MicroRNA-34 Family Enhances Wound Inflammation by Targeting LGR4

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Venous ulcers are the most common type of human chronic nonhealing wounds and are stalled in a constant and excessive inflammatory state. The molecular mechanisms underlying the chronic wound inflammation remain elusive. Moreover, little is known about the role of regulatory RNAs, such as microRNAs, in the pathogenesis of venous ulcers. We found that both microRNA (miR)-34a and miR-34c were upregulated in the wound-edge epidermal keratinocytes of venous ulcers compared with normal wounds or the skin. In keratinocytes, miR-34a and miR-34c promoted inflammatory chemokine and cytokine production. In wounds of wild-type mice, miR-34a–mimic treatment enhanced inflammation and delayed healing. To further explore how miR-34 functions, LGR4 was identified as a direct target mediating the proinflammatory function of miR-34a and miR-34c. Interestingly, impaired wound closure with enhanced inflammation was also observed in Lgr4 knockout mice. Mechanistically, the miR-34–LGR4 axis regulated GSK-3β–induced p65 serine 468 phosphorylation, changing the activity of the NF-κB signaling pathway. Collectively, the miR-34–LGR4 axis was shown to regulate keratinocyte inflammatory response, the deregulation of which may play a pathological role in venous ulcers.

ORIGINAL ARTICLE

INTRODUCTION

Chronic nonhealing wounds present a major and increasing health and economic burden to society. Approximately 1–2% of individuals in developed countries experience a complex chronic wound (Järbrink et al., 2016), which already consumes 2–4% of health care budgets nowadays (Frykberg and Banks, 2015; Gottrup et al., 2001; Richmond et al., 2013). Chronic wounds commonly occur in patients with one or several underlying disorders, such as venous or arterial insufficiency or diabetes mellitus, among which venous ulcer (VU) is the most common type (Medina et al., 2005). To date, chronic wounds still lack efficient targeted treatment, and better understanding of their pathophysiology is needed (Frykberg and Banks, 2015).

Among the various cell types participating in wound repair, keratinocytes, which constitute 95% of the cells in the epidermis, play a pivotal role (Kolarsick et al., 2008); however, their role in chronic wound inflammation still remains puzzling. Keratinocytes at the front line to sense damage and pathogen-associated signals and respond by producing a variety of cytokines and chemokines to trigger a transient inflammatory response, which prevents infection and facilitates healing progress (Chen and DiPietro, 2017; Strbo et al., 2014). Chronic wounds have been shown to be stalled in a constant and excessive inflammatory state (Landén et al., 2016). Persisting inflammatory cells produce a large amount of proinflammatory cytokines, proteolytic enzymes, and reactive oxygen species that inhibit wound repair (Eming et al., 2014; Landén et al., 2016). Keratinocytes at the chronic nonhealing wound edge have been shown to be hyperproliferative and nonmigratory (Usui et al., 2008); however, their role in chronic wound inflammation still remains puzzling.

MicroRNAs (miRNAs) are small (~22 nucleotides) but powerful gene regulators, which can bring the RNA-induced silencing complex to the 3’ untranslated regions of their target mRNAs, repressing gene expression at post-transcriptional level (Jonas and Izaurralde, 2015). Increasing studies have shown that miRNAs play important roles in diverse physiological and pathological processes. The great potential of miRNAs as diagnostic and therapeutic entities recently has attracted broad interest in understanding their
RESULTS

Increased miR-34a and miR-34c expression in the wound-edge keratinocytes of human venous ulcers

To study the molecular pathology of human chronic wounds, we collected biopsies from edges of chronic nonhealing wounds of 19 patients with VU (Supplementary Tables S1 and S2). As a comparison, we created in vivo surgical wounds in the skin of the lower leg area of seven age-matched healthy volunteers and collected intact skin as well as acute wound-edge tissues one day after skin injury (Supplementary Tables S1 and S3). We next examined the expression of the miR-34 family in these human wound samples using quantitative real-time reverse transcriptase–PCR (qRT-PCR). This microRNA family comprises three homologs, miR-34a, b, and c, localized to two distinct genomic loci, MIR34A (chromosome 1p36) and MIR34B/MIR34C (chromosome 11q23). However, we only detected miR-34a-5p and miR-34c-5p, but not miR-34b, in human skin and wound tissues, which was confirmed by RNA sequencing analysis (Supplementary Figure S1). Importantly, we found that the expression levels of both miR-34a and miR-34c were significantly higher in the VUs than the normal wounds or the intact skin from healthy donors (Figure 1a and b). Furthermore, we isolated epidermis from human skin and wound samples using laser capture microdissection (Supplementary Figure S2, Supplementary Tables S2 and S3). QRT-PCR analysis revealed significantly increased miR-34a and miR-34c expression in the wound-edge epidermis of VUs compared with the epidermis from the intact skin or the normal wounds (Figure 1c and d). As keratinocytes constitute more than 95% of the epidermis (Kolarsick et al., 2011), our study focused on unraveling the functional role of miR-34a and miR-34c in keratinocytes during wound healing.

Characterization of the transcriptome regulated by miR-34a and miR-34c in keratinocytes

The biological function of the miR-34 family in epidermal keratinocytes has remained unexplored. To this end, we transfected miR-34a or miR-34c mimics in human primary keratinocytes, which was confirmed by qRT-PCR analysis (Supplementary Figure S3a and b). Next, we performed a global transcriptome analysis using microarray in keratinocytes transfected with miR-34a or miR-34c mimics (GSE117506). Based on the gene expression profile, principle component analysis clearly separated the control group from the groups treated with miR-34 mimics, whereas the gene expression profiles of miR-34a– and miR-34c–transfected cells were partially overlapped, suggesting the functional similarity between these two miRNAs (Supplementary Figure S4a). In keratinocytes, we identified 1,213 and 1,081 genes significantly (absolute fold change ≥2, false discovery rate <5%) regulated by miR-34a and miR-34c, respectively, among which 694 genes were commonly regulated by both miRNAs (Supplementary Figure S4b). Gene set enrichment analysis (Mootha et al., 2003; Subramanian et al., 2005) revealed that the miR-34 targets predicted by the algorithm Targetscan (Agarwal et al., 2015) were significantly enriched among the genes downregulated by miR-34a or miR-34c, and negative enrichment score curves were generated, suggesting that this microarray analysis was specific and sensitive for detecting the miR-34–regulated genes (Supplementary Figure S4c and d). Gene Ontology analysis (Chen et al., 2013; Kuleshov et al., 2016) revealed that biological processes related to the cell cycle and/or mitosis were enriched among the genes downregulated by miR-34a and
miR-34c, which confirmed previous findings that these miRNAs negatively regulate cell cycle progression in mouse epidermal keratinocytes (Antonini et al., 2010) (Supplementary Figure S4e and f). Interestingly, for the genes upregulated by miR-34a and miR-34c, most enriched processes were related to inflammatory response, which was previously unknown (Supplementary Figure S4e and f). Similar results were obtained in Gene Ontology analysis of these microarray data for molecular functions and signaling pathways (Supplementary Figure S5). These results prompted us to explore further the biological function of miR-34a and miR-34c, in particular, in regulating keratinocyte inflammatory response.

MiR-34a and miR-34c promote keratinocyte inflammatory response

We analyzed the effect of miR-34 family members on keratinocyte inflammatory response by measuring cytokine and chemokine production. To this end, we treated keratinocytes with tumor necrosis factor-α, which is a cytokine highly expressed in human chronic wounds (Wallace and Stacey, 1998) and a strong trigger of innate immune response of keratinocytes. QRT-PCR analysis showed that both miR-34a and miR-34c significantly upregulated the mRNA expression levels of tumor necrosis factor-α, IL-1β, IL-8, CXCL1, CXCL5, CCL2, and CCL20 in keratinocytes under both basal and inflammatory conditions (Figure 2a). Accordingly, the amounts of IL-8 and CXCL5 proteins secreted into the culture medium were also increased by transfection of these two miRNAs mimics, as shown by ELISA (Figure 2b). Of note, cotransfection of miR-34a and miR-34c mimics enhanced IL-8 expression to a similar level as transfection of miR-34a or miR-34c alone, suggesting that these two miRNAs are unlikely to act in a synergistic manner (Supplementary Figure S6a). In addition, we performed a loss-of-function study by transfecting keratinocytes with miRNA-specific inhibitors (Supplementary Figure S7). Inhibition of either of these two miRNAs significantly reduced the expression of IL-8, CXCL1, and CXCL5 in keratinocytes (Figure 2c and d). Furthermore, we found that the combination of miR-34a and miR-34c inhibitors reduced IL-8 expression to a lower level than the inhibition of miR-34a or miR-34c separately, indicating that these two miRNAs are not functionally redundant (Supplementary Figure S6b). In line with previous findings, we also showed that treatment with miR-34a or miR-34c inhibited keratinocyte proliferation and migration and the expression of proliferation marker gene Ki67, while promoting cell apoptosis (Supplementary Figure S8a–d). Collectively, the in vitro functional study revealed that miR-34a and miR-34c can trigger and enhance the production of inflammatory mediators by keratinocytes, in addition to their antiproliferative, antimigratory, and proapoptotic effects.

MiR-34a delays wound closure in vivo

To study the effect of miR-34 in skin wound healing in vivo, we injected miR-34a mimics intradermally into the wound edges on the dorsal skin of C57BL/6N mice immediately following skin injury (Figure 3a). QRT-PCR showed that this local injection led to an effective but transient increase of miR-34 level in the wounds, which lasted less than 6 days (Figure 3b). Interestingly, we found that the miR-34a treatment significantly delayed wound closure macroscopically (Figure 3c and d), which was confirmed by a histomorphometry analysis showing that the lengths of the newly formed epithelial tongues were significantly decreased in the miR-34a–treated wounds compared with the wounds injected with control oligos (Figure 3e and f). Moreover, in miR-34a–treated wounds, increased expression of inflammatory cytokine and chemokine genes Tnfα, Il1a, Il1b, Cxcl5, and Ccl2, and decreased expression of Mki67, was detected by qRT-PCR (Figure 3g). In line with this, more CD45+ immune cells were observed in miR-34a–treated wounds (Figure 3h). Taken together, these data demonstrate that miR-34 treatment delayed wound closure and enhanced the inflammatory response in vivo.

MiR-34a and miR-34c target LGR4 in keratinocytes

To probe into the molecular mechanism underlying the biological effect of the miR-34 family, we sought for their target genes in keratinocytes. To this end, we performed an integrated analysis using 11 independent miRNA target prediction algorithms (Dweep et al., 2011), which generated a short list of putative miR-34 targets. Comparing this list with the miR-34–downregulated genes in keratinocytes shown by microarray analysis (Supplementary Figure S4), we identified five overlapping genes, including SYT1, SGPP1, LGR4, PACS1, and TAF5 (Supplementary Figure S9a–d). Surveying their expression in human skin, normal wound, and VU samples, we found that LGR4 exhibited a significant negative-correlated expression pattern with miR-34 in vivo (Figure 4a–c, Supplementary Figure S9e–h). LGR4, a leucine-rich, G protein–coupled receptor, carried one conserved binding site for miR-34 in its 3′ untranslated region (Figure 4d). Luciferase reporter assays showed that both miR-34a and miR-34c decreased the expression of the reporter gene construct containing the 3′ untranslated region of LGR4 mRNA, whereas this effect was abolished by mutation of the miR-34 binding site, demonstrating that LGR4 is a direct target of miR-34a and miR-34c (Figure 4e). In line with this, transfection with miR-34a or miR-34c decreased LGR4 expression at both mRNA and protein levels, whereas their inhibition increased LGR4 expression in keratinocytes (Figure 4f–h). Furthermore, immunohistochemistry staining of human skin and wound samples revealed that LGR4 protein was mainly present in the epidermis and lower LGR4 expression was detected in the VUs than the normal wounds and intact skin (Figure 4i). The reciprocal pattern of LGR4 and miR-34 expression both in human keratinocytes in vitro and in wound biopsies in vivo further support that LGR4 is a direct target of miR-34.

LGR4 mediates the biological function of miR-34a and miR-34c in keratinocytes

LGR4 has been shown to promote keratinocyte proliferation through epidermal growth factor receptor signaling (Lin et al., 2008; Kato et al., 2007; Wang et al., 2010), whereas its role in the innate immune response of keratinocytes remained unexplored. To determine whether the biological function of miR-34 in keratinocytes is, at least partially, mediated through LGR4, we analyzed the effects of LGR4 silencing on inflammatory response in
keratinocytes. QRT-PCR analysis showed that the reduced LGR4 expression led to significantly increased keratinocyte production of neutrophil-attracting chemokines, such as IL-8, CXCL1, and CXCL5, under both basal and inflammatory conditions (Supplementary Figure S10a–c). This finding was confirmed further by ELISA of IL-8 and CXCL5 proteins in the conditioned medium from keratinocytes treated as described (Supplementary Figure S10d). In line with this, chemotaxis assays showed that the conditioned medium from LGR4-silenced keratinocytes attracted more neutrophils, which were isolated from human peripheral blood, than the medium from control-treated cells (Supplementary Figure S10e). Interestingly, we found that the inhibitory effect of blockage of miR-34a on chemokine expression was reversed after silencing LGR4 expression, supporting that miR-34s exert their proinflammatory function through targeting LGR4 in keratinocytes (Supplementary Figure S10f).

Figure 2. MiR-34a and miR-34c promote keratinocyte inflammatory response. (a) QRT-PCR analysis of cytokine and chemokine expression in keratinocytes transfected with miR-34a or miR-34c mimics followed by TNF-α treatment (n = 3). (b) Quantification of IL-8 and CXCL5 protein levels by ELISA in conditioned medium of keratinocytes treated as in (a). (c) QRT-PCR analysis of chemokine expression in keratinocytes transfected with In-miR-34a or In-miR-34c (n = 3). (d) ELISA of IL-8 protein in conditioned medium of keratinocytes upon inhibition of miR-34a or miR-34c followed by TNF-α treatment (n = 3). The data are presented as mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test. In, inhibitor; miR-34a, microRNA-34a; miR-34c, microRNA-34c; miR-con, microRNA control; QRT-PCR, quantitative real-time reverse transcriptase–PCR; s.d., standard deviation; TNF-α, tumor necrosis factor-α.

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Figure 3. Treatment with miR-34a delays wound closure in vivo. (a) MiR-34a or miR-con were injected intradermally into the wound edges of C57BL/6N mice immediately after wounding. (b) QRT-PCR analysis of miR-34a expression in the wound edges at the indicated time points after injury (5 hour and Day 4: n = 4/group, Day 2: n = 6/group, Day 6: n = 11–13/group). (c) Wound closure was quantified and presented as % wound closure = 100% – the percentage of the initial wound area size (n = 10–13/group). (d) Representative photographs of wounds on days 0–6 postwounding. (e) Representative pictures of H&E-stained wound tissues 6 days after wounding. Dashed lines mark the newly formed epithelial tongue. (f) Quantification of the length of epithelial tongues (n = 5 wounds/group; three noncontinued sections were evaluated for each wound). (g) QRT-PCR analysis of Tnf, Il1a, Il1b, Cxcl5, Ccl2, and Mki67 expression in the day 6 wounds treated with miR-34a (n = 11) or control mimics (n = 13). (h) Representative photographs and quantification of immunostaining of CD45+ cells in the day 6 wounds treated with miR-34a. The data are presented as mean ± s.e.m. (b, c, f, g) or mean ± s.d. (h). *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test (b, f, g, h) or two-way ANOVA (c). ANOVA, analysis of variance; H&E, hematoxylin and eosin; miR-34a, microRNA-34a; miR-34c, microRNA-34c; miR-con, microRNA control; QRT-PCR, quantitative real-time reverse transcriptase–PCR; s.d., standard deviation; s.e.m., standard error of the mean. (e) Bar = 500 µm. (h) Bar = 100 µm.
Figure 4. LGR4 is targeted by miR-34a and miR-34c in keratinocytes. (a) QRT-PCR analysis of LGR4 in the skin and NW biopsies from healthy donors (n = 7) and in VUs (n = 19). (b, c) Spearman correlation of LGR4 with (b) miR-34a or (c) miR-34c expression values in the skin, NWs (black dots), and VUs (red dots). (d) Nucleotide resolution of the predicted miR-34 binding site in the 3′-UTR of LGR4 mRNA: seed sequence (green letters), mutated binding sites (red letters). (e) Luciferase activity was measured in keratinocytes transfected with reporter plasmids containing WT or Mut LGR4 3′-UTR together with miR-34a, miR-34c, or control mimics (n = 3). (f, g) LGR4 (f) mRNA and (g) protein in keratinocytes transfected with miR-34a or miR-34c were analyzed by qRT-PCR and western blot.
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Figure 5. Skin wound healing is impaired in Lgr4 KO mice. (a) QRT-PCR analysis of Lgr4 in the skin of Lgr4 KO mice (n = 4) and the littermate controls (WT, n = 4). (b) Representative photographs of wounds on the back of Lgr4 KO (n = 4) and WT mice (n = 4) on days 0–13 postwounding. (c) Wound closure was quantified and presented as % wound closure = 100% – the percentage of the initial wound area size. (d) Immunostaining of PCNA in wound-edge skin from WT and Lgr4 KO mice (n = 4) 3 days after injury. (e) QRT-PCR analysis of Tnf, Il1b, Il6, Cxcl1, Cxcl5, and Cxcl10 expression in the wound edges of WT and Lgr4 KO mice 3, 8, and 13 days after injury (n = 3). (f) Representative photographs and quantification of immunostaining of CD45+ cells in day 3 wound edges of Lgr4 KO mice (n = 3). The data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ****P < 0.0001 by Student’s t-test (a, e, f) or by two-way ANOVA (c). ANOVA, analysis of variance; KO, knockout; PCNA, proliferating cell nuclear antigen; QRT-PCR, quantitative real-time reverse transcriptase—PCR; s.e.m., standard error of the mean; WT, wild-type. Bar = 100 μm.

western blotting (n = 3). (h) QRT-PCR of LGR4 in keratinocytes with inhibited miR-34a or miR-34c. (i) Immunostaining of LGR4 in skin, NWs, and VUs (n = 3). The data are presented as mean ± s.e.m. (a) or mean ± s.d. (e, f, h). *P < 0.05, **P < 0.01, ****P < 0.0001 by Mann-Whitney U test (a) or Student’s t-test (e, f, h). FC, fold change; In, inhibitor; miR-34a, microRNA-34a; miR-34c, microRNA-34c; miR-con, microRNA control; Mut, mutated; NW, normal wound; QRT-PCR, quantitative real-time reverse transcriptase—PCR; s.d., standard deviation; s.e.m., standard error of the mean; UTR, untranslated region; VU, venous ulcer; WT, wild-type. Bar = 100 μm.
Lack of LGR4 impairs wound healing in vivo

To determine the role of LGR4 in skin wound repair in vivo, we examined the healing capacity of Lgr4 knockout (KO) mice (Luo et al., 2009; Weng et al., 2008). Absence of Lgr4 expression in the skin of this mouse model was confirmed by qRT-PCR (Figure 5a). We found that wound closure of Lgr4 KO mice was delayed significantly compared with the littermate controls (Figure 5b and c). Moreover, fewer proliferating cells presented in the wound-edge epidermis of Lgr4 KO mice than the control mice, shown by immunostaining of a cell proliferation marker PCNA (Figure 5d) (Hall et al., 1990; Mazerbourg et al., 2004). We also performed qRT-PCR to analyze the expression of cytokines (Tnf, Il1b, and Il6) and chemokines (Cxcl1, Cxcl5, and Cxcl10) in mice skin wounds 3, 8, and 13 days after injury. Although there were different expression dynamics during wound healing, the levels of all these cytokines and chemokines were increased in the wounds of Lgr4 KO mice compared with the control mice (Figure 5e). In line with this, more CD45+ immune cells were observed in the wound-edge dermis of Lgr4 KO mice than in control mice (Figure 5f). Together, these data showed that lack of Lgr4 resulted in impaired wound closure with enhanced inflammation, which was similar to the miR-34 mimics–treated skin wounds (Figure 3).

MiR-34–LGR4 axis regulates the activity of NF-κB signaling

We sought to address the question of how the miR-34–LGR4 axis regulates keratinocyte inflammatory response. Gene set enrichment analysis of the microarray data of miR-34a– or miR-34c–transfected keratinocytes revealed that a set of NF-κB signaling–related genes (http://www.bu.edu/nf-kb/gene-resources/target-genes/) were enriched significantly among the miR-34–upregulated genes (Figure 6a and b, Supplementary Tables S4 and S5). This suggested that miR-34 might regulate the NF-κB pathway, which is a central signaling pathway controlling the expression of multiple genes important for keratinocyte inflammatory response, such as Tnf, Il1b, Cxcl1, Cxcl5, Cxcl8, Ccl2, and Ccl20 that were analyzed in our study (Tak and Firestein, 2001). In line with the results of the gene set enrichment analysis, miR-34a and miR-34c increased both basal and tumor necrosis factor–α–induced luciferase activity in keratinocytes transfected with the NF-κB–dependent luciferase reporter, demonstrating that these two miRNAs upregulate the activity of NF-κB signaling (Figure 6c). Enhanced expression of the luciferase reporter gene was also observed in keratinocytes with silenced Lgr4 expression, suggesting that LGR4 negatively regulates NF-κB signal (Figure 6d).

We next investigated how the miR-34–LGR4 axis modulated the activity of the NF-κB signaling pathway, which has been known to be controlled at several levels, including phosphorylation of the DNA-binding subunit p65 (Christian et al., 2016). Previous studies have shown that activation of LGR4 signaling inhibited GSK-3β Ser9 phosphorylation, thus increasing the proportion of the unphosphorylated GSK-3β that is the active form (Luo et al., 2016; Zhang et al., 2003). GSK-3β has been identified as a protein kinase phosphorylating p65 at Ser468, which negatively regulates basal NF-κB activity (Buss et al., 2004). Based on this knowledge, we hypothesized that LGR4 might suppress NF-κB signaling activity via the increase of p65 Ser468 phosphorylation. This model was supported by the results of Western blot analysis of Lgr4 KO mouse skin (Figure 6e). Upon absence of Lgr4 expression, we found increased GSK-3β Ser9 phosphorylation and decreased p65 Ser468 phosphorylation. Interestingly, we also observed a higher protein level of total p65 in the skin of Lgr4 KO mice, which is in line with previous findings showing that phosphorylation of p65 Ser468 led to p65 ubiquitination and proteasomal degradation (Geng et al., 2009). Moreover, we found higher amounts of phosphorylated p65 at Ser536 in Lgr4 KO mice, which is required for the activation of the NF-κB signaling pathway (Christian et al., 2016). Furthermore, in human keratinocytes, Western blot analysis showed that either transfection with miR-34 or silencing LGR4 reduced phosphorylation of p65 at Ser468 while increasing phosphorylation at Ser536, enhancing NF-κB signal (Figure 6f–i). Collectively, our data supported a model that LGR4 via GSK-3β negatively regulated NF-κB signal activity. Increased miR-34a and miR-34c with decreased LGR4 expression in the wound-edge keratinocytes of human VUs may contribute to the excessive inflammation (Supplementary Figure S11).

DISCUSSION

Our study identified miR-34a and miR-34c as two upregulated miRNAs in VUs compared with the normal human skin or acute wounds under healing. Increased miR-34 expression leads to enhanced keratinocyte inflammatory response and delayed wound closure, suggesting their pathological role in chronic nonhealing wounds.

MiR-34 family members are well known for their anti-proliferative functions, particularly in a wide range of cancer types (Maroof et al., 2014; Misso et al., 2014); however, their role in inflammation has been much less investigated. Recently, a clinical trial of miR-34 replacement therapy in cancer patients was terminated because of severe immune-related adverse events, which presents an urgent need to understand the impact of miR-34 in immune response. To this end, emerging studies have shown that miR-34a perturbs B lymphocyte development (Rao et al., 2010) while facilitating dendritic cell differentiation (Hashimi et al., 2009). In neutrophils, miR-34a impairs cell migration (Cao et al., 2015) but increases tumor necrosis factor production (Shikama et al., 2016). In addition, it has been shown to inhibit the efferocytosis capacity of tissue macrophages (McCubbrey et al., 2016). Here we found that miR-34a and miR34c increased keratinocyte production of proinflammatory cytokines and chemokines, potentially enhancing their capacity to recruit and activate immune cells in the wounds. Based on this knowledge, we propose that the high expression of miR-34 in VUs may contribute to the deregulated wound inflammation, including the excessive and constant presence of neutrophils and macrophages, and overproduction of proinflammatory cytokines but inefficiency in clearing infection (Eming et al., 2014).

Further mechanistic study identified LGR4 as a major target gene mediating the biological function of miR-34 in epidermal keratinocytes. LGR4 plays important roles in organ development (Hoshii et al., 2007; Kinzel et al., 2014;
Figure 6. MiR-34–LGR4 axis regulates NF-κB signaling. (a, b) GSEA-evaluated enrichment within the microarray data of keratinocytes transfected with (a) miR-34a or (b) miR-34c for the reported target genes of NF-κB pathway. (c, d) Luciferase activity was measured in keratinocytes transfected with NF-κB reporter plasmids together with (c) miR-34a, miR-34c, or miR-con mimics, or (d) si-LGR4 or si-Con, followed by TNF-α treatment (n = 3). (e) Total and Ser9-phosphorylated GSK-3 and total and Ser536-phosphorylated p65 were detected by western blotting in the skin of Lgr4 KO and WT mice (n = 4). (f, g) Total and Ser536-phosphorylated p65 were detected by western blotting in keratinocytes transfected with (f) miR-34a, miR-34c, or miR-con or (g) si-LGR4 or si-Con for 48 hours followed with TNF-α and Calyculin A treatment for 15 minutes. β-actin was used as a loading control. The data are presented as mean ± s.d. (c, d). *P < 0.05, **P < 0.01 by Student’s t-test. GSEA, gene set enrichment analysis; KO, knockout; miR-34a, microRNA-34a; miR-34c, microRNA-34c; miR-con, microRNA control; NES, normalized enrichment score; Ser, serine; si-Con, control small interfering RNA; si-LGR4, LGR4 small interfering RNA; TNF-α, tumor necrosis factor-α; WT, wild-type.
Luo et al., (2013), stem cell functions (Wang et al., 2013), and tumor growth (Gong et al., 2015; Liang et al., 2015). In the skin, it has been shown to positively regulate keratinocyte proliferation (Jin et al., 2008; Kato et al., 2007; Wang et al., 2010) and hair placode formation (Mohri et al., 2008). Importantly, we discovered that LGR4 was crucial in restricting the inflammatory response of keratinocytes. Of note, the anti-inflammatory role of LGR4 has been reported in macrophages treated with bacterial lipopolysaccharide (Du et al., 2013). LGR4 also promotes M2 polarization of tumor associated—macrophages, keeping an immunosuppressive environment to facilitate tumor progression (Tan et al., 2018). As macrophages are major players in innate immune response during wound healing (Bonakowski et al., 2017), it would be interesting to examine the role of the miR-34–LGR4 axis in wound macrophages. Our in vivo studies using mice models with miR-34 treatment or Lgr4 KO further demonstrated that an aberrant miR-34–LGR4 axis impaired proper inflammatory response and re-epithelialization during wound repair. The enhanced miR-34 with deficient LGR4 expression in wound-edge keratinocytes of human VUs supports their pathological role in hard-to-heal wounds.

We next investigated the molecular basis of the anti-inflammatory effect of LGR4 in keratinocytes. As a leucine-rich, G-protein–coupled receptor (Weng et al., 2008), several endogenous ligands have been identified to bind to LGR4 and activate different signaling pathways; R-spondin and norrin enhance Wnt signaling (Carmon et al., 2011; Deng et al., 2013), whereas TNFSF11 induces heterotrimERIC G-protein signaling (Luo et al., 2016). Activation of LGR4 increases the activity of its downstream GSK-3β via the inhibition of GSK-3β Ser9 phosphorylation (Luo et al., 2016; Zhang et al., 2003). As a multifunctional Ser/threonine kinase, GSK-3β has been shown to phospho-rylate p65 Ser468, which negatively regulates NF-κB signaling (Buss et al., 2004; Geng et al., 2009). Here we integrated these clues and proved the LGR4-induced GSK-3β regulation of p65 Ser468 phosphorylation both in human keratinocytes in vitro and in mouse skin in vivo. Interestingly, the negative regulation of NF-κB signaling by LGR4 has also been found in osteoclast differentiation (Luo et al., 2016) and in macrophages treated with lipopolysaccharide (Du et al., 2013). In osteoclasts, LGR4 competes with TNFRSF11A for TNFSF11 binding, thus reducing the canonical TNFRSF11A cascade including NF-κB signaling (Luo et al., 2016). In macrophages, via classical G-protein signaling, LGR4 downregulates the expression of CD14, which is a coreceptor for the detection of lipopolysaccharide (Du et al., 2013). However, these molecular mechanisms are unlikely to act in epidermal keratinocytes because of a lack of TNFSF11 and CD14 expression (Uhlén et al., 2015).

Collectively, our study revealed that miR-34a and miR-34c, by targeting LGR4, enhanced the inflammatory response of epidermal keratinocytes. As upregulated miRNAs in human VUs, miR-34a and miR-34c may play a pathological role by contributing to the persistent inflammation in chronic wounds. It warrants further study if miR-34 may be targeted transiently and locally for VU treatment.

MATERIALS AND METHODS

Human wound samples

Both healthy donors (n = 21, Supplementary Table S3) and patients with VU (n = 24, Supplementary Table S2) were enrolled at the Dermatology Clinic at Karolinska University Hospital (Stockholm, Sweden). The exclusion criteria for healthy donors were diabetes, skin disease, unstable heart disease, infections, bleeding disorder, and immune suppression. One or two full-thickness excisional wounds were created using a 4-mm biopsy punch on the skin of each healthy 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 one or seven days later. Patients with nonhealing VUs (C6 venous disease) that persisted for more than 3 months were enrolled in this study. Excluded were patients with apparent soft tissue infection and need of systemic antibiotics, patients taking systemic antibiotics 24 hours before biopsy, and immunocompromized patients. Tissue samples were taken using a 4-mm biopsy punch at the nonhealing edges of chronic wounds. Local lidocaine injection was used for anesthesia while sampling.

Statistics

Statistical significance was determined by two-tailed Student’s t-test, Wilcoxon matched pairs signed rank test, or Mann-Whitney U test. Differences between groups were computed using two-way repeated-measures analysis of variance. The correlation between the expressions of different genes in the same sample was made using Pearson’s correlation test. For all statistical tests, P-values < 0.05 were considered to be statistically significant.

Study approval

The clinical materials were obtained with written, informed patient consent, and the study was approved by the Stockholm Regional Ethics Committee and conducted according to the Declaration of Helsinki’s principles. All procedures involving mice were approved by the North Stockholm Ethical Committee for Care and Use of Laboratory Animals.

The experimental protocols for cell culture and treatments, laser capture microdissection, RNA extraction and qRT-PCR, small RNA sequencing and data analysis, gene expression microarray, leukocyte chemotaxis assay, protein detection, histological analysis, luciferase reporter assays, in vivo wound model, cell proliferation assay, and cell migration and cell apoptosis analysis are detailed in Supplementary Materials and Methods.

Data availability statement

Datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE117506, hosted at National Center for Biotechnology Information’s Gene Expression Omnibus.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: JW, XL, XY, NXL; Data Curation: JW, XY, NXL; Formal Analysis: XY, NXL; Investigation: JW, XL, DL, XR, YL, EKH, MQ, MAT, AMW, IGS, OR, MS, JDW; Methodology: XY, NXL; Writing - Original Draft Preparations: NXL, JW, XL; Writing - Review and Editing: JW, XL, DL, XR, YL, EKH, MQ, MAT, AMW, IGS, OR, MS, JDW, XY, 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.2019.07.694.

REFERENCES

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SUPPLEMENTARY MATERIALS AND METHODS

Cell culture and treatments
Human adult epidermal keratinocytes were purchased from Cascade Biologics (Portland, OR) and cultured in EpiLife serum-free keratinocyte growth medium supplemented with Human Keratinocyte Growth Supplement at a final calcium concentration of 0.06 mM and Pen Strep (100 units/ml penicillin and 100 µg/ml streptomycin) (Thermo Fisher Scientific, Waltham, MA) at 37 °C in 5% CO2.

Forth-passage keratinocytes at 50% confluency were transfected with 20 nM mimics of microRNA (miR)-34a-5p, miR-34c-5p, or microRNA mimic negative control (Thermo Fisher Scientific); 40 nM miR-34a-5p or miR-34c-5p miRCURY LNA miRNA Power Inhibitor or negative control A (Exiqon, Hilden, Germany); and 20 nM Silencer select pre-designed small interfering RNA for LGR4 (si-LGR4) or small interfering RNA negative control (Thermo Fisher Scientific) designed for LGR4 or small interfering RNA negative control for 48 hours using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Bowtie2 (2.2.2) was used for mapping the reads. Differential expression analysis was performed using the EdgeR statistical software package (Bioconductor, http://bioconductor.org/). For normalization, the trimmed mean of M-values method based on log-fold and absolute gene-wise changes in expression levels between samples (TMM normalization) was used.

Gene expression microarray
Expression profiling of primary human keratinocytes transfected with 20 nM miR-34a-5p, miR-34c-5p, or control mimics for 48 hours (at least triplicates) was performed using Affymetrix Genechip system at the Microarray core facility of Karolinska Institute. The data have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE117506. In brief, total RNA was extracted using miRNeasy Mini Kit (Qiagen) and RNA quality and quantity were determined using an Agilent 2100 Bioanalyzer (Agilent). Following the Affymetrix IVT Express Kit labeling protocol, standardized array processing procedures recommended by Affymetrix including hybridization, fluidics processing, and scanning were used. Genes showing at least two-fold regulation and false discovery rate less than 5% were considered to be significantly differentially expressed. Gene Ontology analysis was performed using Enrichr (Chen et al., 2013; Kuleshov et al., 2016). Gene set enrichment analysis was performed using public software from Broad Institute (Subramanian et al., 2005).

Leukocyte chemotaxis assay
Human primary neutrophils were isolated from 0.2% EDTA-anticoagulated whole blood collected by venipuncture from healthy donors. Erythrocytes were removed using dextran sedimentation (1:1 mixture of blood and 6% dextran/0.9% NaCl) followed by one or two rounds of hypotonic lysis using double-distilled H2O. Neutrophils were isolated from the resulting cell suspension using Ficoll-Histopaque (Sigma, St. Louis, MO) density centrifugation. Purified neutrophils were suspended in EpiLife serum-free keratinocyte growth medium and 3 × 105 cells were added to the inner chamber of a 3-µm polyethylene terephthalate membrane cell culture insert (BD Biosciences, San Diego, CA). The outer chamber contained conditioned medium from keratinocytes transfected with siLGR4 or small interfering RNA negative control for 48 hours. After incubation for 1.5 hours at 37 °C in 5% CO2, the samples were treated with 50 ng/ml rh-TNF-α (R&D Systems, Minneapolis, CA), IL1B, IL6, CXCL1, CXCL8, CCL2, CCL20, LGR4, TNF, IL11a, IL1b, IL6, Cxcl1, Cxcl5, Cxcl10, Ccl2, Mki67, and Lgr4 was analyzed by TaqMan-based predesigned quantitative PCR assays (Integrated DNA Technologies, Coralville, IA). Target gene expression levels were normalized based on the values of 18S ribosomal RNA in humans and Gapdh in mice. Information for all the primers and probes used in this study can be found in Supplementary Table S6.

Small RNA sequencing and data analysis
The small RNA next-generation sequencing was conducted at Exiqon Services (Vedbaek, Denmark). Briefly, the library preparation was done using the NEBNext Small RNA Library preparation kit (New England Biolabs, Ipswich, MA). A total of 500 ng of total RNA was converted into microRNA next-generation sequencing libraries. Adapters were ligated to the RNA. Then RNA was converted to cDNA. The cDNA was amplified using PCR (12 cycles) and indices were added during the PCR. After PCR, the samples were purified. Library preparation quality control was performed using either Bioanalyzer 2100 (Agilent, Santa Clara, CA) or TapeStation 4200 (Agilent). Based on quality of the inserts and the concentration measurements, the libraries were pooled in equimolar ratios. The pool was then size selected using the LabChip XT (PerkinElmer, Waltham, MA) aiming to select the fraction with the size corresponding to microRNA libraries (~145 nucleotides). The library pool(s) were quantified using the qPCR KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA). The library pool was then sequenced on a NextSeq 500 sequencing instrument according to the manufacturer instructions (Illumina, San Diego, CA). Fastq files were sorted and filtered to remove low-quality reads and adaptors. Removal of reads mapping to human, mouse, or rat genomes was performed using the Picard tool. A minimum mapping quality of 30 was required. Each sequencing run was split across multiple SRR files. Reads were质量 normalized using the trimmed mean of M-values method based on log-fold and absolute gene-wise changes in expression levels between samples (TMM normalization) was used.

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migrated neutrophils in the outer chamber were quantified using a FACScan flow cytometer (CyAn ADP, Beckman Coulter, Brea, CA).

**Protein detection**
Conditioned medium from keratinocytes culture was collected, and protein levels of IL-8 and CXCL5 were measured by ELISA (BioLegend, San Diego, CA) according to the manufacturer’s instructions. Protein lysates were extracted from keratinocytes using RIPA buffer (Thermo Fisher Scientific). LGR4, phosphorylated and total p65, phosphorylated and total GSK-3β, and β-actin were analyzed by western blotting using antibodies listed in Supplementary Table S6. Immune complexes were detected by Amersham ECL Prime Western Blotting Detection Reagent according to the manufacturer’s protocol (GE Healthcare, Buckinghamshire, United Kingdom).

**Histological analysis**
Paraffin-embedded tissue sections (6 μm in thickness) were deparaffinized and rehydrated followed by hematoxylin and eosin staining. The length of newly-formed epithelial tongue in mice wounds was quantified using ImageJ (National Institutes of Health, Bethesda, MD). Expression of LGR4, in mice wounds was quantified using ImageJ (National Institutes of Health), corrected for the area of the reference circle and expressed as a percentage of the original area. The mice were killed at the specified time points after injury, and wound edges were collected for histology and gene expression analysis. For wound closure analysis, to see the wound edge clearly, the scab was removed just before photographing every other day until euthanized. For histology analysis, to keep an intact epithelial tongue, we did not interrupt the wound healing process by removing the scab.

Lgr4 constitutive knockout mice were generated in previous studies (Luo et al., 2009; Weng et al., 2008). In brief, the Lgr4 (Gpr48) gene trap ES cell clones (LST020) were injected into C57BL/6 blastocysts and transferred to ICR females. Male chimera mice were mated with C57BL/6 females, resulting in transmission of the inserted allele to the germ line. Positive mice were interbred and maintained on a mixed 129 x C57BL/6 background. 11-week-old male Lgr4 knockout mice and the litterate controls were used in wound assays as described previously.

**Cell proliferation assay**
Cell proliferation was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, Promega) according to the manufacturer’s instructions.

**Analysis of cell migration**
For scratch assay, keratinocytes transfected with 20 nM miR-34a or miR-34c mimics were grown to full confluence, and a scratch was made with a 10-μl pipette tip. The cells were incubated with Epilife medium without Human Keratinocyte Growth Supplement and photographed at the indicated time points. The wound areas were measured using ImageJ.

**Cell apoptosis analysis**
Treated cells were harvested at 80% confluence and washed twice with ice-cold phosphate buffered saline. For cell apoptosis analysis, treated cells were harvested by trypsin without EDTA and stained with an FITC/Annexin V Apoptosis Detection Kit (BD Biosciences) for 15 minutes at room temperature following the manufacturer’s instructions. Flow cytometry was then performed to determine the percentage of apoptotic cells.

**SUPPLEMENTARY REFERENCES**


Supplementary Figure S1. The expression of the miR-34 family in normal human skin and wound. The expression of the miR-34 family was analyzed by small RNA sequencing in the skin and day 7 NWs from seven healthy donors. miR-34, microRNA-34; miR-34a, microRNA-34a; miR-34b, microRNA-34b; miR-34c, microRNA-34c; NW, normal wound; TPM, transcripts per kilobase million.
Supplementary Figure S2. Representative images of hematoxylin staining of tissue sections before and after LCM. The epidermis was isolated by LCM from the skin and NWs of seven healthy donors and from the wound edges of seven VUs. Red arrows indicate wound-edges. LCM, laser capture microdissection; NW, normal wound; VU, venous ulcer. Bar = 320 μm.

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Supplementary Figure S3. Treatment of keratinocytes with miR-34a or miR-34c. (a, b) QRT-PCR analysis of (a) miR-34a or (b) miR-34c in human primary keratinocytes transfected with 20 nM miR-34a, miR-34c, or control mimics for 24 hours followed with TNF-α treatment for another 24 hours (n = 3). The data are presented as mean ± s.d. **P < 0.01, ***P < 0.001, ****P < 0.0001 by Student’s t-test. miR-34a, microRNA-34a; miR-34c, microRNA-34c; miR-con, microRNA control; QRT-PCR, quantitative real-time reverse transcriptase-PCR; s.d., standard deviation; TNF-α, tumor necrosis factor-α.
Supplementary Figure S4. Transcriptome analysis of keratinocytes transfected with miR-34a or miR-34c. (a) PCA was performed on the microarray data of keratinocytes transfected with miR-34a or miR-34c ($n = 3$–$4$ per group). (b) Graphs show the numbers of significant genes (FDR < 0.05) with more than two-fold increase (red) or decrease (blue) in expression levels between the indicated groups; the Venn diagram shows the extent of overlap between the miR-34a- and miR-34c-regulated genes. (c, d) GSEA-evaluated enrichment within the microarray data of keratinocytes transfected with (c) miR-34a or (d) miR-34c for the predicted miR-34a/c targets. Vertical bars along the x-axis denote the positions of predicted target genes within the ranked list. (e, f) Top 10 Gene Ontology biological process terms for the genes up- (red) or downregulated (blue) by (e) miR-34a and (f) miR-34c in keratinocytes. P-values were determined by Fisher’s exact test. FDR, false discovery rate; GSEA, gene set enrichment analysis; miR-34a, microRNA-34a; miR-34c, microRNA-34c; miR-con, microRNA control; NES, normalized enrichment score; PCA, principal component analysis.
Supplementary Figure S5. GO analysis of miR-34-regulated genes in keratinocytes. Top 10 GO molecular function and KEGG terms for the genes up-regulated or downregulated by miR-34a or miR-34c in keratinocytes. P-values were determined by Fisher’s exact test. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; miR-34, microRNA-34; miR-34a, microRNA-34a; miR-34c, microRNA-34c.
Supplementary Figure S6. MiR-34a and miR-34c promote keratinocyte IL-8 expression. (a) QRT-PCR analysis of IL-8 expression in keratinocytes transfected with miR-34a or miR-34c mimics or a combination of miR-34a and miR-34c (n = 3). (b) QRT-PCR analysis of IL-8 expression in keratinocytes transfected with In-miR-34a or In-miR-34c (n = 3) or a combination of In-miR-34a and In-miR-34c. The data are presented as mean ± s.d.. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test. In, inhibitor; miR-34, microRNA-34; miR-34a, microRNA-34a; miR-con, microRNA control; QRT-PCR, quantitative real-time reverse transcriptase–PCR; s.d., standard deviation.

Supplementary Figure S7. Inhibition of miR-34 in keratinocytes. Human primary keratinocytes were transfected with 20nM or 40nM In-miR-34a, In-miR-34c, or In-miR-con. (a, b) The levels of (a) miR-34a and (b) miR-34c were examined using qRT-PCR (n = 3). The data are presented as mean ± s.d.. *P < 0.05, **P < 0.01 by Student’s t-test. con, control; In, inhibitor; miR-34, microRNA-34; miR-34a, microRNA-34a; miR-34c, microRNA-34c; qRT-PCR, quantitative real-time reverse transcriptase–PCR; s.d., standard deviation.
Supplementary Figure S8. MiR-34 suppresses keratinocyte proliferation and migration while promoting cell apoptosis. Keratinocytes were transfected with miR-34a or miR-34c mimics for 48 hours. (a) Cell proliferation was determined at the indicated time points by MTS assay (n = 3). (b) The expression data of proliferation marker Ki67 were extracted from a microarray analysis of miR-34a or miR-34c-transfected keratinocytes. (c) Keratinocyte migration was evaluated by scratch assays (n = 3). (d) Apoptosis was measured in miR-34a- or miR-34c-transfected keratinocytes by Annexin V-FITC/PI double staining and then analyzed with flow cytometry of cells (n = 3). The data are presented as mean ± s.d., *P < 0.05, **P < 0.01, ****P < 0.0001 by two-way ANOVA (a, c) or Student’s t-test (b, d). ANOVA, analysis of variance; con, control; FITC, fluorescein isothiocyanate; miR-34, microRNA-34; miR-34a, microRNA-34a; miR-34c, microRNA-34c; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PI, propidium iodide; s.d., standard deviation.
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Supplementary Figure S9. A search of a clinically-relevant miR-34 target in keratinocytes. (a, c) Venn diagram depicts the number of the genes significantly up- (blue) or downregulated (pink) (absolute fold change ≥ 2, \( P < 0.05 \)) by miR-34a or miR-34c shown in microarray analysis and the number of potential targets of miR-34a or miR-34c predicted by 11 microRNA target prediction algorithms (yellow). The genes downregulated by miR-34a or miR-34c and predicted to contain their binding sites are listed in (b, d). (e–h) The expression data of SYT1, SGPP1, PACS1, and TAF5 were extracted from a microarray analysis of the skin and NW biopsies from seven healthy donors and wound-edge biopsies from six VUs. miR-34, microRNA-34; miR-34a, microRNA-34a; miR-34c, microRNA-34c; n.s., not significant by Mann-Whitney U test; NW, normal wound; VU, venous ulcer.
**Supplementary Figure S10. LGR4 mediates the biological functions of miR-34a and miR-34c in keratinocytes.** (a, b) LGR4 expression was analyzed by (a) qRT-PCR and (b) western blotting in keratinocytes transfected with si-LGR4 or si-Con followed by TNF-α treatment (n = 3). (c) QRT-PCR analysis of chemokine expression in keratinocytes treated as described (n = 3). (d) ELISA of IL-8 and CXCL5 proteins in the conditioned medium of keratinocytes treated as described (n = 3). (e) Human neutrophil chemotaxis toward the conditioned medium from keratinocytes transfected with si-Con or si-LGR4. The migrating cells were quantified by flow cytometry (n = 3). Plots showing FSC/SSC of the migrated cells. (f) QRT-PCR analysis of LGR4 and chemokine expression in keratinocytes cotransfected with si-LGR4 and miR-34a inhibitors followed by TNF-α treatment (n = 3). The data are presented as mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test. FSC, front scatter; miR-34a, microRNA-34a; miR-34c, microRNA-34c; qRT-PCR, quantitative real-time reverse transcriptase–PCR; s.d., standard deviation; si-Con, control small interfering RNA; si-LGR4, LGR4 small interfering RNA; SSC, side scatter; TNF-α, tumor necrosis factor-α.
Supplementary Figure S11. Schematic summary of the proinflammatory role of the miR-34–LGR4 axis in wound-edge keratinocytes. LGR4 via GSK-3β negatively regulated NF-κB signal activity. By targeting LGR4, miR-34 enhanced the inflammatory response of keratinocytes. Increased miR-34a and miR-34c, whereas decreased LGR4 expression in the wound-edge keratinocytes of human VUs may contribute to the excessive inflammation. miR-34, microRNA-34; miR-34a, microRNA-34a; miR-34c, microRNA-34c; NW, normal wound; Ser, serine; VU, venous ulcer.