miR-19a/b and miR-20a Promote Wound Healing by Regulating the Inflammatory Response of Keratinocytes

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Persistent and impaired inflammation impedes tissue healing and is a characteristic of chronic wounds. A better understanding of the mechanisms controlling wound inflammation is needed. In this study, we show that in human wound-edge keratinocytes, the expressions of microRNA (miR)-17, miR-18a, miR-19a, miR-19b, and miR-20a, which all belong to the miR-17∼92 cluster, are upregulated during wound repair. However, their levels are lower in chronic ulcers than in acute wounds at the proliferative phase. Conditional knockout of miR-17∼92 in keratinocytes as well as injection of miR-19a/b and miR-20a antisense inhibitors into wound edges enhanced inflammation and delayed wound closure in mice. In contrast, conditional overexpression of the miR-17∼92 cluster or miR-19b alone in mice keratinocytes accelerated wound closure in vivo. Mechanistically, miR-19a/b and miR-20a decreased TLR3-mediated NF-κB activation by targeting SHCBP1 and SEMA7A, respectively, reducing the production of inflammatory chemokines and cytokines by keratinocytes. Thus, miR-19a/b and miR-20a being crucial regulators of wound inflammation, the lack thereof may contribute to sustained inflammation and impaired healing in chronic wounds. In line with this, we show that a combinatory treatment with miR-19b and miR-20a improved wound healing in a mouse model of type 2 diabetes.

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Abbreviations: cKI, conditional knockin; cKO, conditional knockout; DFU, diabetic foot ulcer; KC, keratinocyte; miR, microRNA; NW7, day-7 wound; poly(I:C), polyinosinic:polycytidylic acid; PU, pressure ulcer; TLR, toll-like receptor; VU, venous ulcer; WT, wild type

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INTRODUCTION

Wound healing is a dynamic, sequential process comprising inflammation, proliferation, and remodeling phases. Among various cellular types participating in wound repair, epidermal keratinocytes (KCs) not only perform re-epithelialization but also play a central role in innate immune response (Strbo et al., 2014). Upon skin injury, the damaged tissue releases RNAs to trigger toll-like receptor (TLR) 3 activation on neighboring KCs, which respond by producing proinflammatory cytokines (e.g., TNF-α, IL-1) and chemokines (e.g., CXCL5 and CXCL8) (Grimstad et al., 2013; Kim et al., 2019; Kinoshita et al., 2009; Lai et al., 2009; Nelson et al., 2015; Yang et al., 2013).

Neutrophils are among the first circulating immune cells recruited by these chemokines to the injured site, where they play a significant role in microbial clearance (Wilgus et al., 2013). However, in chronic nonhealing wounds, the inflammation becomes persistent, which causes tissue damage but cannot efficiently combat infection (MacLeod and Mansbridge, 2016; Ramirez et al., 2018). Therefore, it is important to understand the molecular mechanisms that control the inflammatory response during wound healing, which may provide new insights into chronic wound treatment.

MicroRNAs (miRs) are short (∼22 nucleotides) noncoding RNAs that may regulate about 90% of the protein-coding genes in humans (Miranda et al., 2006). Current research has revealed that miRs function as critical regulators of

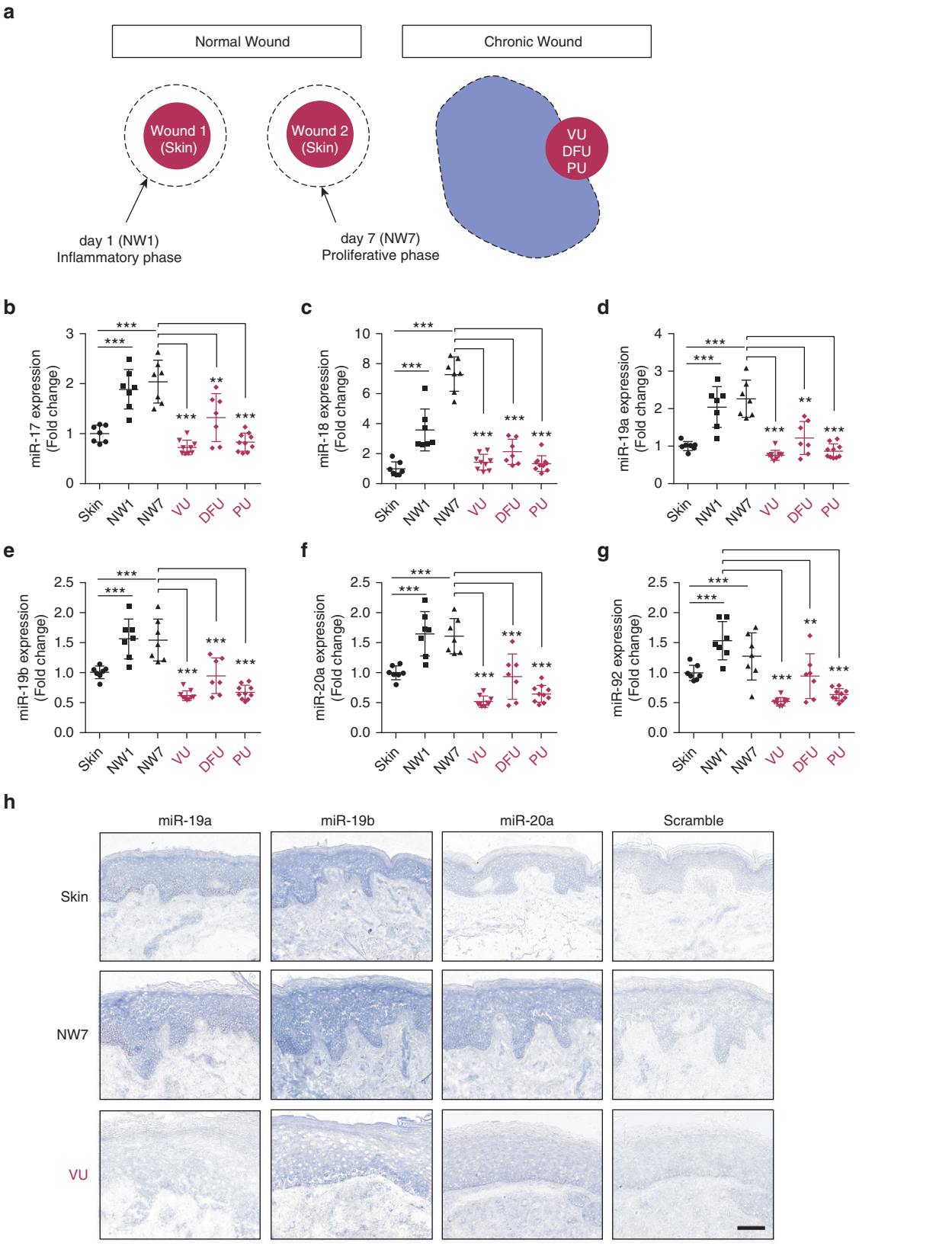

Figure 1. miR-17∼92 expression in human skin wounds. (**a**) Two 4-mm wounds were created, and the wound-edge tissues were excised 1 (NW1) and 7 days (NW7) later. Biopsies were taken from the nonhealing edges of VUs, DFUs, and PUs. (**b**–**g**) QRT-PCR of miR-17∼92 cluster in the skin, NW1, and NW7 from seven healthy donors as well as in VUs (n = 10), DFUs (n = 7), and PUs (n = 10). (**h**) In situ hybridization of miR-19a/b and miR-20a in the skin (n = 3), NW7 (n = 3), and VU (n = 2). Bar = 100 μm. The data are presented as mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$; one-way ANOVA multiple comparisons test. DFU, diabetic foot ulcer; miR, microRNA; NW1, day-1 wound; NW7, day-7 wound; PU, pressure ulcer; QRT-PCR, quantitative real-time reverse transcriptase–PCR; VU, venous ulcer.

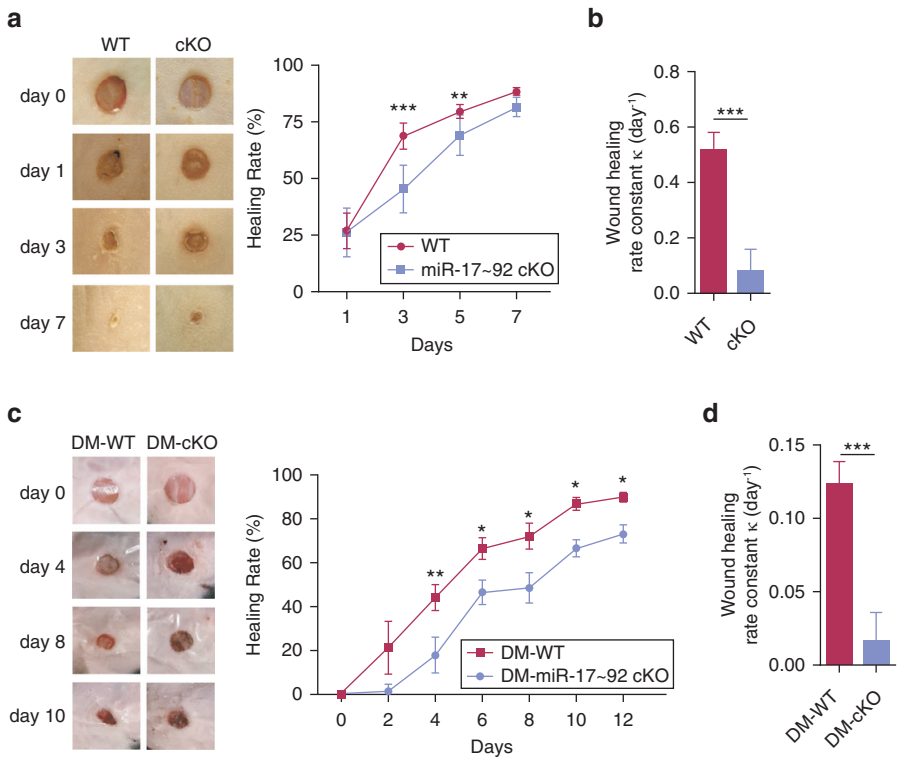

Figure 2. Lack of miR-17∼92 impairs wound healing in vivo. (**a**) Wounds of miR-17 to -92 cKO mice (n = 10) and WT mice (n = 10) on days 0–7 after wounding. Wound closure was quantified and presented as a healing rate = 100% – the percentage of the initial wound size. (**b**) Wound healing rate constant κ (day^{-1}) was calculated using a one-phase decay model. (**c**) Wounds on days 0–10 after injury in WT (n = 8–10) and miR-17∼92 cKO (n = 8–10) mice under diabetic conditions, and wound closure was quantified as in **a**. (**d**) Wound healing rate constant was calculated as in **b**. The data are presented as (**a**, **c**) mean ± SEM or (**b**, **d**) mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; (**a**, **c**) Bonferroni post-hoc test or (**b**, **d**) Student's *t*-test. cKO, conditional knockout; DM, diabetes mellitus; miR, microRNA; WT, wild-type.

numerous processes associated with cellular physiology and pathology, hence making them promising as both therapeutic and diagnostic targets. MiR-17∼92 is a highly conserved, polycistronic miR cluster comprising six mature miRs, which belong to three miR families, that is, the miR-17 family (miR-17, miR-18a, and miR-20a), the miR-19 family (miR-19a and miR-19b), and miR-92a. MiR-17∼92 plays critical roles in normal development, aging, and various diseases, such as cancer, immune disorders, and cardiovascular and neurodegenerative diseases (Mogilyansky and Rigoutsos, 2013). Recently, we identified miR-19a as one of the upregulated miRs during wound healing of human skin (Li et al., 2015b). Aberrant miR-20a expression has also been reported in venous ulcer (VU) previously (Pastar et al., 2012). Moreover, miR-92a has been found to negatively regulate angiogenesis (Gallant-Behm et al., 2018; Lucas et al., 2017), and miR-92a inhibitor is currently under clinical investigation as a new wound treatment in part through enhancing angiogenesis (ClinicalTrials.gov NCT03603431). In light of its ever-increasing importance in a wide variety of biological processes, we decided to investigate the role of miR-17∼92 in skin wound healing. Our study identified miR-19a/b and miR-20a as crucial regulators restricting the inflammatory response of epidermal KCs during wound repair. Their deficiency may contribute to sustained inflammation and impaired healing in chronic wounds.

RESULTS

Characterization of miR-17∼92 expression in human skin wounds

To study miR-17∼92 expression in human skin wounds in vivo, we created wounds in the skin of 18 healthy volunteers (Supplementary Table S1) and collected the wound edges at 1 and 7 days later, whose time points were selected to represent the inflammatory and proliferative phases of wound healing (Figure 1a). In addition, we collected nonhealing wound edges from 10 patients with VU, seven patients with diabetic foot ulcer (DFU), and 10 patients with pressure ulcer (PU), which are the three most common types of chronic wounds (Supplementary Figure S1a and Supplementary Table S1). Quantitative real-time reverse transcriptase–PCR results showed that the expression of miR-17∼92 cluster members was upregulated in acute wounds at the inflammatory phase (day-1 wound [NW1]) or the proliferative phase (day-7 wound [NW7]) compared with the skin from the same healthy donors (Figure 1b–g). Interestingly, compared with the acute wounds under healing (NW1 or NW7), the expression of miR-17∼92 cluster was significantly lower in VUs, DFUs, and PUs (Figure 1b–g). Using laser capture microdissection, we isolated the skin and wound-edge epidermis (Supplementary Figure S1b and Supplementary Table S1) and found that the levels of miR-19a, miR-19b, and miR-20a were significantly lower in the epidermis of VUs than in the normal wounds (Supplementary Figure S1c–h). This was further confirmed by in situ hybridizations, showing that in the wound-edge epidermal KCs, the expression of miR-19a/b and miR-20a was elevated during healing, whereas in the wound-edge epidermis of VU, their levels were overtly lower than those in the normal wounds (Figure 1h). Thus, we focused on epidermal KCs to study the role of miR-19a/b and miR-20a in wound healing.

Lack of miR-17∼92 in KCs impairs wound healing in vivo

To determine the impact of abnormal miR-17∼92 expression in KCs on wound healing in vivo, we examined the healing

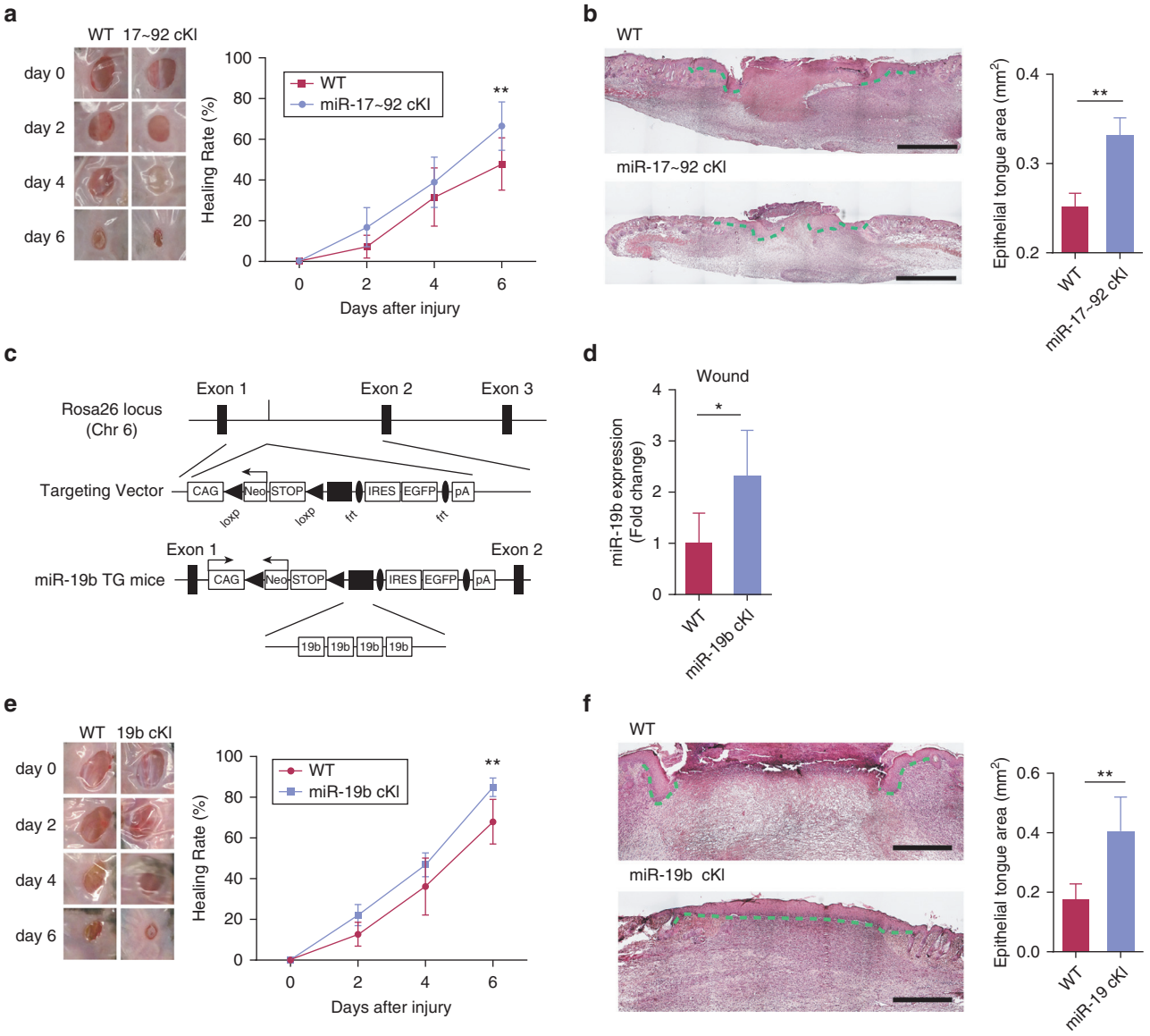

Figure 3. Improved wound healing in miR-17∼92 or miR-19b cKI mice. (**a**) Days 0–6 wounds of miR-17∼92 cKI mice (n = 7) and littermate controls (WT, n = 4) under diabetic condition. Wound closure was quantified as a healing rate. (**b**) Day-6 wounds of miR-17∼92 cKI and WT mice. Dashed lines mark the newly formed epithelial tongues, whose area was quantified (n = 3 per group). (**c**) The strategy of generating miR-19b cKI mice. (**d**) QRT-PCR of miR-19b in the wound-edge epidermis of WT (n = 4) and miR-19b cKI mice (n = 4) at 2 days after injury. (**e**) Days 0–6 wounds of miR-19b cKI (n = 7) and WT mice (n = 4). (**f**) Day-6 wounds of miR-19b cKI and WT mice. The epithelial tongues area was quantified (n = 3 per group). The data are presented as (**a, e**) mean ± SEM or (**b**, **d**, **f**) mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$; (**a**, **e**) Bonferroni post-hoc test or (**b**, **d**, **f**) Student's *t*-test. Chr, chromosome; cKI, conditional knockin; miR, microRNA; QRT-PCR, quantitative real-time reverse transcriptase–PCR; WT, wild type.

capacity of mice with KC-specific miR-17∼92 conditional knockout (cKO) (Wu et al., 2017). We found that wound closure was significantly delayed in the miR-17∼92 cKO mice compared with that in the littermate controls (wild type [WT]) (Figure 2a and b). We also induced diabetes in miR-17∼92 cKO and WT mice with multiple injections of low-dose streptozocin. In the hyperglycemic state, the lack of miR-17∼92 in KCs led to a more profound inhibition of wound healing than in the nondiabetic condition (Figure 2c and d).

To complement the data obtained from the mouse model with constitutive miR-17∼92 deletion, where compensatory events may occur, and also to imitate human chronic wounds in which miR-17∼92 expression was low but not absent, we established a mouse model with transient inhibition of miR expression. For this, we injected a mixture of miR-19a/b and miR-20a inhibitors intradermally into the wound edges of C57BL6 mice immediately after a skin injury, which reduced the levels of these miRs to a similar extent as the difference between human chronic and normal wounds (Supplementary Figure S2a–d). Importantly, inhibition of miR-19a/b and miR-20a significantly delayed wound closure (Supplementary Figure S2e). Together, we show that both miR-17∼92 cKO mice and transient inhibition of miR-19a/b and miR-20a in wounds of WT mice exhibited delayed wound closure.

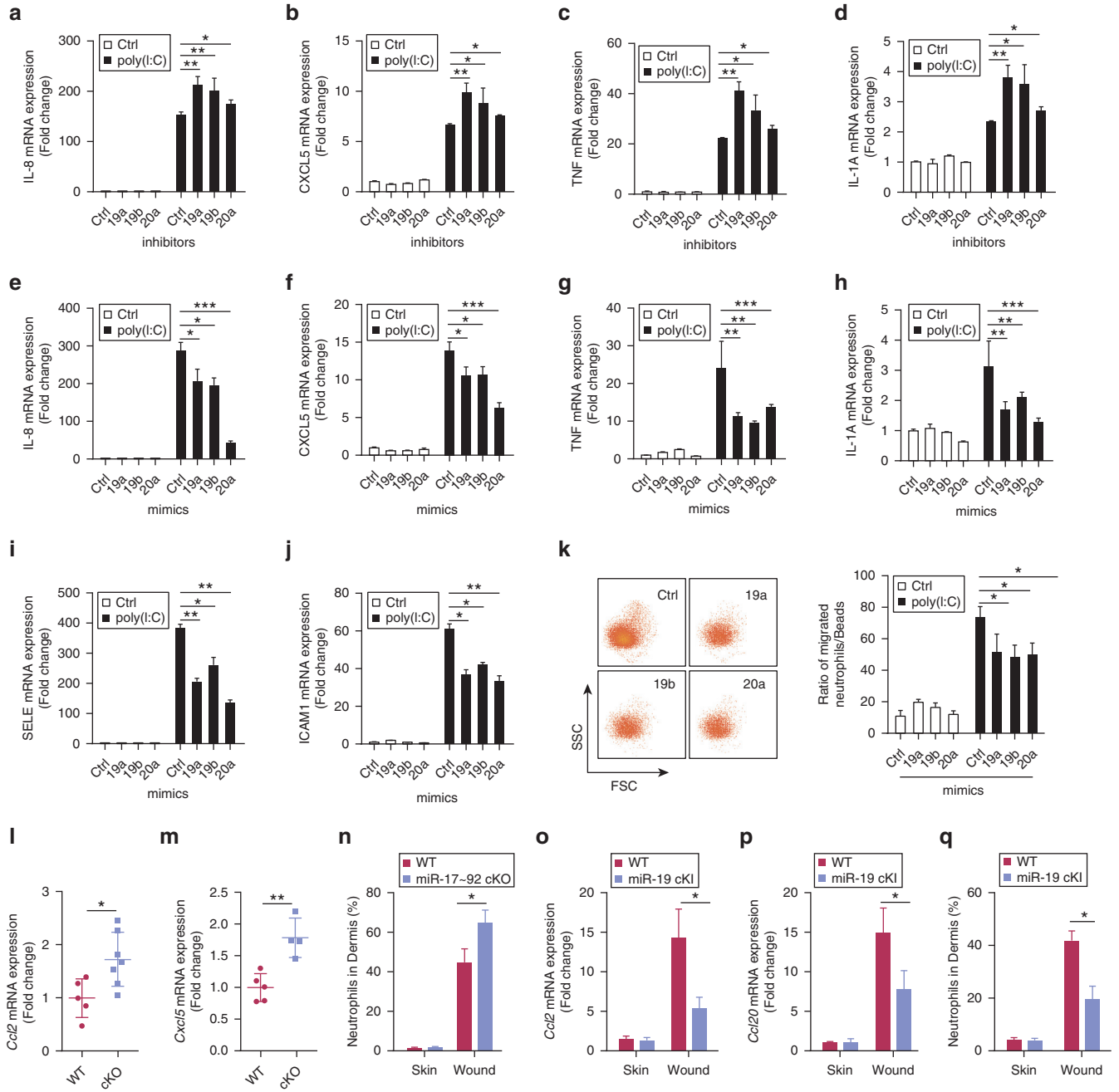

Figure 4. miR-19a/b and miR-20a suppress poly(I:C)-induced inflammation. QRT-PCR of IL-8, CXCL5, TNF, and IL-1A in KCs upon miR-19a/b or miR-20a (**a**–**d**) inhibition or (**e**–**h**) overexpression followed by poly(I:C) treatment (n = 3). Blood vessel endothelial cells were incubated with conditioned medium from the KCs treated as above. (**i**) SELE and (**j**) ICAM1 were analyzed by QRT-PCR (n = 3). (**k**) Neutrophil chemotaxis toward the conditioned medium from the KCs treated as above (n = 3) was quantified by flow cytometry. QRT-PCR of (**l**) *Ccl2* and (**m**) *Cxcl5* in day-5 wound-edge epidermis of WT (n = 5) and miR-17∼92 cKO mice (n = 4–7). Skin and wound neutrophils in (**n**) WT (n = 4) versus miR∼17-92 cKO mice (n = 4), (**q**) WT (n = 3) versus miR-19 cKI mice (n = 3) were quantified by flow cytometry and presented as the percentage of dermal CD45+ cells. QRT-PCR of (**o**) *Ccl2* and (**p**) *Ccl20* in day-5 wound-edge epidermis of WT (n = 3) and miR-19 cKI (n = 3) mice. The data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student's *t*-test. cKI, conditional knockin; cKO, conditional knockout; Ctrl, control; FSC, forward scatter; KC, keratinocyte; miR, microRNA; poly(I:C); polyinosinic:polycytidylic acid; QRT-PCR, quantitative real-time reverse transcriptase–PCR; SSC, side scatter; WT, wild type.

Increased miR-17∼92 expression in KCs promotes wound healing in vivo

To test whether enhanced miR-17∼92 expression promotes wound healing, we examined the healing capacity of mice with KC-specific miR-17∼92 conditional knockin (cKI) (Wu et al., 2017). Under diabetic conditions induced by multiple injections of streptozocin, we observed faster wound closure in miR-17∼92 cKI mice than in the littermate controls (Figure 3a). This was confirmed by histomorphometry analysis, showing that the areas of the newly formed epithelial tongues were significantly increased in the miR-17∼92 cKI mice compared with that in the controls (Figure 3b). We also

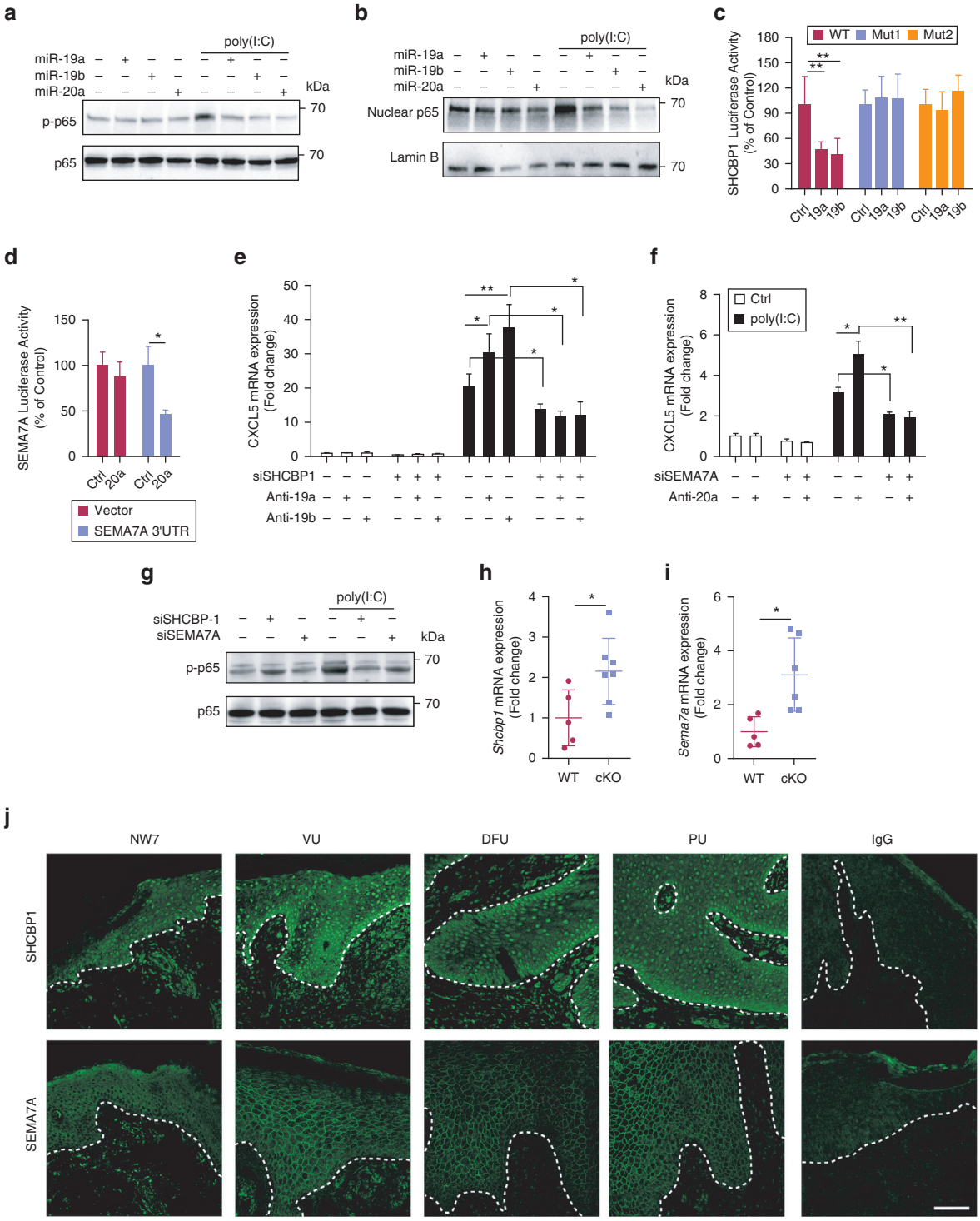

Figure 5. MiR-19a/b targets SHCBP1, whereas miR-20a targets SEMA7A. (**a**) KCs transfected with miR mimics were treated with poly(I:C) for 10 minutes, p-p65 and total p65 were detected by WB (n = 3). (**b**) KCs transfected with miR mimics were stimulated with poly(I:C) for 4 hours. Nuclear p65 and Lamin B were detected by WB. Luciferase activity was measured in (**c**) KCs transfected with reporter plasmids containing WT or Mut SHCBP1 3′-UTR, together with miR-19a/b or control mimics (n = 9), or (**d**) KCs transfected with vector or reporter plasmid containing SEMA7A 3′-UTR, together with miR-20a or control mimics (n = 4–5). CXCL5 was measured in (**e**) poly(I:C)-treated KCs transfected with SHCBP1 siRNA and miR-19a/b inhibitors (n = 3) or (**f**) KCs transfected with SEMA7A siRNA and miR-20a inhibitors (n = 3). (**g**) KCs transfected with SHCBP1 or SEMA7A siRNAs were treated with poly(I:C) for 10 minutes; p-p65 and p65 were detected by WB. QRT-PCR of (**h**) Shcbp1 and (**i**) Sema7a in day-5 wound-edge epidermis of WT (n = 5) and miR-17∼92 cKO (n = 4–7) mice. (**j**) Immunofluorescence staining of SHCBP1 and SEMA7A in NW7, VU, DFUs, and PU (n = 3 per group). Bar = 100 μm. The data are presented as mean ± SD. **P* < 0.05, ***P* < 0.01; Student's *t*-test. 3′-UTR, 3′ untranslated region; cKO, conditional knockout; Ctrl, control; DFU, diabetic foot ulcer; KC, keratinocyte; kDa, kilodalton; miR, microRNA; Mut, mutant; NW7, day-7 wound; poly(I:C); polyinosinic:polycytidylic acid; p-p65, phosphorylated p65; PU, pressure ulcer; QRT-PCR, quantitative real-time reverse transcriptase–PCR; siRNA, small interfering RNA; siSEMA7A, siRNA specific to SEMA7A; siSHCBP1, siRNA specific to SHCBP1; VU, venous ulcer; WB, western blotting; WT, wild type.

established a mouse line with KC-specific miR-19b cKI (Figure 3c). We observed a two-fold upregulation of miR-19b expression in the wound-edge epidermis of this mouse line compared with that of the littermate controls (Figure 3d). Similar to the miR-17∼92 cKI mice, miR-19b cKI mice healed significantly faster than the controls (Figure 3e and f). Together, these in vivo data highlight the importance of the upregulation of miR-17∼92 expression in KCs in wound repair and suggest that miR-17∼92 deficiency may contribute to the pathogenesis of chronic wounds.

MiR-19a/b and miR-20a suppress polyinosinic:polycytidylic acid–induced inflammation

To investigate the impact(s) of miR-19a, miR-19b, and miR-20a on KC functions critical for wound repair, we either overexpressed or inhibited one of them in human primary KCs by transfecting miR-specific mimics or inhibitors, whose effects were confirmed by quantitative real-time reverse transcriptase–PCR (Supplementary Figure S3a and b). We showed that the aberrant miR-19a/b and miR-20a expression did not impact KC growth and migration (Supplementary Figure S3c and d). To examine their impact on KC inflammatory response after injury, we treated the transfected cells with polyinosinic:polycytidylic acid (poly[I:C]), a synthetic double-stranded RNA activating TLR3 signal, because it has been shown that the skin epithelium requires TLR3 activation for normal inflammation after injury (Lai et al., 2009). We found that the inhibition of miR-19a/b and miR-20a increased, whereas their overexpression decreased poly(I:C)-induced expression of several proinflammatory chemokines and cytokines, for example, CXCL8 and/or IL-8, CXCL5, TNF-α, and IL-1A at the mRNA level (Figure 4a–h and Supplementary Figure S4a–c). The reduced production of IL-8 and CXCL5 by KCs overexpressing miR-19a/b or miR-20a was also confirmed at the protein level (Supplementary Figure S4d and e).

These chemokines are key players recruiting neutrophils to the injured skin (Murphy et al., 2012). This is a multistep process starting with the chemokine-induced expression of adhesion molecules, for example, ICAM1, SELE, and VCAM1, on the surfaces of endothelial cells, which mediate attachment of circulating leukocytes to the blood vessel wall, facilitating leukocytes extravasation (Butcher, 1991, Murphy et al., 2012). We measured the expression of these adhesion molecules in the endothelial cells incubated with the conditioned medium from KCs overexpressing miR-19a/b or miR-20a. Our results showed that these miRs decreased the capacity of KCs to induce the expression of adhesion molecules in endothelial cells (Figure 4i and j). Moreover, we performed chemotaxis assays with neutrophils isolated from human peripheral blood using conditioned supernatants from KCs overexpressing miR-19a/b or miR-20a. We found that the supernatants from KCs with increased miR-19a/b or miR-20a expression attracted fewer neutrophils than the medium from control-treated cells (Figure 4k).

In line with the in vitro data, the anti-inflammatory function of miR-19a/b and miR-20a was also observed in vivo. In the wound-edge epidermis of miR-17∼92 cKO mice, the expression of *Ccl2* and *Cxcl5* was significantly upregulated compared with that of the WT mice (Figure 4l and m). Accordingly, more neutrophils infiltrated in the wound dermis of the miR-17∼92 cKO mice than in that of the controls, shown by flow cytometry analysis (Figure 4n). Of note, there was no significant difference in the number of Langerhans cells and γδ T cells in the wound-edge epidermis between the control and the miR-17∼92 cKO mice, excluding the possibility that the changed leukocyte infiltration in wound dermis would result from a different epidermal immune cell composition (Supplementary Figure S5a and b). Moreover, we found that in the mice wounds treated with miR-19a/b and miR-20a inhibitors, the levels of *Cxcl5*, *Ccl20*, and *Ccl2* (Supplementary Figure S5c and e) as well as the number of neutrophils (Ly6G+), macrophages (CD68+), and T cells (CD3+) were also increased compared with those of the controls (Supplementary Figure S5f–i). In contrast, reduced epidermal expression of chemokine genes *Ccl2* and *Ccl20* and decreased dermal infiltration of neutrophils were observed in the wound edges of miR-19b cKI mice (Figure 4o–q). On the basis of these in vitro and in vivo data, we conclude that the increased miR-19a/b and miR-20a expression during wound healing is important to restrict the leukocyte influx through the suppression of epidermal production of inflammatory chemokines.

Furthermore, we detected TLR3 expression as well as the presence of neutrophils (MPO+) and macrophages (CD68+) in human chronic wounds, skin, and day-7 acute wounds by immunofluorescence (Supplementary Figure S5j). For TLR3, we observed its increased expression in the wound-edge epidermis of chronic wounds compared with that of the day-7 acute wounds and the skin (Supplementary Figure S5j). Interestingly, we found a higher TLR3 signal in epidermal cell nuclei in chronic wounds. In contrast, in human skin, TLR3 expression is primarily membranous and cytoplasmic in KCs (Supplementary Figure S5j). In addition, we detected increased infiltration of neutrophils and macrophages in chronic wounds compared with that in the day-7 acute wounds and the skin (Supplementary Figure S5j). Of note, among the three types of chronic wounds we studied, the number of neutrophils in DFU and PU was lower than that in VU (Supplementary Figure S5j). Together, we postulate that the enhanced TLR3 expression as well as increased infiltration of neutrophils and macrophages in VU, DFU, and PU may be at least partially attributed to the low levels of miR-19a/b and miR-20a expression in these chronic wounds (Supplementary Figure S5j).

miR-19a/b and miR-20a regulate NF-κB signaling pathway

Next, we aimed to unravel the signaling pathway(s) mediating the anti-inflammatory function of 19a/b and miR-20a. To this end, we first dissected signaling pathways involved in the poly(I:C)-induced inflammation by treating KCs with pathway-specific chemical inhibitors before poly(I:C) stimulation. Analysis of chemokine expression revealed p38, extracellular signal–regulated kinase, and NF-κB as the key pathways mediating the effects of poly(I:C) in KCs (Supplementary Figure S6a and b). Poly(I:C) treatment increased the phosphorylation of p38, extracellular signal–regulated kinase, and p65 as well as nuclear translocation of p65, indicating activation of these pathways (Figure 5a and b and Supplementary Figure S6c–e).

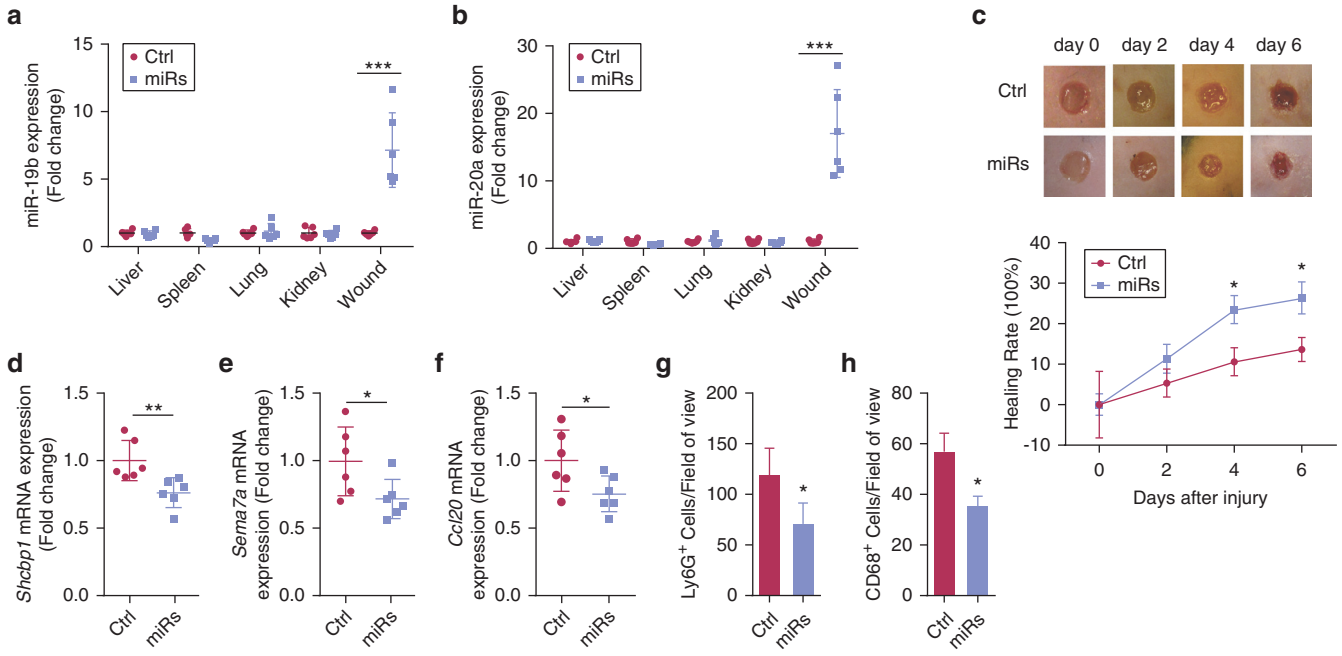

Figure 6. miR-19b and miR-20a exhibit therapeutic potential for chronic wounds. The mixture of miR-19b and miR-20a mimics (miRs) or control oligos was injected into the wound-edges of db/db mice after injury. (**a**) MiR-19b and (**b**) miR-20a were detected by QRT-PCR in wounds and inner organs. (**c**) Days 0–6 wounds in control (n = 6) and miRs-treated group (n = 6). Wound closure was quantified and presented as a healing rate. QRT-PCR of (**d**) *Shcbp1*, (**e**) *Sema7a,* and (**f**) *Ccl20* in the day-6 wounds (n = 6). (**g**, **h**) Ly6G- and CD68-positive cells were counted in the immunostaining of the day-6 wounds (n = 3). Data are presented as (**c**) mean ± SEM or (**a**, **b**, **d**–**h**) mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; (**c**) Bonferroni post-hoc test or (**a**, **b**, **d**–**h**) Student's *t*-test. Ctrl, control; db/db, leptin receptor–deficient; miR, microRNA; QRT-PCR, quantitative real-time reverse transcriptase–PCR.

Interestingly, we found that miR-19a/b and miR-20a mainly reduced the phosphorylation and nuclear translocation of p65 (Figure 5a and b), whereas their impact on p38 and extracellular signal–regulated kinase was minimal (Supplementary Figure S6c–e). In line with this, gene set enrichment analysis of microarray data of KCs overexpressing miR-19a/b or miR-20a revealed that gene sets related to NF-κB signaling pathway were enriched among the genes downregulated by these miRs (Supplementary Figure S6f and g) (Subramanian et al., 2005), further supporting that miR-19a/b and miR-20a suppress poly(I:C)-induced inflammation by regulating NF-κB signaling pathway in KCs.

Identification of target genes important for miR-19a/b's and miR-20a's anti-inflammatory function in KCs

Because miRs act through post-transcriptional regulation of protein-coding genes, identification of target genes is critical to understand miRs' function. To this end, we performed a global transcriptomic analysis in KCs overexpressing miR-19a/b or miR-20a**.** Gene set enrichment analysis of the microarray data revealed the significant enrichment of the predicted targets of miR-19a/b and miR-20a among the downregulated genes, indicating the high specificity of the assay (Supplementary Figure S7a–c). Among the 64 putative targets that were commonly predicted by all the three independent miR-target prediction algorithms, that is, TargetScan (Lewis et al., 2005), miRDB (Wang, 2016), and DIANAT (Vlachos et al., 2015), five were found downregulated by miR-19a/b (fold change of ≥ 1.3, $P < 0.05$, Supplementary Figure S7d–f) shown by the microarray. In this experiment, SHCBP1 was demonstrated as a direct target of miR-19a/b in KCs on the basis of a series of strong evidence from in silico and experimental analysis: (i) two conserved putative binding sites for miR-19a/b were identified in the 3′ untranslated region of *SHCBP1* mRNA (Supplementary Figure S7g); (ii) miR-19a/b decreased luciferase activity of a reporter gene construct containing the full-length 3′ untranslated region of *SHCBP1* mRNA, whose effect was abolished by the mutation either of these two predicted binding sites (Figure 5c); (iii) miR-19a/b overexpression decreased, whereas their inhibition increased the mRNA level of *SHCBP1* shown by quantitative real-time reverse transcriptase–PCR (Supplementary Figure S7h and i). Similarly, among the four putative targets that were commonly predicted by all the three algorithms and significantly downregulated by miR-20a in KCs (fold change of ≥ 1.3, $P < 0.05$, Supplementary Figure S8a–d), we identified SEMA7A as a target directly bound and inhibited by miR-20a by luciferase reporter assay (Figure 5d). In KCs, overexpression of miR-20a decreased, whereas its inhibition increased the SEMA7A mRNA level (Supplementary Figure S8e and f).

Of note, silencing of *SHCBP1* or *SEMA7A* with gene-specific small interfering RNAs significantly decreased poly(I:C)-induced IL-8, CXCL1, and CXCL5 production by KCs (Supplementary Figure S9a–g), phenocopying miR-19a/b or miR-20a overexpression (Figure 4). In addition, enhancement of chemokine expression by the inhibitors of miR-19a/b or miR-20a was completely reversed by the silencing of *SHCBP1* or *SEMA7A*, respectively (Figure 5e and f). Moreover, we found that poly(I:C)-induced p65 phosphorylation was decreased by silencing *SHCBP1* or *SEMA7A*, indicating that these two genes function as positive regulators

of NF-κB signaling (Figure 5g). These findings provide compelling evidence that SHCBP1 and SEMA7A are the key targets mediating the anti-inflammatory function of miR-19a/b and miR-20a in KCs.

In line with the above in vitro data, we found increased *Shcbp1* and *Sema7a* mRNA levels in the wound-edge epidermis of miR-17∼92 cKO mice (Figure 5h and i). In the mice wounds treated with miR-19a/b and miR-20a inhibitors, *Shcbp1* expression was significantly upregulated, whereas *Sema7a* showed a slight increase (Supplementary Figure S9h and i). On the contrary, the level of *Shcbp1* was decreased in the wounds of miR-19b cKI mice (Supplementary Figure S9j). More interestingly, immunofluorescence staining detected more SEMA7A and SHCBP1 proteins in human DFUs, VUs, and PUs than in the normal NW7, which may correspond to the miR-19a/b and miR-20a deficiency in these chronic wounds (Figure 5j). In addition, we observed a significant negative correlation between the levels of SEMA7A mRNA and miR-20a in human acute and chronic wounds (Supplementary Figure S9k). These findings suggest that SHCBP1 and SEMA7A may be regulated by miR-19a/b and miR-20a in human wounds in vivo.

Local application of miR-19b and miR-20a mimics promotes wound healing in a mouse model of type 2 diabetes

Owing to the anti-inflammation and prohealing effects of miR-19a/b and miR-20a and their deficiency in chronic wounds, we hypothesized that the replenishment of miR-19a/b and miR-20a might be a therapeutic approach to promote healing. We tested this in leptin receptor–deficient mice, a mouse model of type 2 diabetes with impaired wound healing capacity (Scherer et al., 2008). Similar to human chronic wounds, the expressions of miR-19a and miR-20a were also lower in the wounds of leptin receptor–deficient mice than in those of the WT mice (Supplementary Figure S10a–f). We injected a mixture of miR-19b and miR-20a mimics encapsulated within a phospholipid-oil emulsion intradermally into the wound edges of leptin receptor–deficient mice immediately after an injury. This treatment specifically and efficiently increased the levels of miR-19b and miR-20a in the wounds but not in inner organs, for example, liver, spleen, lung, and kidney (Figure 6a and b). We observed significantly accelerated wound closure (Figure 6c) as well as decreased expression of miR-19a/b and miR-20a targets, that is, *Shcbp1* and *Sema7a*, and proinflammatory chemokines, for example, *Ccl20*, in the miR-19b/20a mimics–treated wounds compared with those of the controls (Figure 6d–f). Moreover, a reduced number of leukocytes, in particular, neutrophils (Ly6G+) and macrophages (CD68+), was found in the wounds treated with miR-19b/20a mimics (Figure 6g and h and Supplementary Figure S10g). The prohealing effect of miR-19b and miR-20a was not due to better metabolic control because blood glucose and weight of mice were unaffected by the treatment (Supplementary Figure S10h and i). Together, our data highlight the therapeutic potential of local administration of miR-19b and miR-20a mimics for hard-to-heal wounds.

DISCUSSION

At the early phase of wound healing, inflammatory response defends us from invading pathogens and accumulation of dead tissue in the wound. The inflammation ceases once these danger signals are removed (Landén et al., 2016). However, in chronic nonhealing wounds, persistent and impaired inflammation has been observed (MacLeod and Mansbridge, 2016). Chronic wound inflammation has been characterized as overpersistence of neutrophils that cause collateral tissue damage (Wilgus et al., 2013), a reduced capability of macrophages to clear neutrophils, and deficient transition from a proinflammatory to a reparative phenotype of macrophages (Hesketh et al., 2017; Krzyszczyk et al., 2018). In line with previous findings (Diegelmann and Evans, 2004; Khanna et al., 2010; Krzyszczyk et al., 2018; Larouche et al., 2018; Loots et al., 1998; MacLeod and Mansbridge, 2016; Wilgus et al., 2013; Wu et al., 2016), the increased infiltration of neutrophils and macrophages was also detected in our VU, DFU, and PU samples (Supplementary Figure S5j). Interestingly, our immunofluorescence staining also detected fewer neutrophils in DFU and PU than in VU (Supplementary Figure S5j), which may be due to differences in chronic wound etiologies (e.g., venous insufficiency, diabetes, and mechanical pressure) or infection status.

It is crucial to understand the molecular mechanisms that regulate the inflammatory response during normal wound healing and to examine whether any of these mechanisms are dysregulated in nonhealing ulcers. In this study, we identified miR-19a/b and miR-20a as negative regulators of KC inflammatory response; their increased expression during wound healing is important to restrict the leukocyte trafficking to the skin through the suppression of epidermal production of inflammatory chemokines. Compared with the human normal wounds at the inflammatory phase (NW1) or the proliferative phase (NW7), we found that the expression of miR-19a/b and miR-20a were significantly downregulated in all the three major types of chronic wounds, that is, VU, DFU, and PU, which may contribute to sustained inflammation and impaired healing there. Interestingly, recent studies have revealed beneficial effects of converting chronic wound microenvironment to a healing milieu similar to that in an acute wound (Stone et al., 2020, 2017). In line with this, our study suggested that the replenishment of miR-19b and miR-20a in wounds with a deficiency of these miRs has therapeutic potential.

TLRs are the first sensors to danger signals after a skin injury, including invading pathogens and molecules released by stressed cells undergoing necrosis (Kawai and Akira, 2010). TLR engagement by these ligands activates MAPKs and NF-κB signaling, which leads to the expression of proinflammatory cytokines and chemokines important for triggering innate immune responses and priming antigen-specific adaptive immunity (Kawai and Akira, 2010). However, it is critical to turn off the TLR-induced inflammation after the removal of danger signals. Loss of negative regulation of TLR-signaling has been involved in the pathogenesis of inflammatory diseases (Liew et al., 2005). Moreover, persistent activation of TLR signaling has been found in human venous or diabetic ulcers (Dasu and Martin, 2014;

Pukstad et al., 2010). In line with previous findings, in this study, we detected increased TLR3 expression in the wound-edge epidermis of VU, DFU, and PU compared with that of NW7 and the skin (Supplementary Figure S5j). In addition, we observed different subcellular localization of TLR3 in epidermal KCs of chronic wounds (nuclear), in comparison with those of the skin (membranous and cytoplasmic) (Supplementary Figure S5j). Similarly, nuclear localization of TLR3 protein has been recently reported in the lesions of patients with psoriasis, atopic dermatitis, and prurigo nodularis (Szöllősi et al., 2019). We postulate that the altered subcellular localization of TLR3 may be involved in the pathogenesis of chronic inflammatory skin diseases, which underlying molecular mechanism warrants further investigation.

In this study, we identified miR-19a/b and miR-20a as negative regulators of TLR signaling in epidermal KCs, which inhibit the phosphorylation and nuclear translocation of P65, resulting in reduced inflammatory chemokine production in wound healing. Increased expression of miR-19a/b and miR-20a with healing progress may reduce the responsiveness of TLRs on KCs, repress the capacities of KCs to recruit neutrophils, and thus contribute to the resolution of inflammation in the wound repair process. Of note, the miR-17∼92 cluster was found to be upregulated in psoriasis lesions (Zhang et al., 2018), where TLR3 was also induced and with the nuclear localization (Szöllősi et al., 2019). This is probably because the strong activation of TLR3 signaling could not be completely reversed by miR-17-92 elevation in psoriasis lesions or because of quite different roles of miR-17-92 in psoriasis compared with those in wound healing, which warrants further investigation.

Furthermore, we found that the anti-inflammatory function of miR-19a/b was mediated at least partially through the downregulation of their target SHCBP1, a binding partner of Shc, and acting as an adaptor of numerous cell surface receptors (Schmandt et al., 1999). Previous studies have linked SHCBP1 mainly to cell proliferation and tumorigenesis (Asano et al., 2013; Feng et al., 2016; Peng et al., 2016; Schmandt et al., 1999; Tao et al., 2013). Our study reveals that SHCBP1 also regulates NF-κB signaling and inflammatory chemokine production in KCs. In parallel, we identified SEMA7A as a direct target of miR-20a. SEMA7A is a semaphorin important for both innate and adaptive immunity (Garcia-Areas et al., 2013) and is expressed on the cell membrane of basal and suprabasal human epidermal KCs (Scott et al., 2008). Interestingly, the expression of both SHCBP1 and SEMA7A was aberrantly upregulated in VU, DFU, and PU, suggesting their pathological roles in chronic wounds.

In this study, we focused on the role of miR-19a/b and miR-20a in wound-edge KCs. Of note, another member of the miR-17∼92 cluster, miR-92a, has been identified as an important negative regulator of angiogenesis (Bonauer et al., 2009). Inhibition of miR-92a enhances angiogenesis and accelerates wound healing in diabetic mice (Gallant-Behm et al., 2018; Lucas et al., 2017). Currently, the miR-92a inhibitor is being tested as a new wound treatment (clinicaltrials.gov NCT03603431). These pieces of evidence suggested that multiple cellular processes during wound healing could be coordinated by different members of a miR cluster; therefore, it is important to dissect the functional roles of each member.

As a significant health and economic burden globally, there is a continued search toward more effective treatment for chronic wounds. Intensive studies have led to the development of GFs- and stem cell–based therapies, however with limited clinical success (Borena et al., 2015). As potent gene regulators, miRs are becoming promising tools in the diagnostic and therapeutic fields of medicine. Recent clinical trials demonstrate that modulation of miRs has beneficial effects on a variety of diseases, including virus infection, cancer, and diabetes (Chakraborty et al., 2017; Rupaimoole and Slack, 2017). The progress of miR therapy provides opportunities to develop efficient and targeted wound treatments (Eming et al., 2014; Meng et al., 2018). To this end, it is a requisite to understand the functions of miRs in wound healing, and much efforts have been invested in solving this challenge in recent years (reviewed in Herter and Landén [2017]; Li and Landén [2017]; Meng et al. [2018]). In human skin wounds in vivo, a miR expression profile has been reported (Li et al., 2015b). Among the miRs regulated during wound healing, many have been shown to play functional roles in wound repair, for example, miR-21 (Das et al., 2014), miR-27b (Wang et al., 2014), miR-31 (Li et al., 2015a), miR-99 family (Jin et al., 2013), miR-132 (Li et al., 2017a, 2017b, 2015b), miR-146a (Roy et al., 2014), miR-155 (van Solingen et al., 2014), and miR-210 (Biswas et al., 2010). It is not surprising that the complex process of wound healing involving the dynamic interaction of diverse biological processes requires complex regulation. In this study, evidence derived from several complementary experimental models demonstrates a significant anti-inflammatory role of miR-19a, miR-19b, and miR-20a in wound-edge KCs. Because miR-19a and miR-19b have very similar expression and function patterns as well as a highly overlapped target spectrum, in this study, we used one of them, that is, miR-19b, together with miR-20a to treat wounds of diabetic mice. We showed that the combination of miR-19b and miR-20a mimics reduced wound inflammation and accelerated wound closure. A further study to compare the therapeutic potential of miR-19a and miR-19b mimics head to head in vivo is warranted before recommending that either one could serve as a therapeutic to accelerate human chronic wound repair. In addition, although the oncogenic role of miR-17∼92 has been reported in multiple hematopoietic and solid cancers (Mogilyansky and Rigoutsos, 2013; Olive et al., 2013), we did not observe obvious effects of miR-19a/b and miR-20a on the growth and motility of human primary KCs (Supplementary Figure S3c and d), and along this line, miR-20a has previously been shown to inhibit the proliferation and metastasis of squamous cell carcinoma (Zhou et al., 2014). Therefore, the primary effect of local and transient replenishment of miR-19b and miR-20a in wounds is likely to be anti-inflammatory. However, for patients with chronic wounds, multiple or long-term treatments may be required. Thus, the oncogenesis risk of miR-19/20a treatment needs to be carefully evaluated.

In summary, our study identified miR-19a/b and miR-20a from the miR-17∼92 cluster as molecular brakes suppressing the inflammatory response of KCs through the regulation of the NF-κB signaling pathway (Supplementary Figure S10j). With the increased expression in KCs during wound healing, miR-19a/b and miR-20a play an important role in resolving inflammation, whereas their deficiency in KCs, as observed in chronic wounds, impairs wound healing in vivo. Thus, our study emphasizes the importance of the properly controlled innate immune response of KCs in wound repair. Moreover, our data highlight the therapeutic potential of recovery of the brake system of inflammation, for example, miR-19a/b and miR-20a, in non-healing wounds, which may help to break the vicious circle and reactivate the healing program.

In conclusion, miR-19a/b and miR-20a restrict KC inflammatory response and promote skin wound healing.

MATERIALS AND METHODS

Human wound samples

Human samples were collected at the Department of Dermatology, the Department of Reconstructive Plastic Surgery, and the Department of Endocrinology and Diabetes, Karolinska University Hospital (Stockholm, Sweden), the Dermatology clinic of Academic University Hospital (Uppsala, Sweden), and the Second Hospital of Dalian Medical University (Dalian, China). Patients with nonhealing VUs, DFUs, or PUs that, despite conventional therapy, persisted for more than 2 months were enrolled in this study. Tissue samples were taken using a 4-mm biopsy punch at the nonhealing edges of chronic wounds. Healthy donors without diabetes, skin diseases, unstable heart disease, infections, bleeding disorder, immune suppression, and any on-going medical treatments were recruited. One or two full-thickness excisional wounds were created using a 4-mm biopsy punch at the lower leg area or the upper buttock area of each donor. The central skin excised from these surgical wounds were saved as intact skin control. The wound-edge skin was collected using a 6-mm biopsy punch 1 and 7 days later. Local lidocaine injection was used for anesthesia while sampling.

Statistical analysis

Statistical significance was determined by two-tailed Student's *t*-test or Wilcoxon-matched pairs signed-rank test or Mann–Whitney *U* test. Differences between groups were computed using Bonferroni post-hoc test in GraphPad Prism 7 (GraphPad Software, San Diego, CA). The correlation between the expressions of different genes in the same sample was made using Pearson's correlation test on log-transformed data. For all statistical tests, P-values < 0.05 were considered to be statistically significant.

Study approval

Written informed consent was obtained from all the donors for the collection and use of clinical samples. The study was approved by the Stockholm Regional Ethics Committee (Stockholm, Sweden) and the Ethics Committee of The Second Hospital of Dalian Medical University (Dalian, China). The study was conducted according to the Declaration of Helsinki's principles. All animal procedures were reviewed and approved by the North Stockholm Ethical Committee for Care and Use of Laboratory Animals (Stockholm, Sweden) and the Henry Ford Hospital Institutional Animal Care and Use Committee (Detroit, MI). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996).

The experimental protocols for in vivo wound models, RNA extraction and quantitative real-time reverse transcriptase–PCR, laser capture microdissection, in situ hybridization, cell culture and treatments, leukocyte chemotaxis assay, proliferation assay, scratch assay, protein detection, histological analysis, microarray, luciferase reporter assay, and flow cytometry are detailed in the Supplementary Materials and Methods available online.

Data availability statement

Datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE93110, hosted at the National Centre for Biotechnology Information Gene Expression Omnibus database (GSE93110).

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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AUTHOR CONTRIBUTIONS

Conceptualization: DL, HP, QSM, LZ, NXL; Funding Acquisition: DL, QSM, LZ, NXL; Investigation: DL, XL, HP, LQ, XB, WL, XZ, QL; Methodology: JG, CX, QSM, LZ, AP, ES, SBC, MS; Project Administration: NXL, DL, QSM, LZ; Resources: MS, IGS, OR, PS, SBC, SEA, AW, TC; Supervision: AP, ES, SBC, MS, NXL, QSM, LZ; Writing - Original Draft Preparation: DL, NXL; Writing - Review and Editing: DL, HP, LQ, PS, AW, TC, XL, XB, QL, IGS, OR, WL, XZ, SEA, JG, AP, ES, SBC, CX, MS, QSM, LZ, NXL

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.06.037.

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