this study (Fig. 1D). Future studies are needed to determine the roles of keratins 24 and 77 in wound healing.

In addition to keratins, kallikreins, expressed widely in the epidermis, regulate epidermal barrier function, tissue integrity, and innate immune responses (Borgono and Diamandis, 2004; Kishibe et al., 2012; Paliouras et al., 2007). Kallikreins are a family of secreted serine proteases comprising kallikrein 1 (KLK1) and 14 kallikrein-related peptidases (KLK2–KLK15) (Borgono and Diamandis, 2004; Paliouras et al., 2007). Kallikrein-related peptidase 8 (KLK8) is required for maintenance of skin homeostasis (Kishibe et al., 2012). Following skin wounding, *Klk8* expression is increased by more than ten-fold during both the early and the late stages of healing, and mice lacking *Klk8* show impaired wound healing due to delayed keratinocyte proliferation, migration, and differentiation (Kishibe et al., 2012). Indeed, we observed that the kallikrein proteases *Klk7* and *Klk8* were significantly upregulated in *Rhbdf2^{cub/cub}* relative to *Rhbdf2^{+/+}* mice at all sampling times (Fig. 1C & 1D).

Chemokines also play an important role in various stages of wound healing by promoting restoration of tissue homeostasis (Coppinger et al., 2004; Keeley et al., 2008). Chemokines are signaling proteins that regulate chemotaxis, orchestrate the host immune response, and promote angiogenesis (Akhtar et al., 2013; Gillitzer and Goebeler, 2001; Zhang and Xu, 2014). Upon injury, chemokines including CXCL2, CXCL8, and CXCL16 promote activation of neutrophils, macrophages, keratinocytes, and endothelial cells (Polverini, 2011; Tonnesen et al., 2000). CXCL16, a membrane-bound chemokine, is cleaved by ADAM10 (a disintegrin and metalloproteinase 10) into a soluble form, which promotes proliferation and migration of endothelial cells through activation of the ERK pathway, and subsequent re-epithelialization (Zhuge et al., 2005). *In vitro*, keratinocytes produce CXCL16 upon stimulation with tumor necrosis factor A, IL1A or IFNG (Tohyama et al., 2007). In this study, we observed a two-fold increase in mRNA levels of *Cxcl16* in *Rhbdf2^{cub/cub}* mice compared with wild-type mice (Fig. 1C, D). Taken together, these results reveal differential expression of a collection of genes that are likely to accelerate cutaneous healing in *Rhbdf2^{cub/cub}* mice. As the re-epithelialization process is impaired in wound-healing disorders, a better understanding of the growth factors, keratins, kallikrein serine proteases, and chemokines involved in re-epithelialization process may lead to the development of novel therapeutic strategies for non-healing wounds.

NRF2-mediated oxidative stress response pathway genes are activated immediately after wounding

Next, to identify the biological functions and predicted pathways associated with each of the four (0h, 15 min, 2h, and 24h) datasets, we used Ingenuity Pathway Analysis (IPA) software. Each analysis per time point revealed unique biological functions.

The wound repair process involves three overlapping phases: inflammation, proliferation and maturation, and tissue remodeling (Gurtner et al., 2008; Martin, 1997). During the inflammatory phase, neutrophils and macrophages, in addition to secreting proteolytic enzymes and pro-inflammatory cytokines, contribute to the production of reactive oxygen species (ROS), to protect the wound site from infections (Steiling et al., 1999). Although ROS generation is required for defense against invading pathogens and mediating angiogenesis, it must be followed by efficient detoxification of ROS. Low levels of ROS are required for angiogenesis (Breton-Romero and Lamas, 2014; Roy et al., 2006; Sen et al., 2002), and persistence of excessive levels of ROS leads to severe cell damage, and eventually to chronic non-healing wounds. To maintain homeostasis and prevent superoxide-mediated damage to the surrounding tissues, antioxidative pathways activate to scavenge ROS, a process referred to as redox homeostasis. For instance, superoxide is dismutated to hydrogen peroxide (H_2O_2) and molecular oxygen by superoxide dismutases (SOD), and H_2O_2 is detoxified by glutathione peroxidases (GPX), preventing generation of hydroxyl radicals (Fridovich, 1995; Steiling et al., 1999).

The SOD family members SOD1, a cytosolic Cu/Zn-dependent SOD; SOD2, a mitochondrial Mn-dependent SOD; and SOD3, an extracellular SOD, mediate detoxification of superoxide radical anions generated by NADPH oxidase (Fridovich, 1995). In the present study, we observed significant downregulation of *Sod2* and upregulation of *Sod3* immediately after injury (Fig. 2A). SODs are differentially expressed and play important roles not only in the wound healing process but also during skin development (Ceradini et al., 2008; Chiumiento et al., 2006; Schafer and Werner, 2008; Shukla et al., 1997; Steiling et al., 1999). Increased transcript levels of *Sod1* and *Sod2* are commonly observed in healing skin wounds in mice. In *Sod1*-deficient mice, wound healing is delayed (Iuchi et al., 2010). Concordantly, *Sod2* overexpression rescues delayed healing in streptozotocin-induced type I diabetes rats through suppression of ROS (Luo et al., 2004). Interestingly, SOD3 is associated with age-related defects in cutaneous wound healing; a recent study showed that delayed healing of 6-mm circular full-thickness wounds on the dorsal skin of 22-month-old aged male mice is associated with reduced levels of SOD3 (Fujiwara et al., 2016). Taken together, these findings suggest that SOD-mediated detoxification might be beneficial in impaired cutaneous wound healing.

The glutathione peroxidase (GPX) family members GPX1-8 mediate detoxification of H_2O_2 (Rasik and Shukla, 2000). GPX1-4 contain a selenocysteine residue in the catalytic center (Brigelius-Flohe and Maiorino, 2013). Upon injury via full-thickness excisional mouse wounds, *Gpx1* mRNA and protein levels are upregulated (Iuchi et al., 2010; Steiling et al., 1999). GPX2 is reported to be a downstream target of the NRF2 oxidative stress response pathway (Banning et al., 2005). Also, GPX2 is induced by the WNT signaling pathway and plays a role in mucosal homeostasis (Esworthy et al., 2005). Mice deficient for either the

Gpx1 or the *Gpx2* gene appear normal; however, *Gpx1* and *Gpx2* double-knockout mice present with significant mucosal inflammation, suggesting that GPXs also play an important role in regulating inflammation (Banning et al., 2005; Chu et al., 2004). In line with the results of these studies, we observed significant upregulation of *Gpx2* in *Rhbdf2^{ub/cub}* mice relative to *Rhbdf2^{+/+}* mice (Fig. 2A). Additionally, *Rhbdf2^{ub/cub}* mice showed significant downregulation of glutathione transferases *Gsta3* (glutathione s-transferase a3), *Gsto1* (glutathione s-transferase omega 1), and *Mgst1* (microsomal glutathione s-transferase 1), and of *Gsr* (glutathione reductase), relative to controls, suggesting the significance of these antioxidative enzymes in redox homeostasis immediately after injury (data not shown).

Heme oxygenases, a downstream target of NRF2, also play a protective role in healing, by degrading heme to iron, carbon monoxide, and biliverdin (Lee et al., 1997; Stocker et al., 1987). The transcript and protein levels of heme oxygenase 1 (HMOX1), an inducible isoform, are significantly upregulated in full-thickness excisional wounds in mice (Hanselmann et al., 2001). Additionally, *Hmox1* knockout mice show significantly delayed wound re-epithelialization and angiogenesis, resulting in impaired wound closure (Deshane et al., 2007). In the present study, we observed significant upregulation of *Hmox1* immediately after injury (Fig. 2A). Particularly, HMOX1 is also implicated in regulation of expression of several genes involved in dermal tissue remodeling in human patients (Deshane et al., 2007; Hanselmann et al., 2001).

Taken together, these results showing differential expression of antioxidative enzymes including glutathione peroxidases, glutathione transferases, heme oxygenase 1, and superoxide dismutases indicate that the NRF2-mediated antioxidant response element pathway (Fig. 2B) plays a role in ear-hole closure immediately and shortly after injury in *Rhbdf2^{ub/cub}* mice. Notably, recent studies identify an essential role for NRF2 in diabetic wounds and premature aging (Bitar and Al-Mulla, 2011; Kubben et al., 2016; Long et al., 2016).

Integrin receptor aggregation signaling pathway genes are activated 2h after wounding

The second phase of wound repair involves proliferation and maturation of different cell types, including keratinocytes. In the epidermis, integrin-receptor signaling is essential for maintenance of cutaneous structure and integrity. For instance, integrin beta 1 is key for keratinocyte migration during wound closure (Schlaepfer et al., 1994; Schlaepfer and Hunter, 1997). Further, integrin receptors mediate interactions between keratinocytes and the extracellular matrix during the other phases of wound repair—re-epithelialization and tissue remodeling (Koivisto et al., 2014).

In this study, several integrin genes showed significant upregulation 2h post-injury in *Rhbdf2^{ub/cub}* mice relative to control mice (Fig. 3A). Platelet activation during the initial plug formation phase of wound healing is known to activate integrins (Golebiewska and Poole, 2015; Rowley et al., 2011). Integrins are heterodimeric glycoproteins that are essential for adhesion of cells to extracellular matrix (ECM) proteins including collagen and fibronectin (Broughton et al., 2006; Miyamoto et al., 1996). Integrins mediate inside-out and outside-in signaling across the plasma membrane. During inside-out signaling, intracellular proteins including paxillin and vinculin mediate integrin binding to actin microfilaments,

whereas during outside-in signaling, binding of ligands including fibronectin and laminins initiates intracellular signaling (Kenny and Connelly, 2015). In addition to bi-directional signaling, integrins aggregate with growth-factor receptors to synergistically modulate intracellular signaling (Kenny and Connelly, 2015; Koivisto et al., 2014). Notably, expression of several integrin genes in *Rhbdf2^{cup/cup}* mice showed similar times of induction (Fig. 3B), suggesting that the integrin receptor aggregation pathway might have a critical role not only in facilitating adhesion to the ECM post-injury but also in accelerating cytoskeletal rearrangement and cell motility.

Fc-gamma receptor (Fc γ R) -mediated phagocytosis pathway genes are activated 24h after wounding

Phagocytosis occurs during all phases of wound repair (Adamson, 2009; Brancato and Albina, 2011). In our present study, the 24h data revealed that several genes within the Fc γ R-mediated phagocytosis pathway were differentially expressed (Fig. 4A,B), implicating immune-cell-mediated phagocytosis in playing a role during this stage of cutaneous regeneration. The immune system plays a key role in elimination of foreign antigens and other pathogens via phagocytosis. Phagocytic cells including macrophages, neutrophils, and dendritic cells detect pathogens through their cell-surface receptors and launch an immune attack (Aderem, 2003; Aderem and Underhill, 1999). Phagocytic Fc γ Rs on macrophages detect immunoglobulin G (IgG)-coated foreign particles and activate intracellular signaling pathways by re-organizing the actin cytoskeleton and engulfing the foreign object to form a vacuole, commonly known as phagosome. The phagosome fuses with lysosomes to degrade or clear foreign particles (May and Machesky, 2001).

Fc γ Rs transduce signals through immunoglobulin tyrosine activation motif (ITAM) domains (Ravetch and Bolland, 2001). The interaction between Fc γ R and the Fc (fragment crystallizable) region of IgG on the foreign antigen induces phosphorylation of tyrosine residues in the Fc γ R ITAM domain, leading to recruitment of actin cytoskeletal proteins (ARP). After engulfing the foreign particle, a multitude of downstream effector pathways, including phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), phospholipase-C (PLC), the Rho family of GTPases (Rac1, Cdc42, and ADP-ribosylation factor), and protein kinase-C (PKC) participate in formation of the phagosome (Garcia-Garcia and Rosales, 2002; Kedzierska et al., 2001). Indeed, we observed a significant increase in expression of several genes in the Fc γ R phagocytosis pathway including, *Fcgr3A*, *Arpc5*, *Actg1*, *Fgr*, *Prkcb*, *Ptk2b*, and *Pxn*, 24h post-wounding in *Rhbdf2^{cup/cup}* mice (Fig. 4B), reiterating the significance of innate immune cell-orchestrated phagocytosis not only in degradation of pathogens but also in wound repair and tissue remodeling.

In summary, the focus of this study was to identify early-stage events in cutaneous wound healing in *Rhbdf2^{cup/cup}* distinct from those in *Rhbdf2^{+/+}* mice. We demonstrated that increased baseline expression of genes acting within the NRF2 anti-oxidative pathway (0h and 15 min), followed by gene alterations associated with the integrin receptor aggregation pathway (2h) and the Fc γ R-mediated phagocytosis pathway (24h), contribute to accelerated ear-hole closure in *Rhbdf2^{cup/cup}* mice. Therapeutic modulation of these signaling pathways, especially the NRF2 anti-oxidative pathway or modulation of RHBDF2 activity to induce

the NRF2 pathway in the epidermis of the skin, might prove beneficial to the repair of non-healing cutaneous wounds.

Materials and Methods

Mice

(B6.Cg-*Rhbf2*^{xcub}/J, stock number 003826 and its control C57BL/6J, stock number 000664) mice were obtained, bred and maintained in a barrier facility at The Jackson Laboratory (JAX) with a 12-hour light to 12-hour dark cycle. Mice were provided food pellets (Purina LabDiet 5K52; 6% fat, autoclaved) and acidified water *ad libitum*. The Jackson Laboratory Animal Care and Use Committee approved all the experimental procedures. Three female 4-week-old mice per group were used for transcriptome analysis and three female 4-week-old mice were used for qPCR validation. Female mice were used because male mice fight, which would complicate these studies, and because there is no sexual dichotomy in the phenotype of the B6.Cg-*Rhbf2*^{xcub}/J mice.

Tissue collection

Mice were wounded using a surgical ear punch device (Napox KN-292B; Natsume Seisakusho Co.) to make 2-mm through-and-through holes in the center of the ears (Hosur et al., 2014). At the indicated times, mice were euthanized and a 3-mm biopsy punch (ThermoFisher Scientific) was used to collect 1-mm of tissue adjacent to the 2-mm through-and-through holes. Skin tissue was then washed with PBS and stored in *RNAlater* solution (ThermoFisher Scientific, Waltham, MA).

Affymetrix gene arrays

A total of 23 *Affymetrix Mouse Gene 1.0 ST Arrays* were used for gene expression analysis (Affymetrix, Santa Clara, CA). This procedure was performed as described previously (HogenEsch et al., 2016). Ear skin samples collected in *RNAlater* were homogenized in TRIzol (ThermoFisher Scientific). After total RNA isolation, quality assessment was performed using a 2100 Bioanalyzer. Subsequently, cDNA was synthesized using the GeneChip Expression 3' – Amplification One-cycle kit (Affymetrix). Following cDNA synthesis, it was amplified, labeled with biotinylated nucleotides (Affymetrix), and hybridized onto 1.0 ST GeneChip arrays. Slides were scanned and quantified using GeneChip Scanner 3000 and GCOS 1.0 software, respectively.

Microarray analysis

Average signal intensities for each probe set within arrays were calculated and exported using Affymetrix's Expression Console (Version 1.1) software. Probes were summarized into probe sets using the RMA (Robust Multi-Array Average) method, which incorporates convolution background correction, sketch quantile normalization, and summarization based on a multi-array model fit robustly using the median polish algorithm. For this experiment, pairwise comparisons were used to statistically resolve gene-expression differences between genotype groups at 0h, 15 min, 2h, and 24h using the R/maanova analysis package (Cui and Churchill, 2003). Specifically, differentially expressed genes were detected using F_s , a modified F-statistic that incorporates shrinkage estimates of variance components from

within the R/maanova package (Cui et al., 2005). Statistical significance levels of the pairwise comparisons were calculated by permutation analysis (1,000 permutations) and adjusted for multiple testing using the false discovery rate (FDR), q-value, method (Cui and Churchill, 2003). Differentially expressed genes were declared at an FDR q-value threshold of 0.05. We have submitted our dataset to GEO (Accession number: GSE95250).

Ingenuity Pathway Analysis

Ingenuity Pathway Analysis software (IPA; Ingenuity Systems, www.ingenuity.com, Winter 2016 release) was used to compare networks and biological functions associated with four data sets, i.e., differential expression at 0h, 15 min, 2h, and 24h post-wounding of the ear in *Rhbd2^{cut/cut}* versus *Rhbd2^{+/+}* mice. Expression results for all comparisons were limited to probe sets having a q-value < 0.05. Affymetrix Mouse Gene 1.0 ST probe set IDs were used as molecule identifiers in IPA. Each identifier was mapped to its corresponding object in the IPA Knowledge Base. These objects were overlaid onto a global gene network developed from information contained in the IPA Knowledge Base. Pathways of genes were then algorithmically generated based on their connectivity to each of the comparison data sets. In each pathway, molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the IPA Knowledge Base. Nodes are displayed using various shapes that represent the functional class of the gene product.

Quantitative real-time PCR (qRT-PCR)

Purified RNA was extracted from skin according to the manufacturer's instructions using the TRIzol reagent (ThermoFisher Scientific). We used a NanoDrop ND-1000 spectrophotometer to determine the quality and to quantify the amount of purified RNA. Realtime PCR was performed using a ViiA7 instrument. Gene-expression levels were normalized to the housekeeping gene *18s rRNA*. The delta-delta-Ct ($2^{-\Delta\Delta C_t}$) method was used to analyze the relative changes in gene expression. Data represents log₂-transformed mean $\pm$ S.D. The following list of predesigned TaqMan gene-expression assays were obtained from ThermoFisher Scientific.

Gene	Assay
<i>Cxcl16</i>	<i>Mm00469712 m1</i>
<i>Klk7</i>	<i>Mm01197332 g1</i>
<i>Klk8</i>	<i>Mm00501179 m1</i>
<i>Krt77</i>	<i>Mm02343485 m1</i>
<i>18s rRNA</i>	<i>Mm03928990 g1</i>

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Figure 1.

Comparative transcriptome analysis between *Rhbdf2^{cub/cub}* and *Rhbdf2^{+/+}* mice at 0h, 15 min, 2h, and 24h post-wounding. A) Venn diagram detailing the number of significantly differentially expressed (q < 0.05) probe sets identified among the four pairwise comparisons of sample groups (*Rhbdf2^{cub/cub}* versus *Rhbdf2^{+/+}* mice at 0h, 15 min, 2h, and 24h time points post-wounding) and their intersections. The maximum number of gene-expression changes was observed at 15 min post-injury (1,492). B) Heatmap displaying the expression profile of 32 of 33 significantly differentially expressed (q < 0.05) probe sets (mapping to known genes) present at the intersection of the four pairwise comparisons of sample groups (*Rhbdf2^{cub/cub}* versus *Rhbdf2^{+/+}* mice at 0h, 15 min, 2h, and 24h time points). Duplicate gene symbols indicate the presence of multiple probe sets mapping to the same gene but at different locations. C) qRT-PCR validation of gene expression changes that are commonly altered at all times. RNA was isolated from ear pinnae obtained after punching 2-mm through-and-through holes in the center of the left ear (n=3 per group). While 18S rRNA served as an internal control, data are normalized to the corresponding *Rhbdf2^{+/+}* control. D) Fold changes for individual genes showing significant alterations (upregulation or downregulation) in *Rhbdf2^{cub/cub}* versus *Rhbdf2^{+/+}* mice over the 24h period. Data represent mean ± S.D. *p<0.05, ***p<0.001.

Figure 2.

A) Fold changes for individual genes showing significant alterations (upregulation or downregulation) specifically at 0h and 15 min post-wounding. B) NRF2-mediated oxidative stress pathway. Networks generated using Ingenuity Pathway Analysis (IPA) suggest that the NRF2-mediated oxidative stress pathway is activated immediately after injury in *Rhbd2^{cup/cup}* mice. Nodes are displayed using various shapes that represent the functional class of the molecule. Nodes are colored red for upregulation and green for downregulation with respect to comparison of *Rhbd2^{cup/cup}* versus *Rhbd2^{+/+}* mice at the corresponding time point. Gray nodes represent molecules included by IPA to complete the network (red, upregulation; green, downregulation).

A

Figure 3.

A) Fold changes for individual integrin genes showing significant upregulation specifically at 2h post-wounding, $q < 0.05$ compared with *Rhbdf2*^{+/+} control mice. B) Ingenuity Pathway Analysis (IPA) suggests that the integrin receptor aggregation pathway promoting cytoskeletal rearrangement and cell motility is significantly activated at 2h in *Rhbdf2*^{ub/cub} relative to *Rhbdf2*^{+/+} mice (red, upregulation).

Figure 4.

A) IPA analysis suggests the involvement of signaling by ITAMs (immunoreceptor tyrosine-based activation motifs) in the Fc γ R pathway at 24h. Fc γ R2A and Fc γ R3A are the predominant receptors that activate phagocytosis in monocytes/macrophages (red, upregulation; green, downregulation). B) Fold changes for individual genes showing significant alterations specifically at 24h post-wounding. $q \leq 0.05$ compared with *Rhbd2*^{+/+} controls.