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***MiR-31* mediates inflammatory signaling to promote re-epithelialization during skin wound healing**

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Short title: *miR-31* promotes epidermal wound healing

Abbreviations used: PWD, post-wounding; WT, wild type; NC, negative control; cKO, conditional knockout.

Key words: microRNA, keratinocyte proliferation and migration, wound healing, *miR-31*, RAS/MAPK

ABSTRACT

Wound healing is essential for skin repair after injury, and consists of hemostasis, inflammation, re-epithelialization and remodeling phases. Successful re-epithelialization, which relies on proliferation and migration of epidermal keratinocytes, requires reduction in tissue inflammation. Therefore, understanding the molecular mechanism underlying the transition from inflammation to re-epithelialization will help to better understand the principles of wound healing. Currently, the *in vivo* functions of specific microRNAs in wound healing are not fully understood. We observed that *miR-31* expression is strongly induced in wound edge keratinocytes, and is directly regulated by the activity of NF- κ B and STAT3 signaling pathways during inflammation phase. We utilized *miR-31* loss-of-function mouse models to demonstrate that *miR-31* promotes keratinocyte proliferation and migration. Mechanistically, *miR-31* activates the RAS/MAPK signaling by directly targeting *Rasa1*, *Spred1*, *Spred2* and *Spry4*, which are negative regulators of the RAS/MAPK pathway. Knockdown of these *miR-31* targets at least partially rescues the delayed scratch wound re-epithelialization phenotype observed *in vitro* in *miR-31* knockdown keratinocytes. Taken together, these findings identify *miR-31* as an important cell-autonomous mediator during the transition from inflammation to re-epithelialization phases of wound healing, suggesting a therapeutic potential for *miR-31* in skin injury repair.

INTRODUCTION

Wound healing is the physiological response of skin to injury, which restores its integrity and some of its functions. Wound healing consists of hemostasis, inflammation, re-epithelialization and remodeling phases (Reinke and Sorg, 2012). Acute wounds are able to heal rapidly by properly executing all of the phases. However, repair process becomes defective in chronic wounds, which fail to heal and pose an increasingly urgent clinical challenge worldwide (Mustoe et al., 2006). Elucidating the molecular mechanism underlying normal wound healing can help understand the principle of skin repair and provide new insight into the pathogenesis of chronic wounds. Re-epithelialization is the crucial phase during wound healing (Pastar et al., 2014), when keratinocytes migrate from the edge towards the wound center, and form new epidermis over the provisional dermal scar. Many molecular factors governing wound re-epithelialization have been identified, including integrins (Grinnell, 1992, Koivisto et al., 2014), matrix metalloproteinases (MMPs) (Krampert et al., 2004), growth factors (Le et al., 2012), cytokines (Werner and Grose, 2003) and extracellular matrix (ECM) (Zhou et al., 2016). However, several aspects of the mechanism for re-epithelialization, including the role that microRNAs (miRNAs) play, remain incompletely understood.

MiRNAs are ~22nt-long non-coding RNAs that negatively regulate gene expression by either degrading mRNA of their target genes or repressing their translation. It has been reported that microRNAs are extensively involved in a variety of normal and pathological biological processes (Fahs et al., 2015). Recently, miRNAs have been shown to modulate wound re-epithelialization by regulating pathway activities tied with keratinocyte

proliferation and migration. Many microRNAs dynamically change during the wound healing process (Banerjee and Sen, 2015, Horsburgh et al., 2017). Among them, *miR-31* is of great interest, because it has been implicated in promoting epidermal hyperplasia in psoriasis (Xu et al., 2013, Yan et al., 2015), epithelial stem cell regulation in the mammary gland (Lv et al., 2017) and intestine (Tian et al., 2017), and because it functions as an oncogene in lung and colorectal cancers (Edmonds, 2016, Sun et al., 2013). Furthermore, *in vitro* evidence suggests that *miR-31* likely promotes skin wound healing in humans by directly regulating epithelial membrane protein-1 (EMP-1) (Li et al., 2015). However, the *in vivo* functions of *miR-31* during the key phases of wound inflammation and re-epithelialization have not been comprehensively accessed.

Here we found that *miR-31* is induced in wound edge keratinocytes in response to inflammatory signaling through the activity of NF- κ B and STAT3 pathways. We utilized *miR-31* loss-of-function mouse models to demonstrate that *miR-31* is the key regulator in promoting migration and proliferation of keratinocytes by targeting RAS/MAPK signaling pathway. Our data identifies *miR-31* as an important cell-intrinsic regulator of transition from inflammation to re-epithelialization phases in wound keratinocytes *in vivo*.

RESULTS

MiR-31 expression pattern during skin wound healing.

We compared *miR-31* expression in whole tissue between heart, skin, spleen, lung, liver, kidney and duodenum in mice and found that it is expressed in the skin at a moderate level, compatible to that in the lung and liver (Figure S1a). We then examined *miR-31* expression levels in skin at different time points after wounding. *MiR-31* expression increased in the wound edge tissue during the inflammatory phase (PWD 1-3) and reached its highest level during the proliferative phase as compared to its level two hours after wounding (Figure 1a). *In situ* hybridization revealed that *miR-31* signal primarily localizes to the keratinocytes at the wound edge (Figure 1b). Normal epidermis away from the wound edge was largely negative. Positive *in situ* signal was also seen in some cells in the dermis.

MiR-31 is directly regulated by the NF- κ B and STAT3 signaling pathways.

Next, we examined how *miR-31* is regulated after wounding. Our previous work has shown that *miR-31* promoter contains p65 and STAT3 binding sites (Tian et al., 2017). Therefore, we tested if *miR-31* is induced upon activation of NF- κ B and STAT3 signaling pathways. Multiple inflammatory cytokines, including *Ifn γ* , *Tnf α* , *Cxcl1*, *Il6* and *Il1b* were induced two days after wounding (Figure 1c) and NF- κ B and STAT3 signaling pathways as judged by p-p65 and p-STAT3 levels respectively, were activated in the wound edge tissue (Figure 1d and 1e), where *miR-31* also becomes highly induced. In agreement with *miR-31* upregulation at PWD 6, we found that NF- κ B and STAT3 signaling pathway are still activated at this time point (Figure S1b and S1c). It has also been reported that TNF α can

activate NF- κ B (Zhu et al., 2012), while IL-6 can activate STAT3 pathway (Venkatesan et al., 2017, Wang et al., 2003). Thus, we tested whether TNF α or IL-6 can trigger *miR-31* expression *in vitro*. Indeed, both TNF α and IL-6 were able to induce *miR-31* expression in mouse primary keratinocytes (Figure 1f), concomitant with upregulation of p-p65 and p-STAT3 (Figure S1d and S1e). In contrast, siRNA-mediated knockdown of *p65* and *Stat3* significantly repressed *miR-31* expression, accompanied by the repression of NF- κ B and STAT3 signaling (Figures 1f, S1f and S1g). We also found that both TNF α and IL-6 induced *miR-31* up-regulation in HaCaT cells, while siRNA-mediated knockdown of *p65* and *Stat3* repressed *miR-31* expression (Figure S1h and S1i). Moreover, luciferase reporter assay revealed that mutation of the p65 and STAT3 binding sites in *miR-31* promoter suppressed luciferase activity. Consistently, upon TNF α (10 ng/mL) or IL-6 (20 ng/mL) stimulation, the above mutations also blocked luciferase activity induced by these cytokines (Figure 1g). Together, these data suggest that *miR-31* expression is induced in keratinocytes after wounding by NF- κ B and STAT3 signaling pathways.

Conditional deletion of *miR-31* delays skin wound healing.

To investigate the function of *miR-31* in wound healing *in vivo*, we generated *K14-Cre;miR-31^{fl/fl}* (cKO) mice, in which *miR-31* is specifically deleted in skin epithelia. The specificity and efficiency of *miR-31* deletion in cKO epidermis was confirmed by qRT-PCR analysis, while no change in *miR-31* levels was detected in cKO duodenum and liver (Figure 2a). *Mir-31* cKO mice were fertile without apparent gross phenotypes. Skin histology in day P49 *miR-31* cKO mice was comparable to that in littermate controls. There were no

significant differences in epidermal thickness between cKO and control mice (Figure S2a). We also tested whether loss of *miR-31* affects differentiation, apoptosis and cell proliferation. Immunofluorescence for K14, K10 and Loricrin showed that epidermal differentiation is not altered in P49 cKO epidermis (Figure S2b and S2c). Cleaved Caspase3 staining revealed no apoptotic cells at P49 both in cKO and control epidermis (Figure S2d). Immunofluorescence for PCNA and Ki67 showed that *miR-31* deletion does not significantly affect proliferation dynamics in the basal layer of the epidermis (Figure S2e, S2f and S2g). Collectively, these findings suggest that *miR-31* is not essential for key physiological functions of epidermis under normal conditions.

Considering prominent upregulation of *miR-31* after wounding, we studied its role during skin wound healing. When animals were challenged with large, 1x1.5 cm² full-thickness wounds, healing was delayed in *miR-31* cKO mice as compared to control on PWD 20, evidenced by the retention of scab in mutants (n = 5 biological replicates for both control and cKO mice) (Figure 2b). We then compared healing dynamics of smaller, 6mm round wounds over time between cKO and control mice (n = 6 biological replicates for both control and cKO mice). Area measurements show that wound closure process is moderately, yet significantly delayed in cKO as compared to control mice (Figure 2c). On histology at PWD 6, epidermal edge was shorter and thinner in cKO mice (n = 6 biological replicates for both control and cKO mice) (Figure 2d), suggesting impaired keratinocyte proliferation and/or migration. In agreement, analysis of tail skin wounds, which have minimal contraction (Falanga et al., 2004), also revealed delayed wound closure in cKO mice (n = 3 biological replicates for both control and cKO mice) (Figure S3a and S3b). Keratins K6 and K17 are

normally induced in response to injury and are highly expressed in migrating keratinocytes near the wound edge (McGowan and Coulombe, 1998, Wevers et al., 1990). Consistently, we observed fewer K6- and K17- positive cells in cKO epidermis around the wound (Figure 2e, 2f and 2g). To understand the cellular basis of delayed wound healing in cKO mice, we examined proliferation by the wound edge keratinocytes on PWD 6. Following 90 min BrdU pulse, much fewer BrdU-positive keratinocytes were found in the cKO mice. Consistently, the numbers of Ki67-positive proliferating keratinocytes were also decreased in cKO mice (Figure 3a and S3c). Furthermore, WST-8 assays showed that *miR-31* promotes proliferation of HaCaT keratinocytes *in vitro* (Figure 3b). These findings demonstrate that loss of *miR-31* represses proliferation of keratinocytes around the wound edge.

Next, we examined whether altered levels of *miR-31* influences keratinocyte migrations. The *in vitro* scratch assay revealed that *miR-31* inhibitor indeed significantly repress migration of HaCaT keratinocytes, whereas *miR-31* mimics significantly promoted it (Figure 3c). High transfection efficiency for *miR-31* mimics and inhibitor was confirmed by qRT-PCR analysis (Figure 3d). F-actin polymerizes to form a contractile cable at the cell's leading edge, and drives epithelial sheet movement in a purse string manner (Martin and Parkhurst, 2004, Shimizu et al., 2005). In agreement with its role in promoting cell migration, phalloidin staining showed that *miR-31* mimics upregulate F-actin fiber formation and, conversely, *miR-31* inhibitor decreases it (Figure 3e). Taken together, these findings indicate that *miR-31* accelerates the process of wound healing by promoting both the proliferation and migration of keratinocytes.

MiR-31 activates the RAS/MAPK (ERK1/2) pathway.

To gain a mechanistic insight into the molecular events underlying the delayed wound healing in cKO mice, we examined the effect of *miR-31* alterations on the status of RAS/MAPK signaling pathway, previously shown to be essential for promoting cell proliferation and migration (Falanga et al., 2004, Lin et al., 2016). We examined phosphorylation status of ERK at the wound edge at PWD 6, and found p-ERK to be prominently downregulated in *miR-31* cKO mice (Figure 4a). Cyclin D1 and c-Myc are the two downstream components of the RAS/MAPK (ERK1/2) pathway (Lavoie et al., 1996, Li et al., 2014, Luo et al., 2012). We observed that the expression levels for both Cyclin D1 and c-Myc were decreased at the edges of PWD 6 cKO wounds (Figure 4b and 4c). Slug, another downstream component of the RAS/MAPK pathway, which triggers epithelial-mesenchymal transition of keratinocytes (Aomatsu et al., 2012, Hwang et al., 2017, Kusewitt et al., 2009, Savagner, 2001), was also downregulated in *miR-31* cKO wounds at PWD 6 (Figure 4d). Downregulation of *Cyclin D1* (*Ccnd1*), *c-Myc* and *Slug* at the RNA level was further confirmed by qRT-PCR (Figure 4e). Western blot assay showed decreased phosphorylation of ERK, p-MEK, Cyclin D1, c-Myc and Slug in cKO mice (Figure 4f), supporting the notion that *miR-31* upregulates RAS/MAPK pathway activity. To learn if *miR-31* can activate RAS/MAPK pathway *in vitro*, we analyzed the effects of *miR-31* alterations on RAS/MAPK signaling status in HaCaT cells. *MiR-31* mimics increased the number of cells with nuclear p-ERK expression, while *miR-31* inhibitor had an opposite effect (Figure 4g). Consistently, Western blot assay also showed that *miR-31* mimics increased p-ERK protein level both with and without EGF addition, while *miR-31* inhibitor decreased it (Figure 4h). Furthermore,

protein levels of p-MEK, c-Myc, Cyclin D1 and Slug also increased after *miR-31* mimics treatment, and reduced in response to *miR-31* inhibitor (Figure 4i). U0126 is extensively used as the inhibitor of RAS/MAPK signaling (Marampon et al., 2011, Tong et al., 2003). To further test whether the effect of *miR-31* on keratinocyte proliferation and migration is through the regulation of RAS/MAPK signaling pathway, we treated HaCaT cells *in vitro* under conditions of scrambled RNA (negative control, NC), *miR-31* mimics and *miR-31* mimics with U0126. We found that U0126 repressed cell migration induced by *miR-31* mimics (Figure S4a), concomitant with the repression of RAS/MAPK pathway (Figure S4b). In addition, WST-8 assay showed that U0126 at least partially attenuates increased cell proliferation caused by *miR-31* mimics (Figure S4c). Collectively, our results demonstrate that *miR-31* promotes wound healing by activating the RAS/MAPK signaling pathway.

***miR-31* directly targets negative regulators of RAS/MAPK signaling pathway.**

MicroRNAs exert their functions by negatively regulating target genes. To identify *miR-31* targets that may be responsible for its observed functional effects, we analyzed *miR-31*-binding sites in 3'UTRs of transcripts encoding negative regulators of RAS/MAPK pathway. Putative *miR-31*-binding sites were found in several genes including *Rasa1*, *Spred1* and *Spry4* (Figure S5a). We also aligned and analyzed the 3'UTR sequences of other family members of negative regulators of RAS/MAPK signaling, and found that 3'UTR of *Spred2* also has a potential *miR-31*-binding region. To validate whether the above genes are actual targets of *miR-31*, first we examined their expression levels in wounds of *miR-31* cKO mice. We found that *Rasa1*, *Spred1*, *Spred2* and *Spry4* are upregulated in cKO wounds both

at the protein and RNA levels (Figure 5a and 5b). Further, we analyzed their expression levels in the HaCaT cells transfected with either *miR-31* mimics or inhibitor. *RASA1*, *SPRED1*, *SPRED2* and *SPRY4* were downregulated in *miR-31* mimics treated cells both at the protein and RNA levels (Figure 5c and 5d). Conversely, they were increased in response to *miR-31* inhibitor (Figure 5c and 5e). To further test whether they are direct targets of *miR-31*, we constructed luciferase reporters for the *Rasa1*, *Spred1*, *Spred2* and *Spry4* 3'UTRs, as well as reporter constructs in which the predicted *miR-31* binding sites were mutated (Figure S5b). The luciferase reporter assay showed that *miR-31* mimics significantly repressed luciferase activity of constructs containing *Rasa1*, *Spred1*, *Spred2* and *Spry4* 3'UTR elements, while *miR-31* inhibitor significantly up-regulated it.. Mutations in 3'UTRs abolished *miR-31* binding and the repressive effect of *miR-31* mimics, or the potentiating effect of *miR-31* inhibitor (Figure 5f and 5g). Together, these results identify *Rasa1*, *Spred1*, *Spred2* and *Spry4* as the direct molecular targets of *miR-31*. In addition, we also found that , *Emp-1*, a previously reported target gene of *miR-31* (Li et al., 2015) was significantly up-regulated in *miR-31* cKO wounds (Figure S6). This suggests that *miR-31* likely promotes wound healing via several pathways.

Knockdown of *RASA1*, *SPRED1*, *SPRED2* and *SPRY4* phenocopies the effect of *miR-31* overexpression.

Next, we asked whether *RASA1*, *SPRED1*, *SPRED2* and *SPRY4* functionally mediate the observed biological function of *miR-31*. First, we generated siRNAs of *RASA1*, *SPRED1*, *SPRED2* and *SPRY4*. These siRNAs efficiently repressed their gene expression both at the

protein and RNA levels (Figure S7a and S7b), and upregulated p-ERK level (Figure S7b), suggesting activation of RAS/MAPK pathway. *In vitro* scratch and WST-8 assays showed that inhibition of *RASA1*, *SPRED1*, *SPRED2* and *SPRY4* with siRNAs significantly promotes cell migration and proliferation (Figure 5h, S7c and S7d).

We also tested whether silencing of these targets is able to rescue the *miR-31* loss phenotypes. Indeed, siRNA-mediated silencing of any one of these targets was sufficient to rescue reduced cell migration and proliferation caused by *miR-31* inhibitor (Figure 6a-6c). Thus, our results reveal that *RASA1*, *SPRED1*, *SPRED2* and *SPRY4* are functional targets of *miR-31*, mediating its wound healing promoting effect. In summary, our findings demonstrate that inflammation-induced *miR-31* drives keratinocyte proliferation and migration via RAS/MAPK signaling pathway, acting to promote wound re-epithelialization.

DISCUSSION

In this study, we utilized a *miR-31* loss-of-function mouse model to demonstrate that inflammatory signals-responsive *miR-31* promotes keratinocyte proliferation and migration during wound healing by directly regulating RAS/MAPK signaling pathway. To our knowledge, this study is the first to demonstrate the *in vivo*, physiological role of *miR-31* in regulating wound healing using loss-of-function mouse model. Previously, Li et al. showed that *miR-31* becomes upregulated in wound edge keratinocytes in human volunteers (Li et al., 2015). Using *in vitro* assays, Li et al. also showed that *miR-31* promotes primary human keratinocyte proliferation and migration, and that *miR-31* directly targets epithelial membrane protein 1 (EMP-1). Our mouse findings corroborate the expression pattern and the effect of *miR-31* on wound edge keratinocytes, provide crucial *in vivo* functional data from the *miR-31* cKO animal model, and also identify several functionally important *miR-31* targets. Together with Li et al.'s findings, they demonstrate that *miR-31* is able to promote wound healing via several pathways, significantly strengthening the evidence for the importance of *miR-31* in wound healing.

Wound healing comprises four distinct but overlapping phases: hemostasis, inflammation, proliferation and remodeling (Gurtner et al., 2008). After skin wounding, epidermal keratinocytes produce inflammatory cytokines to activate immune response during the inflammatory phase (Strbo et al., 2014, Wilson et al., 2001). Our results also suggest that *miR-31* starts to up-regulated at the leading migrating keratinocytes of wound edge in the inflammatory phase. Our molecular data shows that *miR-31* is upregulated by both NF- κ B and STAT3 pathways. Our findings of a tight association between *miR-31* induction and

NF- κ B and STAT3 pathway activation during wound healing are important, as both of these pathways are activated by the inflammatory signals (Venkatesan et al., 2017, Yang et al., 2017). They suggest that *miR-31* is an important mediator of inflammatory signals in promoting epithelial regeneration during wound healing. In our recent work on the gut, we showed that *miR-31* was upregulated by STAT3 signaling activity upon radiation-induced intestinal injury and that it promoted intestinal epithelium regeneration (Tian et al., 2017). In the mammary gland, we showed that *miR-31* mediates progesterone signaling through the NF- κ B signaling pathway (Lv et al., 2017). Thus, *miR-31* emerged as the stress-responsive microRNA downstream of NF- κ B and STAT3 inflammatory signals. It plays essential role in epithelial remodeling in the context of diverse tissue injuries. In the skin, it has been reported that *miR-31* can be induced by the NF- κ B signaling activity and that it promotes epidermal hyperplasia in psoriasis (Yan et al., 2015). In contrast, *miR-31* could modulate inflammatory signaling by targeting STK40 in psoriasis (Xu et al., 2013). Thus, complex feedback regulatory network between inflammatory signals and *miR-31* likely operates in psoriasis.

Inflammatory phase is followed by the proliferative phase, when keratinocytes proliferate and migrate to execute wound re-epithelialization (Takeo et al., 2015). Here we demonstrate that *miR-31* promotes keratinocyte proliferation and migration by activating RAS/MAPK signaling pathway. Recently, this pathway has been shown to play a crucial role during skin wound healing (Li et al., 2017). In addition, RAS/MAPK activation by *miR-31* has been found in the lung, colorectal and pancreatic cancer cells (Edmonds, 2016, Kent et al., 2016, Sun et al., 2013). It appears that *miR-31* mediates epithelial regeneration and tumorigenesis in different tissues via common molecular mechanism.

In summary, our findings demonstrate that *miR-31* is induced by inflammatory signals and that it promotes keratinocyte proliferation and migration during skin wound healing. We identified *miR-31* as an important mediator of transition from inflammation to re-epithelialization phase (Figure S8), highlighting essential role of *miR-31* in wound healing *in vivo*.

MATERIALS AND METHODS

Additional details are available in the Supplementary Materials.

Mouse tissue collection.

Prior to wounding, 7- to 8-week-old mice were shaved. Biopsy punches were used to inflict 6mm and 2mm full-thickness wounds in the dorsal and tail skin respectively. 1x1.5 cm² full-thickness large wounds were created on dorsal skin for observing the retention of scab on PWD 20. Mice were sacrificed and tissue biopsies at the wound site and intact skin were collected for analysis 6 days after wounding. All mice were kept under specific pathogen-free (SPF) conditions in accordance with the guidelines of the Institutional Animal Care and Use Committee of China Agricultural University (approval number: SKLAB-2011-04-03).

miR-31 in situ hybridizations.

miR-31 in situ hybridization was performed using previously described protocol (Jorgensen et al., 2010, Yuan et al., 2015). Double DIG-labeled *miR-31* and scrambled LNA probes (Exiqon) were hybridized at 61°C. *In situ* signal was detected by staining with Anti-Digoxigenin-AP antibody (Roche) and developed using BM purple substrate (Roche).

Luciferase assay for miR-31 promoter activity.

For promoter reporter assay, pGL3-basic vector (Promega) was used to clone the promoter of *miR-31* as previously described (Tian et al., 2017). HaCaT cells were seeded in a 24-well plate at the density of 5×10⁴ cells per well one day before transfection and then each well was transfected with a mixture of 500 ng pGL3 luciferase vector and 50 ng pRL-TK

renilla vector using Lipofectamine 2000 Transfection Reagent (Invitrogen). 24 hours post transfection, cells were treated with inflammatory cytokines for another 24 hours before being lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The ratio of firefly luciferase to renilla luciferase was calculated for each well.

Statistical analysis.

The data were analyzed in triplicate or greater and the means obtained were used for student's *t*-tests. Asterisks denote statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). All data are reported as mean $\pm$ SD. Means and standard errors were from at least three independent biological replicates experiments.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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FIGURE LEGENDS:

Figure 1. *MiR-31* is induced by the NF- κ B and STAT3 signaling. (a) qRT-PCR for *miR-31* in the wound edge tissue at indicated time points. (b) *In situ* hybridization for *miR-31* in skin wounds. Scale bar: 50 μ m. (c-e) qRT-PCR analysis for *Ifn γ* , *Tnfa*, *Il6*, *Il1b* and *Cxcl1* (c); Western blot for p65, p-p65, STAT3 and p-STAT3 (d); immunostaining for p65 and p-STAT3 (e) in skin wounds at PWD 2. (f) qRT-PCR for *miR-31* in keratinocytes upon treatments of siCtrl, siCtrl+IL6, siCtrl+TNF α , sip65, siStat3, TNF α +sip65, TNF α +siStat3, IL6+siStat3 and IL6+sip65 for 24 hours. (g) Luciferase reporter assay for *miR-31* promoter in HaCaT cells at indicated conditions. *p<0.05; **p <0.01; ***p<0.001. n $\geq$ 3 biological replicates for **a**, and **c-h**.

Figure 2. *MiR-31* conditional deletion delays skin wound healing. (a) qRT-PCR for *miR-31* in epidermis, duodenum and liver of *miR-31* cKO and control. (b) Representative images of 1x1.5 cm² skin wounds on *miR-31* cKO and control mice on PWD 0 and 20. (c) Representative images and quantification of wound sizes in cKO and control mice on PWD 0, 2, 4, 6 and 8 after 6mm injury. (d) Schematics showing wound tissue sample collection. Histology and quantification of newly formed epidermis in *miR-31* cKO and control wounds on PWD 6. Scale bar: 50 μ m. (e,f,g) Immunostaining for keratins K17 (e) and K6 (f) and their quantification (g) in *miR-31* cKO and control wounds on PWD 6. *p<0.05; **p <0.01; n $\geq$ 3 biological replicates for **a-g**.

Figure 3. *MiR-31* promotes proliferation and migration of keratinocytes. (a) Immunostaining for BrdU in the wound edge keratinocytes in *miR-31* cKO and control wounds on PWD 6 following 90 min BrdU pulse. Scale bar: 25 μ m. (b) WST-8 assay showing human HaCaT keratinocyte growth changes in response to *miR-31* mimics (blue) and inhibitor (red). (c) *In vitro* scratch assay showing changes in migration potential of HaCaT cells in response to *miR-31* mimics and inhibitor. (d) qRT-PCR for *miR-31* to test the transfection efficiency for *miR-31* mimics and inhibitor. ** $p < 0.01$. (e) Phalloidin staining (green) in HaCaT keratinocytes transfected with *miR-31* mimics (left) and inhibitor (right). Scale bar: 25 μ m. $n = 3$ biological replicates for **a-e**.

Figure 4. *MiR-31* activates RAS/MAPK signaling pathway. (a-d) Immunostaining for p-ERK, Cyclin D1, c-Myc and Slug in cKO wound edges on PWD 6. Scale bar: 50 μ m. (e,f) qRT-PCR for *Ccdn1*, *c-Myc* and *Slug*, and Western blot for p-MEK, MEK, p-ERK1/2, ERK1, Cyclin D1, c-Myc and Slug in cKO wounds on PWD 6. (g) Immunostaining for p-ERK in HaCaT cells and quantification of p-ERK⁺ cells treated with *miR-31* mimics or inhibitor. Scale bar: 25 μ m. (h) Western blot for phosphorylated and total ERK in HaCaT cells transfected with *miR-31* mimics or inhibitor. (i) Western blot for MEK, p-MEK, c-Myc, Cyclin D1 and Slug in HaCaT cells upon *miR-31* mimics or inhibitor treatment. $n = 3$ biological replicates for **a-i**. * $p < 0.05$; ** $p < 0.01$.

Figure 5. Identification of *miR-31* direct targets. (a,b) Western blot (a) and qRT-PCR (b) for *Rasa1*, *Spred1*, *Spred2* and *Spry4* in *miR-31* cKO and control wounds on PWD 6. (c-e)

Western blot for RASA1, SPRED1, SPRED2 and SPRY4 (c), and qRT-PCR for *RASA1*, *SPRED1*, *SPRED2* and *SPRY4* (d,e) in HaCaT cells transfected with *miR-31* mimics or inhibitor. (f,g) Luciferase reporter activity of WT and mutant 3'UTR constructs of *Rasa1*, *Spred1*, *Spred2* and *Spry4* in HaCaT cells transfected with *miR-31* mimics (f) and *miR-31* inhibitor (g). (h) Statistical analysis on wound closure in *in vitro* scratch assay shown in supplementary Figure S6c. n = 3 biological replicates for a-h. *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 6. Repression of *RASA1*, *SPRED1*, *SPRED2* and *SPRY4* can rescue the effects of *miR-31* knockdown in keratinocytes. (a) *In vitro* scratch assays assessing the migration rate of HaCaT cells transfected with *miR-31* inhibitor, inhibitor control and/or siRNAs for *RASA1*, *SPRED1*, *SPRED2* or *SPRY4* for 24 hours. Representative images are shown at indicated time points. (b) Statistical analysis of scratch assay wound closure from panel (a). (c) WST-8 assays on HaCaT cells transfected with *miR-31* inhibitor, inhibitor control and/or siRNA for *RASA1*, *SPRED1*, *SPRED2* or *SPRY4*. n = 3 biological replicates for a-c. ** p < 0.01 and * p < 0.05.

Figure 1

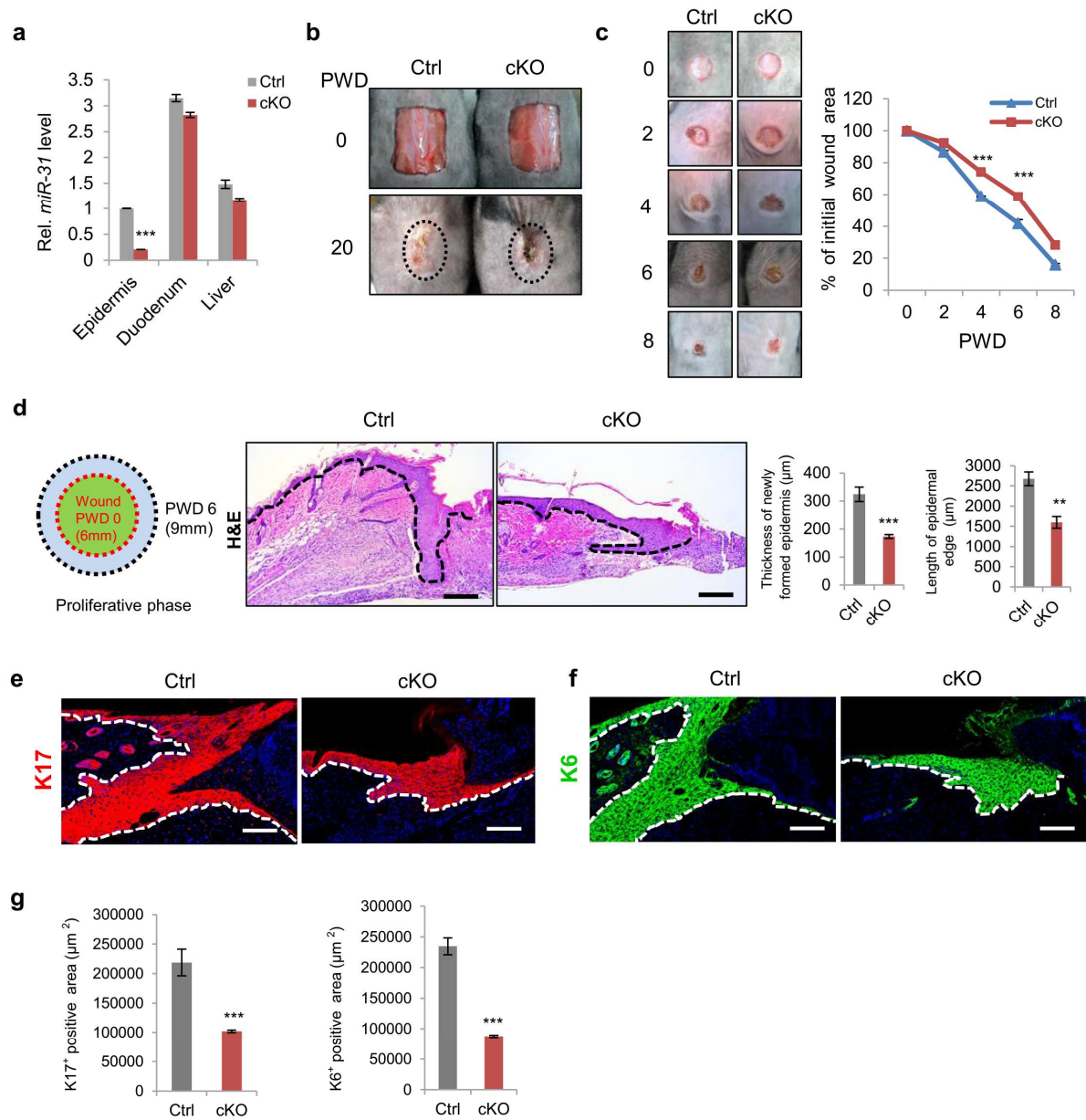


Figure 2

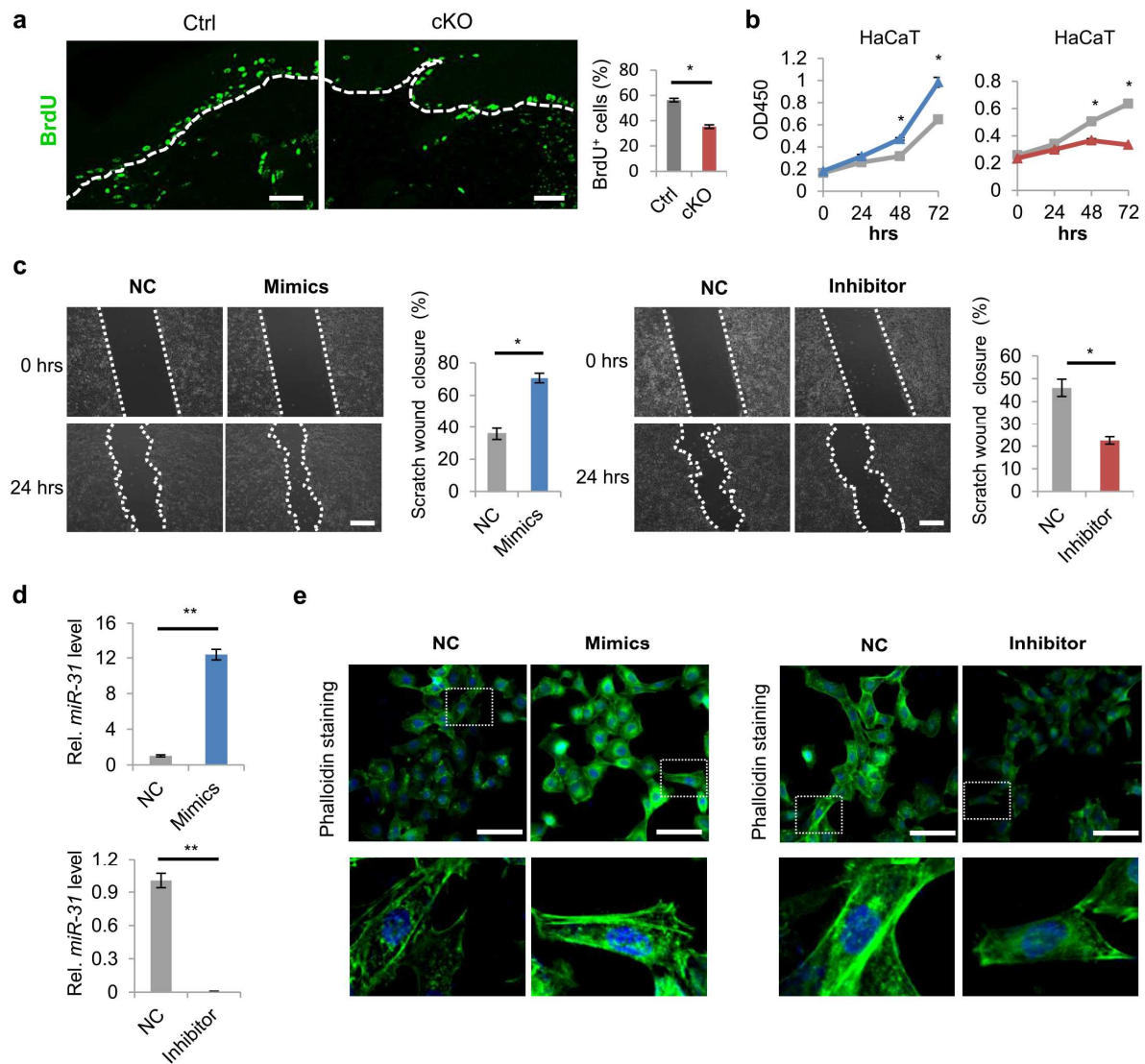


Figure 3

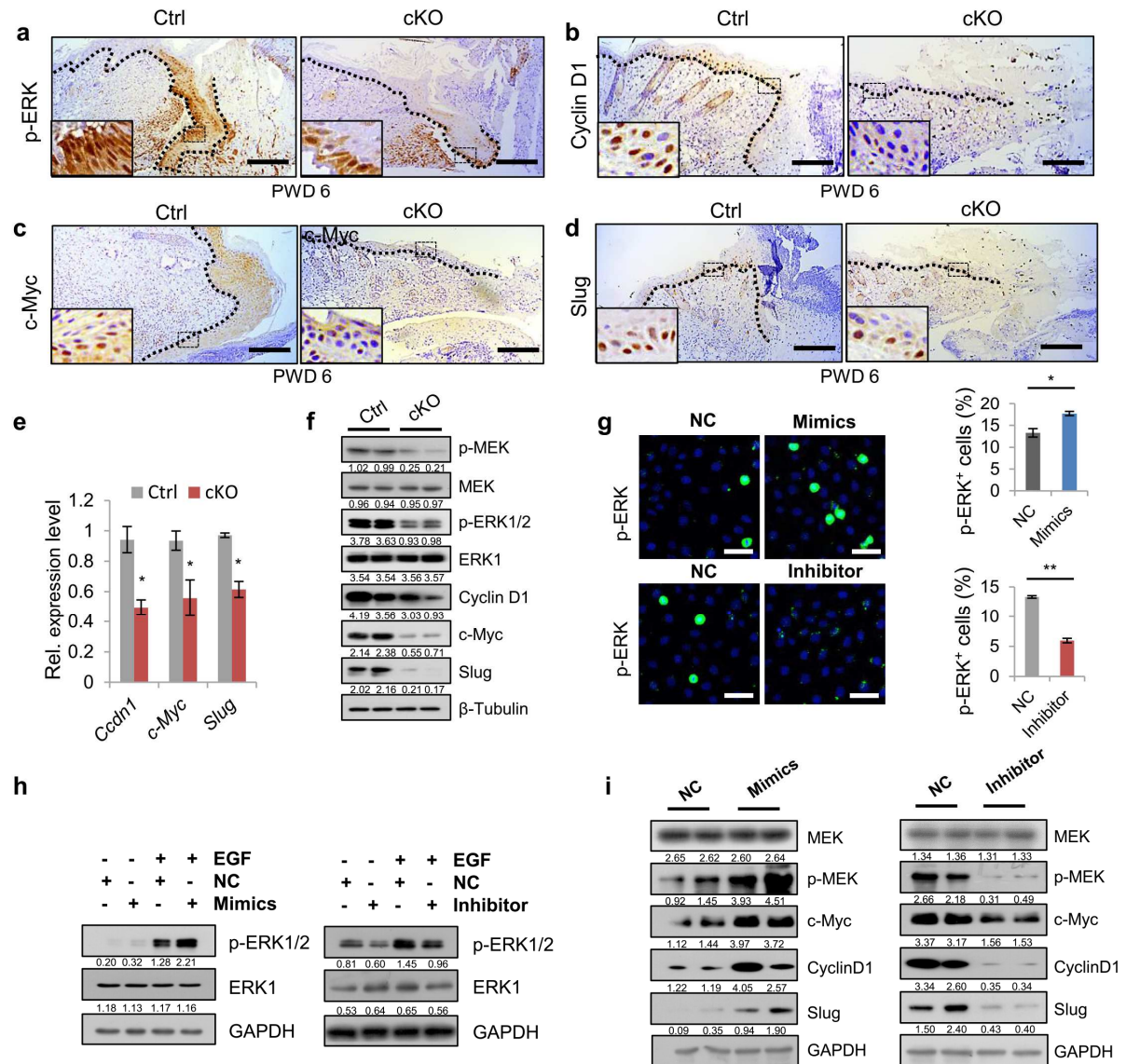


Figure 4

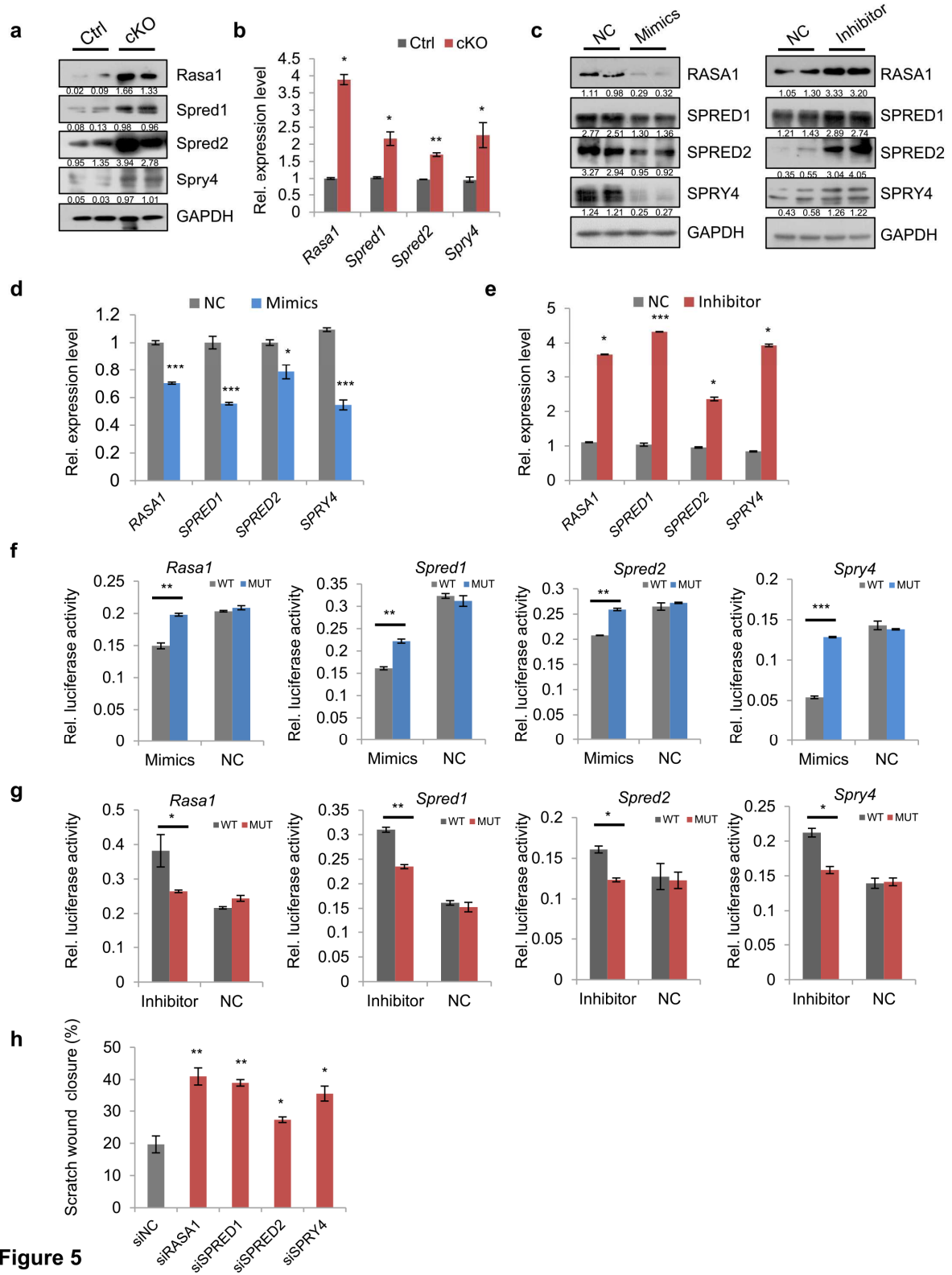


Figure 5

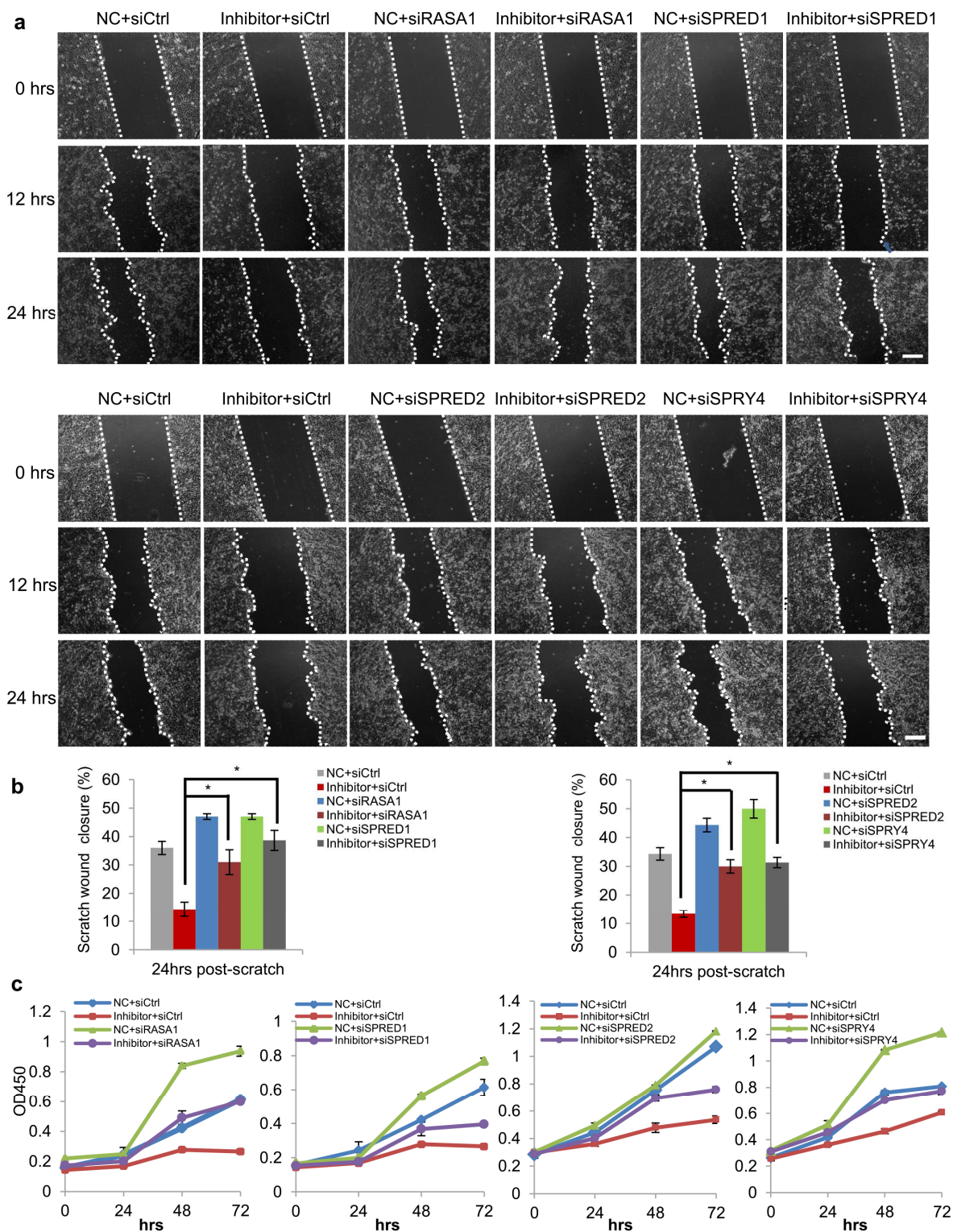


Figure 6