WAKMAR2, a Long Noncoding RNA Downregulated in Human Chronic Wounds, Modulates Keratinocyte Motility and Production of Inflammatory Chemokines

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Chronic wounds represent a major and growing health and economic burden worldwide. A better understanding of molecular mechanisms of normal as well as impaired wound healing is needed to develop effective treatment. Herein we studied the potential role of long noncoding RNA LOC100130476 in skin wound repair. LOC100130476 is an RNA polymerase II–encoded polyadenylated transcript present in both cytoplasm and nucleus. We found that its expression was lower in wound-edge keratinocytes of human chronic wounds compared to normal wounds of healthy donors and intact skin. In cultured keratinocytes, LOC100130476 expression was induced by TGF-β signaling. By reducing LOC100130476 expression with antisense oligos or activating its transcription with CRISPR/Cas9 Synergistic Activation Mediator system, we showed that LOC100130476 restricted the production of inflammatory chemokynes by keratinocytes, while enhancing cell migration. In line with this, knockdown of LOC100130476 impaired re-epithelization of human ex vivo wounds. Based on these results, we named LOC100130476 wound and keratinocyte migration–associated long noncoding RNA 2 (WAKMAR2). Moreover, we identified a molecular network that may mediate the biological function of WAKMAR2 in keratinocytes using microarray. In summary, our data suggest that WAKMAR2 is an important regulator of skin wound healing and its deficiency may contribute to the pathogenesis of chronic wounds.


INTRODUCTION

Wound healing is fundamental to keep our skin intact. Normal wound repair process consists of sequential and overlapping phases, that is, inflammation (up to day 3 after injury), proliferation (days 4–21), and remodeling (week 4 to 1 year) (Reinke and Sorg, 2012). Pathologic factors, for example, venous insufficiency and diabetes mellitus, often impair normal wound healing and result in chronic non-healing wounds. Chronic wounds represent a major and growing health and economic burden to our society, affecting 1.3% of the adult population in developed countries, and cost more than $28 billion per year in the United States alone (Margolis, 2013; Nussbaum et al., 2018). Insufficient understanding of the pathophysiology of chronic wounds hampers the development of effective targeted treatment, which is considered a major unmet medical need to date (Frykberg and Banks, 2015).

Keratinocytes, which constitute 95% of the epidermis (Kolarsick et al., 2011), play critical roles in skin wound healing. In the inflammatory phase, keratinocytes produce several cytokines and chemokines, for example, CXCL5, CXCL8/IL8, and CCL20, thus triggering and modulating the inflammatory response (Roupe et al., 2010; Strbo et al., 2014). In the proliferation phase, their growth and migration are essential for re-epithelialization (Pastar et al., 2014). The transition from inflammation to proliferation is critical for wound healing, because excessive and prolonged inflammation has been shown to impede the healing process, as observed in chronic wounds (Landen et al., 2016). In chronic non-healing wound-edges, keratinocytes are hyperproliferative but fail to migrate (Stojadinovic et al., 2005). Long noncoding RNAs (lncRNAs) are a large and diverse class of RNA molecules that are longer than 200 nucleotides
and lack protein-coding capacity (Bhat et al., 2016; Kung et al., 2013). Although more than 100,000 IncRNAs have been detected in humans (Volders et al., 2013, 2015), very few have been characterized in detail, and their importance in regulating gene expression has been recognized only recently (Iyer et al., 2015). Notably, several IncRNAs have been revealed to play pivotal roles in skin biology and diseases. For example, TINCR and ANCR modulate epithelial differentiation (Kretz et al., 2012, 2013; Lopez-Pajares et al., 2015). TSIX (Wang et al., 2016) and PRINS have been shown to be involved in the pathogenesis of systemic sclerosis and psoriasis, respectively (Sonkoly et al., 2005; Szell et al., 2016). However, the expression pattern and biologic functions of IncRNAs in skin wound healing remain largely unexplored (Herter and Xu Landen, 2017).

Recently, Tsai et al. (2015) profiled IncRNA expression in psoriatic skin, revealing LOC100130476 as top downregulated in lesional compared with nonlesional skin of psoriasis patients, which is a chronic inflammatory skin disease sharing some features with wounded skin, for example, epidermis thickening and inflammation (Morhenn et al., 2013; Nickoloff et al., 2006). Also, LOC100130476 was recently reported to be downregulated in esophageal squamous cell carcinoma (Guo et al., 2016a). Among the 53 human tissues characterized in the Genotype-Tissue Expression Project (Lonsdale et al., 2013), the skin has the highest LOC100130476 expression, suggesting its functional role in the skin. Moreover, the genomic locus of LOC100130476 is interesting; it is transcribed in antisense direction and overlaps partially with the gene body and promoter of TNFAIP3, which is a critical brake on NF-kB signaling and its absence in keratinocytes is sufficient to exacerbate inflammatory skin disorders by increasing cytokine and chemokine expression (Devos et al., 2019). Based on these facts, we decided to examine the expression and function of LOC100130476 in human skin wounds. We found that the level of LOC100130476 in wound-edge keratinocytes of human chronic wounds was lower compared with intact skin and normal wounds of healthy donors. In addition, we showed that deficiency of LOC100130476 resulted in enhanced production of inflammatory chemokines and reduced motility of keratinocytes, impairing re-epithelialization of human ex vivo wounds. Therefore, we named LOC100130476, wound and keratinocyte migration–associated IncRNA 2 (WAKMAR2). We propose that lack of WAKMAR2 expression in keratinocytes may contribute to the pathogenesis of chronic wounds.

RESULTS
WAKMAR2 is an RNA polymerase II–encoded polyadenylated transcript present in both cytoplasm and nucleus

WAKMAR2 is a previously annotated but uncharacterized IncRNA, which gene is located on chromosome 6q23.3 (GRCh38/hg38, chr6: 137823657-137868233, NR_049793.1), containing 4 exons and spanning 44,559 base pairs (Supplementary Figure S1a online). We assessed its protein-coding potential using a prediction algorithm Coding Potential Calculator (cpc.cbi.pku.edu.cn) (Kong et al., 2007). We found that WAKMAR2 had a coding potential score of −0.864, which is similar to a known IncRNA XIST (−0.945), but lower compared with the protein-coding transcripts GAPDH (12.25) and ACTB (14.25) (Supplementary Figure S1b). To determine which RNA polymerase transcribes WAKMAR2, we treated human primary keratinocytes with α-amanitin for 2–8 hours at a concentration of 5 μg/ml, which has been shown to specifically inhibit RNA polymerase II (Figure 1a) (Pandey et al., 2008). Accordingly, α-amanitin treatment did not affect the expression of 28S and 18S ribosomal RNA, encoded by RNA polymerase I (Figure 1b). However, the levels of WAKMAR2 and RNA polymerase II–encoded RPL10 mRNA were decreased by α-amanitin, indicating that RNA polymerase II transcribes WAKMAR2. From this assay, we also determined the half-life of 4.7 hours for WAKMAR2 in keratinocytes. This was further confirmed by treating the cells with actinomycin-D (5 μg/ml) for 2–8 hours, which blocks total cellular transcription (Figure 1c) (Pandey et al., 2008). In addition, we characterized subcellular localization of WAKMAR2 by performing quantitative real-time reverse transcriptase PCR (QRT-PCR) on RNA extracted from either nucleus or cytoplasm of keratinocytes. Enrichment of nuclear IncRNA MALAT1 (Gutschner et al., 2013) and GAPDH mRNA in the nuclear and cytoplasmic fractions, respectively, confirmed successful separation of these fractions. We found that WAKMAR2 is present in both fractions, with a slightly higher level in the cytoplasm (57.3% vs. 42.7%) (Figure 1d). Moreover, we checked the polyadenylation status of WAKMAR2 in keratinocytes by separating Poly(A)+ and Poly(A)− RNA with Dynabeads. In line with previous knowledge, GAPDH mRNA was almost exclusively detected in the Poly(A)+ RNA fraction, whereas H2BK RNA was mainly found in the Poly(A)− fraction (Yang et al., 2011). Interestingly, 99% of WAKMAR2 was detected in the Poly(A)+ fraction, indicating it is a polyadenylated transcript (Figure 1e). Collectively, we identified WAKMAR2 as a polyadenylated, RNA polymerase II–encoded transcript that is expressed in both cytoplasm and nucleus of keratinocytes.

WAKMAR2 expression is downregulated in wound-edge keratinocytes of human chronic wounds

Among the 53 human tissues characterized in the Genotype-Tissue Expression Project (Lonsdale et al., 2013), 7 tissues (i.e., skin, spleen, ovary, vagina, lung, small intestine, and testis) have median WAKMAR2 expression above two transcripts per million. The skin has the highest WAKMAR2 expression (median 8.4 transcripts per million in lower leg skin and 7.5 transcripts per million in suprapubic skin), suggesting that this IncRNA may play a functional role in the skin (Supplementary Figure S2 online).

To study WAKMAR2 expression in human skin wounds in vivo, we created excisional wounds in the skin of healthy volunteers (Supplementary Table S1 online) and collected the tissue around the wounds 1, 7, and 30 days later. These time points were selected to represent the different phases of wound healing (Figure 2a). In addition, we collected nonhealing wound-edge biopsies from patients with venous ulcer (VU) (Supplementary Table S2 online) or diabetic foot ulcer (DFU) (Supplementary Table S3 online). WAKMAR2 expression was analyzed with QRT-PCR in these biopsies. During
normal wound healing, we observed a trend of decreased WAKMAR2 expression in the inflammatory phase (NS > NW1), while its level increased gradually in the following proliferative and remodeling phases (NW1 < NW7 < NW30), although these changes were not statistically significant (Figure 2b). Interestingly, a more prominent reduction of WAKMAR2 level was found in VU compared to the intact skin (NS) and the normal wounds in proliferative (NW7) or remodeling phases (NW30) (Figure 2b). The deficiency of WAKMAR2 in VU was further confirmed in another independent cohort including seven healthy donors and six VU patients (Figure 2c, Supplementary Tables S1, S2). We found that WAKMAR2 was also significantly downregulated in DFU compared to NS and NW7 (Figure 2d, Supplementary Tables S1, S3). Moreover, in epidermis isolated from the skin and wound-edges using laser capture microdissection, a prominent reduction of WAKMAR2 expression was observed in VU compared to NS, NW1 and NW7, as was shown for the whole skin biopsies (Figure 2e, 2f, Supplementary Tables S1, S2). Because keratinocytes constitute 95% of the epidermis and are one of the major players in wound healing (Kolarsick et al., 2011), we next focused on the role of WAKMAR2 in keratinocytes.

WAKMAR2 expression is induced by TGF-β signaling in keratinocytes

To understand the molecular mechanisms regulating WAKMAR2 expression, we treated human primary keratinocytes with a variety of cytokines, chemokines, and growth factors present in the wound environment. Several members of the TGF-β superfamily, that is, TGF-β1, TGF-β2, and BMP-2, as well as TNF-α were found to significantly induce WAKMAR2 expression in keratinocytes with TGF-β2 being the most prominent (Figure 2g). Furthermore, we showed that inhibition of TGFBR1 with a chemical inhibitor SB431542 or silencing the expression of SMAD3, a key mediator of TGF-β signaling, impaired the TGF-β2-mediated induction of WAKMAR2 (Figure 2h, 2i). By chromatin immunoprecipitation using SMAD3 antibody

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**Figure 1.** Characterization of the molecular properties of WAKMAR2. Quantitative real-time reverse transcriptase PCR analysis of WAKMAR2 and RPL10 RNAs in keratinocytes treated without or with α-amanitin (5 μg/ml) (**a**, **b**) or actinomycin-D (5 μg/ml) (**c**) for 2–8 hours. WAKMAR2 and RPL10 RNA levels were normalized to 18 ribosomal RNA, their levels in control were set as 100%, and the levels in α-amanitin or actinomycin-D–treated cells were presented relative to control. (**b**) Agarose gel showing equal levels of 18S and 28S ribosomal RNA in control and α-amanitin–treated keratinocytes. (**d**) Quantitative real-time reverse transcriptase PCR of WAKMAR2, MALAT1, and GPADH in nucleus or cytoplasm of keratinocytes. (**e**) Quantitative real-time reverse transcriptase PCR of WAKMAR2, H2BK, and GAPDH in Poly(A)+ and Poly(A)− RNA fractions from keratinocytes. Data are presented as mean ± standard deviation (**a**, **c**, **d**). *P < 0.05, **P < 0.01, ***P < 0.001; test for linear trend after one-way analysis of variance (**a**, **c**). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate buffered saline; WAKMAR2, wound and keratinocyte migration–associated long noncoding RNA 2.
Figure 2. WAKMAR2 expression is downregulated in wound-edge keratinocytes of human chronic wounds. (a) Excisional wounds were created in the skin (NS) of healthy donors and the tissue around the wounds were collected 1 (NW1), 7 (NW7), and 30 days (NW30) later. Non-healing wound-edges were collected from patients with VU or DFU. (b) Quantitative real-time reverse transcriptase PCR (QRT-PCR) analysis of WAKMAR2 expression in skin and wounds of 6 healthy donors and in 4 VUs. (c) WAKMAR2 expression was revealed by microarray analysis of NS and NW7 of 7 healthy donors and 6 VUs. (d) QRT-PCR analysis of WAKMAR2 in skin and wound biopsies of 8 healthy donors and in 17 DFUs. (e) Wound-edge epidermis was isolated from five healthy donors and four VU patients by LCM. Blue arrows indicate the wound-edges. Scale bar = 200 μm. (f) QRT-PCR analysis of epidermal WAKMAR2 expression.
coupled to QRT-PCR analysis, we demonstrated that SMAD3 directly bound to the promoter of WAKMAR2 in human primary keratinocytes, and the binding was significantly enhanced by TGF-β2 treatment (Figure 2j). TGF-β signaling has been found decreased in VU and DFU, which might explain the reduced WAKMAR2 expression in chronic wounds (Jude et al., 2002; Pastar et al., 2010).

**WAKMAR2 regulates inflammatory chemokine production by keratinocytes**

To study the biologic functions of WAKMAR2, we knocked down its expression in human primary keratinocytes by transfection of specific lock nucleic acid modified-long RNA-antisense-oligonucleotides (GapmeR). Two GapmeRs were designed to target different sites at the 4th exon of WAKMAR2 and both significantly decreased its expression in keratinocytes, as shown by QRT-PCR analysis (Figure 3a, Supplementary Figure S1a). Keratinocytes play an important role in the innate immune response during wound healing. By producing inflammatory chemokines (e.g., CXCL8/IL8, CCL20, and CXCL5), keratinocytes attract immune cells to migrate into the injured site, thus defending us from invading pathogens, clearing dead tissue, and contributing to wound repair (MacLeod and Mansbridge, 2016). We studied the effect of WAKMAR2 on keratinocyte chemokine production under inflammatory condition, which was mimicked by TNF-α treatment. QRT-PCR analysis of IL8 and CCL20 expression showed that knockdown of WAKMAR2, by transfection of GapmeR 1 or 2, upregulated the expression of these chemokines under both basal and inflammatory conditions (Figure 3b, 3c). In line with this, the amounts of IL-8 and CXCL5 protein secreted into the cell culture medium were also increased upon WAKMAR2 knockdown (Figure 3d, 3e). In addition, we activated endogenous WAKMAR2 transcription in human primary keratinocytes using CRISPR/Cas9 Synergistic Activation Mediator system (Joung et al., 2017). For this, we designed two single-guide RNAs targeting various regions of the WAKMAR2 promoter, and we found that both single-guide RNAs increased WAKMAR2 expression significantly (Figure 3f). In contrast to the WAKMAR2 knockdown, enhancement of its expression reduced the levels of IL8 and CXCL5 in keratinocytes treated with TNF-α (Figure 3g, 3h). These results suggest that WAKMAR2 may be important in restricting the production of inflammatory chemokines by keratinocytes during wound healing.

**WAKMAR2 regulates keratinocyte motility**

Keratinocyte proliferation and migration are essential for wound re-epithelialization (Pastar et al., 2014). To study the effect of WAKMAR2 on proliferation, we performed 5-ethynyl-2′-deoxyuridine incorporation assay and CyQUANT cell proliferation assay (ThermoFisher Scientific, Waltham, MA) for keratinocytes transfected with GapmeR targeting WAKMAR2. However, we did not find any obvious impact of WAKMAR2 on cell proliferation (Supplementary Figure S4 online). Next, we characterized the role of WAKMAR2 in keratinocyte migration. Using scratch wound assays, we found that knockdown of WAKMAR2, either by GapmeR 1 or GapmeR 2, significantly delayed wound closure (Figure 4a, 4b), whereas enhancement of WAKMAR2 expression using CRISPR/Cas9 Synergetic Activation Mediator system promoted keratinocyte migration (Figure 4d, Supplementary Movie S1 online). In line with this, haptotactic Transwell migration assay showed that knockdown of WAKMAR2 significantly reduced the migratory capacity of keratinocytes (Figure 4c). Together, our results suggest WAKMAR2 as a positive regulator of keratinocyte motility.

**Knockdown of WAKMAR2 inhibits re-epithelialization of human ex vivo skin wounds**

The role of WAKMAR2 in skin wound healing was further evaluated in a human ex vivo wound model. For this, full-thickness excisional wounds were made on human skin obtained from plastic surgery (Supplementary Table S1) and the wounds were excised and cultured as described previously (Heilborn et al., 2003). We treated these wounds topically with WAKMAR2 GapmeR 1 or control oligos immediately after skin injury as well as 3 days later and then collected wound samples 5 days later for RNA and histology analysis (Figure 5a). We found that topical treatment with GapmeR 1 significantly decreased the expression level of WAKMAR2 in wounds (Figure 5b), which led to delayed re-epithelialization compared to wounds treated with control oligos (Figure 5c, 5d). This may be due to the critical role of WAKMAR2 in keratinocyte migration. Moreover, we did not observe a significant change of MKI67 expression, which is a proliferation marker, after WAKMAR2 GapmeR treatment (Supplementary Figure S5 online). This is in line with the results of in vitro assays (Supplementary Figure S4) and suggests that WAKMAR2 does not affect keratinocyte proliferation. Together, our data suggest that as an important regulator of keratinocyte innate immune response and motility, deficiency of WAKMAR2 expression may contribute to the pathogenesis of chronic nonhealing wounds.

**Transcriptomic analysis of keratinocytes with WAKMAR2 knockdown**

To determine the molecular basis for the observed effects of WAKMAR2 in keratinocytes, we performed a global transcriptomic analysis in human keratinocytes upon WAKMAR2 knockdown using microarray and 348 genes was identified to be significantly regulated (fold change ≥1.4 or < −1.4; \( P < 0.01 \)) by WAKMAR2 (Figure 6a). Because approximately half amount of WAKMAR2 is present in the nucleus (Figure 1d), we hypothesize that it may act in a cis-acting manner. To this end, we assessed whether WAKMAR2...
regulates the expression of genes at the local genomic region neighboring its locus (Supplementary Figure S6a online). Although WAKMAR2 is transcribed in antisense direction and overlaps partially with the gene TNFAIP3 (Supplementary Figure S1a), knockdown of WAKMAR2 did not change the level of TNFAIP3 in keratinocytes (Supplementary Figure S6b). Interestingly, we found the expression of PERP, which is 265 kb from WAKMAR2 locus, was significantly downregulated after...
Figure 4. WAKMAR2 regulates keratinocyte migration. Scratch assays were performed for keratinocytes transfected with WAKMAR2-specific GapmeR 1 (a) or GapmeR 2 (b) (n = 3). Photographs were taken at indicated time points after scratch injury. The healing rates were quantified by measuring the area of the injured region. Scale bar = 100 μm. (c) Representative photographs of the Transwell migration assay for keratinocytes with WAKMAR2 knockdown (n = 3). The number of cells passing through the membrane was counted. Scale bar = 300 μm. (d) IncuCyte 96-well real-time cell migration assay was performed for keratinocytes transfected with WAKMAR2 CRISPR/Cas9 Synergistic Activation Mediator plasmids (n = 8–10). Data are presented as mean ± standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.001; two-way analysis of variance (a, b, d) or unpaired two-tailed Student t test (c). sgRNA, single-guide RNA; WAKMAR2, wound and keratinocyte migration-associated long noncoding RNA 2.

Figure 5. Knockdown of WAKMAR2 inhibits re-epithelialization of human ex vivo skin wounds. (a) Scheme of topical treatment of human ex vivo wounds from four healthy donors with WAKMAR2 GapmeR. (b) Quantitative real-time reverse transcriptase PCR analysis of WAKMAR2 in the day-5 wounds. (c) Day-5 wounds were stained with hematoxylin and eosin. Blue arrows demarcate the initial wound edges, while the red arrows indicate a newly formed epidermis. Scale bar = 200 μm. (d) Re-epithelialization was quantified as healing rate = 100% — the percentage of the initial wound area. Data are presented as mean ± standard error of the mean (b, d); *P < 0.05, **P < 0.01; unpaired two-tailed Student t test. WAKMAR2, wound and keratinocyte migration–associated long noncoding RNA 2.
Figure 6. Transcriptomic analysis of keratinocytes with WAKMAR2 knockdown. Expression profiling of keratinocytes transfected with control (Ctr) or WAKMAR2 GapmeR1 for 24 hours (n = 3). (a) Heatmap illustrating expression of significantly changed genes (fold change ≥1.4 or ≤−1.4, P < 0.01). Color intensity is scaled within each row so that the highest expression value corresponds to red and the lowest to blue. Genes related to NF-κB signaling (blue), migration (red), or both (black) are highlighted. GSEA evaluates enrichment for the genes related to migration (b) or NF-κB signaling (c) in the microarray data.
WAKMAR2 knockdown (Supplementary Figure S6c). PERP is a component of desmosomes (Ihrie et al., 2005) and its conditional knockout in mouse keratinocytes delays wound healing (Beaudry et al., 2010). However, we did not observe any obvious effect of silencing PERP on keratinocyte migration and production of inflammatory chemokines, for example, CXCL5, CCL20, and IL-8, indicating that PERP does not mediate the biologic effect of WAKMAR2 (Supplementary Figure S7 online). Together, our data suggest that WAKMAR2 is unlikely to act in a cis-acting manner.

Next, we compared the WAKMAR2-regulated genes to published gene sets related to inflammation and cell motility. Gene set enrichment analysis (Subramanian et al., 2015) of microarray data revealed that the migration-related genes (Supplementary Table S4 online) were significantly ($P < 0.001$) enriched among the downregulated genes after WAKMAR2 knockdown (Figure 6b) (Simpson et al., 2008). In contrast, genes involved in the NF-kB pathway (Supplementary Table S5 online), a central pro-inflammatory pathway, were significantly ($P = 0.016$) enriched among the genes upregulated by WAKMAR2 knockdown, suggesting that WAKMAR2 negatively regulates NF-kB pathway activity (Figure 6c). To test this hypothesis, we analyzed the effect of WAKMAR2 GapmeR on NF-kB-dependent luciferase reporter gene expression in keratinocytes. The results of the luciferase assays showed that WAKMAR2 knockdown increased TNF-α–induced luciferase activity (Figure 6d). In accordance with this finding, Western blotting analysis revealed that WAKMAR2 knockdown enhanced phosphorylation of the NF-kB transcription factor p65, further demonstrating that loss of WAKMAR2 expression upregulates NF-kB signaling, thus enhancing the production of inflammatory mediators by keratinocytes (Figure 6e).

Using the STRING database, we analyzed functional protein association network among the WAKMAR2-regulated genes (Supplementary Figure S8 online) (von Mering et al., 2007). Notably, many genes in this network, in particular the centrally located nodes identified based on topologic features (Supplementary Table S6 online), have been previously involved in regulating cell migration, for example, IRAK4 and CTNND1 (Bouma et al., 2009; Tang et al., 2016), or inflammatory response, for example, IL1B, MDM2, and IRF7 (Hauser et al., 2016; Ren and Torres, 2009; Zhao et al., 2015). Interestingly, the expression levels of 6 among the top 20 central hubs, that is, ACACA, IL1B, ACSF2, MMP3, DSG1, and CTNND1, were found significantly correlated with the expression levels of WAKMAR2 in human skin, normal wounds and VUs, supporting that WAKMAR2 regulates their expression in vivo (Figure 6f–6k). Moreover, we tested biologic functions of a few central hubs, that is IL1B, MMP3, and RRM2B, the expression of which was upregulated after WAKMAR2 knockdown in keratinocytes and in human ex vivo wounds (Supplementary Figure S9a, S9b online). Silencing their expression with gene-specific small interfering RNAs led to reduced IL8 and CCL20 expression and faster migration of keratinocytes, mimicking the effect of WAKMAR2 (Supplementary Figure S9c–S9f). In summary, our study unraveled a molecular network that may mediate the biologic function of WAKMAR2 in keratinocytes.

**DISCUSSION**

Although more than 100,000 lncRNAs have been discovered in humans, the biologic relevance of the vast majority remains puzzling (Volders et al., 2013, 2015). Investigating the noncoding transcriptome may unravel new and unanticipated mechanisms of wound healing and identify novel therapeutic targets. Our current study focuses on the lncRNA WAKMAR2, the expression of which is decreased in human chronic wounds compared to wounds undergoing healing process or the intact skin. Knockdown of WAKMAR2 leads to enhanced chemokine production and reduced motility of human keratinocytes, as well as delayed re-epithelialization of human ex vivo wounds, mimicking the impaired phenotype of keratinocytes in chronic wounds (Stojadinovic et al., 2005). Thus, we propose that the deficiency of WAKMAR2 may contribute to the pathogenesis of chronic wounds.

We show that several members of the TGF-β superfamily, i.e., TGF-β1, TGF-β2, and BMP-2, induce WAKMAR2 expression in keratinocytes. Upon binding to the receptors TGFBR1 and TGFBR2 by TGF-β, or serine/threonine kinase receptor type I and II by BMP, Smad proteins, i.e., Smad2 and 3 for TGF-β signaling and Smad1, 5, and 8 for BMP signaling, are activated and interact with their common partner, Smad4 (Guo and Wang, 2009; Miyazono et al., 2005; Ramirez et al., 2014). The Smad complex then cooperates with a specific subset of transcription factors and induces target gene expression (Massagué et al., 2005). Here we demonstrate that Smad3 directly regulates WAKMAR2 expression by binding to its promoter. Moreover, at the promoter of WAKMAR2, we used PROMO software, version 3.0.2, to identify several putative binding sites of transcription factors known to interact with Smad complexes, for example, AP1, HNF-1B, HOXD9, and HOXD10, as well as nuclear receptors like the glucocorticoid receptors α and β, supporting our findings of TGF-β–induced WAKMAR2 expression (Farre et al., 2003; Messeguer et al., 2002). Of note, WAKMAR2 was recently reported to be downregulated in esophageal squamous cell carcinoma and gastric cardia adenocarcinoma due to aberrant hypermethylation of CpG islands (Guo et al., 2016a, 2016b). We noticed that the potential binding sites of Smad3 and several aforementioned transcription factors, for example, AP-1, glucocorticoid receptor α/β, HOXD9, and HOXD10, co-localized with CpG islands at the WAKMAR2
promoter. Therefore, it would be interesting to determine whether epigenetic regulation, such as DNA methylation, may contribute to the differential WAKMAR2 expression between normal and chronic wounds. As an essential signaling in skin wound healing, TGF-β has been shown to stimulate keratinocyte migration (Zambruno et al., 1995), which effect may be partially due to its induction of WAKMAR2 expression. In line with this, lack of TGF-β signaling has been reported in DFU (Jude et al., 2002) and VU (Pastar et al., 2010), which may contribute to the deficiency of WAKMAR2 in such wounds.

Our study reveals that deficient WAKMAR2 expression upregulates NF-kB signaling, thus enhancing the production of inflammatory chemokines by keratinocytes, which can potentially enhance the capacity of keratinocytes to recruit leukocytes from circulation to the wound site. Interestingly, we observed a transient downregulation of WAKMAR2 during wound repair, likely reflecting the need for enhancing inflammation to remove invading pathogens and dead tissue. Recovery of WAKMAR2 levels at a later phase may contribute to resolution of inflammation and facilitate wound healing by promoting keratinocyte migration. In chronic wounds, excessive and persistent inflammation impedes wound repair and wound-edge keratinocytes fail to migrate (Reinke and Sorg, 2012; Strobo et al., 2014). We propose that lack of WAKMAR2 may contribute to this pathology.

Furthermore, we explored the gene network regulated by WAKMAR2 in keratinocytes. We found that many nodes of this network were involved in cell migration and inflammatory response. Here we validated expression and function of a few central hubs, i.e., IL1B, MMP3, and RRM2B, in keratinocytes. Although it is still unclear how these genes were regulated by WAKMAR2, the change of their expression levels could partially explain the biologic effects of WAKMAR2 in keratinocytes. The inflammatory cytokine IL-1β is highly upregulated in human chronic wounds, for example, VU and DFU (Ortega et al., 2000; Wiegand et al., 2009), and its blockage enhances wound healing in a mouse model of diabetes (Mirza et al., 2013; Thomay et al., 2009).

MMP3 is an indispensable matrix metalloproteinase in wound repair, because mice with MMP3 deficiency exhibit delayed wound healing due to impaired wound contraction (Bullard et al., 1999). However, elevated MMP3 level, as observed in DFUs, has been shown to impair wound healing in human and mice (Lazar et al., 2016; Wall et al., 2002). This highlights the importance of precise and timely regulation of gene expression during wound healing, and regulatory RNA, such as WAKMAR2, may play an important role in this aspect. In addition, many genes in this network, such as RRM2B, ACACA, ACSF2, and DSG1, were, to our knowledge, previously unreported to be linked to skin wound healing and their detailed functions in wound repair warrant further study.

In summary, we identified the IncRNA WAKMAR2 as a critical regulator of keratinocyte inflammatory response and motility. The dynamic expression of WAKMAR2 during normal wound healing, as well as its deficiency in chronic wounds, underscore its indispensable role in wound repair. In general, our study unravels the importance of IncRNA-mediated regulation in skin wound healing and further efforts are needed to understand their precise mechanisms and potential as therapeutic targets.

MATERIALS AND METHODS
RNA extraction, QRT-PCR, cell fractionation, polyadenylation study, laser capture microdissection, cell culture and treatments, ELISA, analysis of cell motility and growth, ex vivo wound model, gene expression microarray, NF-κB luciferase reporter assay, Western blotting, CRISPR–Synergistic Activation Mediator and chromatin immunoprecipitation–Q-PCR are described in the Supplementary Materials and Methods online.

Tissue samples
The human samples used in this study were collected from two centers: the dermatology clinic and the plastic surgery section at Karolinska University Hospital (Stockholm, Sweden) provided normal wounds from healthy donors (nos. 1–18 and nos. 27–30 in Supplementary Table S1) and VU samples (n = 13, Supplementary Table S2) from Caucasian donors; the Second Hospital of Dalian Medical University (Dalian, China) provided normal wounds from healthy donors (nos. 19–26 in Supplementary Table S1) and DFUs (n = 17, Supplementary Table S3) from Asian donors. The DFU and VU patients were always compared with the healthy donors with same ethnicity.

Patients with nonhealing VUs or DFUs that, despite conventional therapy, persisted for more than 2 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, as well as immunocompromised patients. Tissue samples were taken using a 4-mm biopsy punch at the nonhealing edges of chronic wounds. The exclusion criteria for healthy donors were diabetes, skin disease, unstable heart disease, infections, bleeding disorder, immune suppression, and any ongoing medical treatments. One or two full-thickness excisional wounds were created using a 4-mm biopsy punch for each donor. The central skin excised from these surgical wounds was saved as intact skin control. The wound-edge skin was collected using a 6-mm biopsy punch 1, 7, or 30 days later (Figure 2a). Local lidocaine injection was used for anesthesia while sampling.

Written informed consent was obtained from all 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.

Statistics
Statistical significance was determined by paired or unpaired two-tailed Student t test, Mann–Whitney U Test or Wilcoxon matched pairs signed rank test. Differences between groups were computed using two-way repeated-measures analysis of variance. Correlation of the expression of different genes was made using Pearson’s correlation test on log-transformed data. A P value < 0.05 was considered to be statistically significant.

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CONFLICT OF INTEREST
The authors state no conflict of interest.
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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.2018.11.033.

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WAKMAR2 and Wound Healing

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