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MicroRNA-132 with therapeutic potential in chronic wounds

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# Equal contribution

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Short title: Treating chronic wounds with miR-132

Abbreviations: miR, microRNA; DFU, diabetic foot ulcer; qRT-PCR, quantitative real-time PCR; db/db mouse, leptin receptor-deficient mouse; Cxcl1, CXC chemokine ligand 1; Il1b, interleukin 1b; GSEA, gene set enrichment analysis; Hb-egf, heparin binding EGF like growth factor; TGF-β, transforming growth factor beta.

Keywords: MicroRNA, miR-132, chronic wounds, diabetic foot ulcer, therapy
ABSTRACT

Chronic wounds represent a major and rising health and economic burden worldwide. There is a continued search towards more effective wound therapy. We found significantly reduced microRNA-132 (miR-132) expression in human diabetic ulcers compared to normal skin wounds, and also in skin wounds of leptin receptor-deficient (db/db) diabetic mice compared to wild-type mice. Local replenishment of miR-132 in the wounds of db/db mice accelerated wound closure effectively, which was accompanied by increased proliferation of wound-edge keratinocytes and reduced inflammation. The pro-healing effect of miR-132 was further supported by global transcriptome analysis, which revealed that several inflammation-related signaling pathways, e.g. NF-κB, NOD-like receptor, Toll-like receptor, TNF signaling pathways, were the top ones regulated by miR-132 in vivo. Moreover, we applied topically liposome-formulated miR-132 mimics mixed with pluronic F-127 gel on human ex vivo skin wounds, which treatment promoted re-epithelialization. Together, our study demonstrated the therapeutic potential of miR-132 in chronic wounds, which warrants further evaluation in controlled clinical trials.
INTRODUCTION

Chronic non-healing wounds represent a major health and economic burden worldwide. They affect 6.5 million patients in the United States alone (Sen et al., 2009). In Europe and Scandinavia, the associated costs for treating chronic wounds account for 2–4% of the total health care expenses (Sen et al., 2009). This burden is continuously growing, due to population aging, increasing health care cost and rising incidence of diabetes and obesity (Sen et al., 2009). Among different types of chronic wounds, diabetic foot ulcer (DFU) is notoriously difficult to treat and is associated with high amputation rate and mortality. Effective treatment is lacking due to the complicated diabetic wound environment and the limited understanding about underlying pathophysiology (Amin and Doupis, 2016, Naves, 2016). Research aiming at discovering more effective wound therapies is therefore needed.

Chronic wounds are stalled in a constant inflammatory state and fail to progress through the normal healing process (Landen et al., 2016). Persisting inflammatory cells, mainly neutrophils and macrophages, generate proinflammatory cytokines, proteolytic enzymes and reactive oxygen species at the wound site, compromising the environment for wound healing (Landen et al., 2016). Keratinocytes in chronic wounds are hyperproliferative, but are deficient in migration and differentiation, which impairs re-epithelialization (Demidova-Rice et al., 2012). Moreover, high-glucose environment in diabetic skin has been shown to enhance oxidative stress and increase the inflammatory response of keratinocytes, contributing to impaired wound healing (Lan et al., 2013). The molecular mechanism underlying these abnormal cellular functions remains largely elusive, which impedes the development of effective treatment for chronic wounds.

MicroRNAs (miRNAs) are ~22 nt noncoding RNAs, which incorporate into the RNA-induced silencing complex (RISC) and bind to the 3’ untranslated region (UTR) of target
mRNAs, resulting in translational repression or degradation of the target mRNAs (Jonas and Izaurralde, 2015). MiRNAs play important regulatory roles in diverse biological processes and are often deregulated in pathological conditions. Recent clinical trials have demonstrated that modulation of miRNA expression by administration of miRNA specific mimics or inhibitors exhibits promising therapeutic effects on multiple diseases as diverse as cancer, cardiovascular disease and hepatitis C virus infection (van Rooij and Kauppinen, 2014).

Recently, we identified miR-132 as a top up-regulated miRNA in the inflammatory phase of human normal skin wound healing (Li et al., 2015). MiR-132 inhibits inflammation but promotes growth of epidermal keratinocytes, indicating that it may facilitate the inflammatory-proliferative phase transition during wound repair (Li et al., 2015). Following this line of research, we evaluated the therapeutic potential of miR-132 in chronic wounds using mouse in vivo and human ex vivo wound models.

RESULTS

MiR-132 is down-regulated in diabetic wounds

We have previously shown that miR-132 is required for normal skin wound healing (Li et al., 2015). To investigate whether miR-132 plays a role in chronic wounds, we compared the expression of miR-132 in DFU with normal acute wounds. To this end, biopsies were collected from edges of chronic non-healing wounds of 29 patients with type 2 diabetes mellitus (Table 1, Supplementary Table S1, Figure 1a). As a control, we created surgical wounds in the skin of 8 healthy volunteers and collected wound-edge biopsies 7 days after injury (Supplementary Table S2, Figure 1a). In line with our previous findings, quantitative real-time PCR (qRT-PCR) demonstrated that the expression of miR-132 was significantly up-regulated (4.98-fold change, P = 0.0078) in the acute wounds compared to the intact skin.
Importantly, the level of miR-132 in the DFUs was significantly lower (0.61-fold change, P = 0.0081) compared with the normal acute wounds (Figure 1b). This was further confirmed by in situ hybridization using miR-132 specific probes, which showed that the expression of miR-132 was mainly down-regulated in the epidermis of the DFUs compared to the normal wounds (Figure 1c).

We also examined the expression of miR-132 in the skin wound of leptin receptor-deficient (db/db) mice, which have been used as a type 2 diabetic model with impaired wound healing capacity (Scherer et al., 2008). Similar to the situation in human skin, miR-132 expression was up-regulated in the day-6 and day-10 wounds compared to the intact skin in both wild-type and db/db mice (Figure 1d and Supplemental Figure S1). Notably, the expression of miR-132 was significantly lower (0.58-fold change, P = 0.04) in the day-6 wounds of db/db mice compared to the wounds of wild-type mice (Figure 1d). The reduced expression of miR-132 in diabetic wounds suggests that it may have a functional role in this disease and that restoration of miR-132 expression may be beneficial.

*Local replenishment of miR-132 in skin wounds*

To evaluate the therapeutic potential of miR-132 in wound healing, we chose synthetic double-stranded miR-132 mimics encapsulated within a neutral lipid emulsion, an approach shown to enhance delivery and to be well tolerated *in vivo* (van Rooij and Kauppinen, 2014). We first examined the efficiency of miRNA mimic delivery by intradermal injection of 3 different doses of miRNA mimics, i.e. 0.5, 1 or 2 nmol per wound, into the wound-edges of normal C57BL/6N mice immediately after skin injury (Figure 2a). QRT-PCR analysis of wound biopsies six days after injection showed that all three doses effectively increased the level of miR-132 (Figure 2b). Of note, comparing to PBS injection, treatment with miR-132
or control mimics did not enhance inflammatory response in the wounds, shown by CXC chemokine ligand 1 (Cxcl1) and interleukin 1b (Il1b) expression, indicating well tolerance of the local application of miRNA mimics (Supplementary Figure S2).

In db/db mice, we injected 0.5 nmol miR-132 mimic or control oligos into the edge of each wound immediately after skin injury (Figure 2c), which significantly elevated the level of miR-132 in the wound sites, but not in the inner organs, e.g. liver, spleen, lung and kidney (Figure 2d). Accordingly, there was no morphological or histological difference observed in the internal organs between the miR-132- and the control-treated groups, suggesting that there was no systemic adverse side effect for the local miR-132 treatment (Supplementary Figure S3). By qRT-PCR detection of the control oligos, we further confirmed that the injected exogenous oligos remained restricted to the skin (Supplementary Figure S4). Moreover, we separated epidermis and dermis of the wound-edge skin using laser capture microdissection and found that the level of miR-132 was highly up-regulated in both skin compartments of the mice receiving miR-132 mimics (Figure 2e). We concluded that local administration of liposome-formulated miR-132 mimics specifically and effectively enhanced miR-132 levels in skin wounds.

**MiR-132 promotes skin wound healing in a mouse model of type 2 diabetes**

Db/db mice have been widely used as a model of impaired wound healing (Scherer et al., 2008). We found that intradermal injection of miR-132 mimics into the wound-edges of db/db mice significantly (P = 0.0014) accelerated wound closure (Figure 3a-b). Histomorphometry analysis also revealed that both the length (P = 0.0007) and area (P = 0.0202) of the newly formed epithelial tongue were significantly increased in the miR-132-treated wounds compared to the controls (Figure 3c-e). Of note, in the wound-edges of db/db mice injected
with miR-132, we found increased number of proliferating keratinocytes, as shown by Ki-67 staining, compared to the ones receiving control mimics (Figure 3f-g). Moreover, the expression levels of Cxcl1, a chemokine with neutrophil chemoattractant activity, were detected lower in miR-132 treated wounds (Figure 3h); and in line with this, the decreased numbers of neutrophils (Gr-1⁺) were observed in the miR-132-treated wound-edge tissues compared to the control group (Figure 3i-j).

There was no significant difference in weight and blood glucose levels between the miR-132 mimics- and the control oligos- treated groups either before or 6 days after treatment, excluding the possibility that the improved wound healing is secondarily to a better metabolic control (Supplementary Figure S5).

Since hair follicle cycling influences wound healing (Ansell et al., 2011), we also examined the hair follicles adjacent to the wounds of the treated mice by immunohistochemistry of keratin5 (Krt5). We did not find any obvious difference in hair cycle between the miR-132 and the control-treated groups: most hair follicles were in the telogen phase (Muller-Rover et al., 2001), suggesting that the improved wound healing in miR-132 treated-group was not due to the impact of hair follicle cycling (Figure 3k).

Contraction of the dermis plays an important role during mouse skin wound-healing, which is not the primary mechanism in human wounds. We have previously shown that miR-132 did not affect mouse dermal fibroblast contraction in vitro (Li et al., 2015). Moreover, here we found that there was no obvious difference for the presence of myofibroblast (α-SMA+) in the dermis between the miR-132- and control oligo-treated wounds (Supplementary Figure S6), further confirming that miR-132 does not affect wound contraction.

Taken together, our results showed that miR-132 treatment repressed inflammation while increasing growth of keratinocytes in the hard-to-heal wounds of db/db mice.
**Transcriptome analysis of the wounds treated with miR-132 mimics**

To characterize molecular mechanism underlying the pro-healing effect of miR-132, we performed global transcriptome analysis of wound-edge tissues from the db/db mice and identified 122 genes differentially expressed (fold change ≥ 1.5 or ≤ -1.5, P < 0.05) between the miR-132 or control mimics-treated groups (Supplementary Table S3). Among the 364 miR-132 targets predicted by the TargetScan algorithm (Agarwal et al., 2015), 267 genes were detected in the wounds of db/db mice. Gene set enrichment analysis (GSEA) revealed that these miR-132 target genes were significantly (P < 0.001) enriched among the genes down-regulated by miR-132 in the microarray data, and a negative enrichment score curve was generated (Figure 4a) (Subramanian et al., 2005). Of note, the expression of heparin binding EGF like growth factor (Hb-egf), which was previously identified as a key target of miR-132 in wound-edge keratinocytes (Li et al., 2015), was found to be down-regulated in the wounds treated with miR-132 mimics compared to the control group (Figure 4b). Moreover, there was a significant negative correlation of Hb-egf with miR-132 level in the treated mice wounds (Figure 4b).

Interestingly, gene ontological (GO) analysis using the database for annotation, visualization and integrated discovery (DAVID) v6.8 (Huang da et al., 2009a, 2009b) revealed that among the top 10 enriched biological processes and signaling pathways for the genes differentially expressed between the miR-132- and the control-treated wounds, several were related to inflammatory response (Figure 4c). This was further supported by GSEA, showing that the genes involved in the NF-κB pathway were significantly (P < 0.001) enriched among the genes down-regulated by miR-132 treatment (Figure 4d-e, Supplementary Table S3). Together, the results of transcriptome analysis suggested that miR-132 promoted healing of diabetic wounds by inhibiting several central-signaling pathways controlling inflammation.
Application of miR-132 mimics topically promotes re-epithelialization of human ex vivo skin wounds

The therapeutic effect of miR-132 was further evaluated in a human ex vivo wound model. Full-thickness wounds were made on human skin obtained from plastic surgery (n = 4 donors), and the wounds were excised and cultured as previously described (Heilborn et al., 2003). Aforementioned liposome-formulated miR-132 mimics or control oligos were mixed with pluronic F-127 gel, which is a thermo reversible gel commonly used as a vehicle for topical administration of drugs and has several advantages over traditional oleaginous bases in terms of ease of application, cosmetic acceptability and desirable drug release characteristics (Kant et al., 2014). This mixture was topically applied onto the wounds immediately after injury, and the wound tissues were collected 3 and 5 days later for RNA and histological analysis (Figure 5a).

We found that the level of miR-132 in the wounds was significantly increased (4.32-fold change, P = 0.026) at 3 days after a single application of miR-132 mimics (Figure 5b). H&E staining showed that wounds receiving miR-132 mimics were completely re-epithelialized, whereas wounds treated with control oligos were only partially covered by the newly formed epithelium on day 5 after injury (Figure 5c-d). Moreover, we found increased number of Ki-67-positive cells in the wound-edge epidermis treated with miR-132 mimics compared to the control group (Figure 5e-f). Thus we concluded that miR-132 accelerated re-epithelialization of human ex vivo skin wounds. Our data in these pre-clinical wound models suggest that local application of liposome-formulated miR-132 mimics may serve as a potential wound therapy.

DISCUSSION
Accumulating evidence has demonstrated that miRNAs play important roles in the general pathology of diabetes; however, their function in DFU remains largely elusive (Moura et al., 2014). Our study revealed the significant down-regulation of miR-132 in DFUs compared to human normal skin wounds. Moreover, detection of miR-132 in human wounds in situ suggested that the dysregulation of miR-132 mainly existed in the epidermal keratinocytes in DFUs. We have previously shown that transforming growth factor beta (TGF-β), which play a central role in regulation of the wound healing cascade, are the major cytokines inducing miR-132 expression in keratinocytes during wound repair (Li et al., 2015). Interestingly, lack of TGF-β signaling has been found in DFU (Jude et al., 2002). Thus we propose that the reduced miR-132 expression in DFU may be due to the dysregulation of TGF-β signal. Since the gene expression pattern of human skin is diverse across anatomic sites (Rinn et al., 2008) and changes during ageing (Li et al., 2016), it is important to utilize site- and age-matched controls to compare with the chronic wound biopsies, which is a limitation in the current study and needs to be further improved.

We have recently shown that miR-132 was required for normal skin wound healing, since inhibition or knockout of miR-132 in mouse skin led to delayed wound closure accompanied with increased inflammation (Li et al., 2015). In line with this, we demonstrated in this article that local replenishment of miR-132 in skin wounds of diabetic mice effectively accelerated wound closure by reducing inflammation and promoting re-epithelialization. Transcriptome analysis of the wound tissues from the miR-132- or control-treated mice revealed that miR-132 targets were highly enriched in the group of genes decreased by miR-132 treatment. Importantly, we found that the expression of Hb-egf, a key target mediating the anti-inflammatory and pro-proliferative functions of miR-132 in keratinocytes (Li et al., 2015), was down-regulated in the wounds treated by miR-132, which may be responsible for the pro-healing effects of miR-132 observed in the diabetic wounds. Moreover, transcriptome
analysis highlighted the molecular basis of the anti-inflammatory function of miR-132. The genes involved in several key pathways controlling inflammatory response were regulated by miR-132 treatment. In particular, the genes related to the NF-κB signaling were significantly enriched in the group of miR-132 down-regulated genes, which is in line with our previous finding showing that miR-132 suppresses the activity of NF-κB signaling pathway (Li et al., 2015). Excessive and persistent inflammation is a common denominator for all chronic wounds (Ennis et al., 2013, Landen et al., 2016). Although the pro-healing effects of miR-132 was demonstrated in a diabetic mouse model in this study, the anti-inflammatory function of miR-132 suggests that it may exhibit therapeutic potential also in other types of chronic wounds, which needs further investigation.

Intradermal injection of miR-132 mimics leads to increased miR-132 levels in both epidermal and dermal compartments, indicating that multiple cellular players in the wound, e.g. keratinocytes, macrophages, neutrophils and blood vessel endothelial cells, get access to the exogenously added miR-132. Actually miR-132 has the capacity to target the defects of various cell types in chronic wounds. For inflammation, the major pathological factor in chronic wounds, miR-132 has been shown to inhibit the expression of pro-inflammatory cytokines / chemokines by keratinocytes, monocytes and macrophages (Li et al., 2015, Liu et al., 2015, Nahid et al., 2013). Moreover, miR-132 can induce M2 polarization in macrophages, which is linked to immunosuppression and wound repair (Essandoh et al., 2016). In neutrophils, the level of miR-132 is highly up-regulated after extravasation and infiltration into the skin, indicating its potential functional role (Larsen et al., 2013). In chronic ulcers angiogenesis is reduced (Bodnar, 2015). Increased miR-132 expression has been reported to enhance blood vessel endothelial cell (EC) proliferation and tube-forming capacity (Anand et al., 2010, Lei et al., 2015, Mulik et al., 2012). In line with this, sustained delivery of miR-132 by nanoparticle improves EC transplantation and vascularization (Devalliere et al., 2014),
whereas inhibition of miR-132 represses ECs sprouting, thus decreasing neovascularization (Westenskow et al., 2013). The pro-healing functions of miR-132 in all these key players of wound repair may cooperatively contribute to its therapeutic potential in chronic wounds.

To date there lacks of efficient targeted treatment for chronic wounds, due to the limited understanding about their pathogenesis (Sen et al., 2009). Recently, different strategies, e.g. administration of growth factors, tissue reconstruction using stem cells or progenitor cells, have been applied to treat chronic wounds but with limited results (Borena et al., 2015). Due to their important regulatory roles in diverse physiological and pathological conditions, miRNAs have emerged as promising therapeutic targets (van Rooij and Kauppinen, 2014). Aided by the development of in vivo delivery strategies, several miRNA-based therapies have entered clinical trial phase, showing promising results and few side-effects (van Rooij and Kauppinen, 2014). Here we locally applied miR-132 mimics onto skin wounds, which efficiently elevated the miR-132 level in the wounds and accelerated wound closure. The increase of miR-132 level was restricted within the wound area and not observed in the inner organs, suggesting specificity of the local treatment, which achieves high and sustained concentrations of miR-132 without large systemic doses, thus minimizing systemic toxicity and reducing the cost of treatment. In line with this, there was no change in body weight, blood glucose, morphology and histology of internal organs observed in the miR-132- treated group compared with the control group, indicating low systemic toxicity. The potent pro-healing ability coupled with the low toxicity suggests that it is feasible to use this liposome-formulated miR-132 mimics to treat chronic wounds.

Taken together, our study revealed significant down-regulation of miR-132 in chronic diabetic ulcer compared to normal skin wounds. Importantly, we demonstrated that local replenishment of miR-132 effectively enhances wound healing. These findings suggest that
miR-132 replacement treatment should be further evaluated in controlled trials as potential therapy for chronic wounds.

**MATERIALS AND METHODS**

RNA extraction, qRT-PCR, *in situ* hybridization, *in vivo* wound models, histological analysis and gene expression microarray are described in the Supplementary materials and methods online.

*Tissue samples*

All the DFU patients (Asian, n = 29) in this study were enrolled at the Second Hospital of Dalian Medical University (SHDMU, Dalian, China) between October 2015 and March 2016 (Table 1, Supplementary Table S1). Tissue samples were taken using 4 mm biopsy punch at the edge of chronic wound area (Figure 1a). Healthy donors (Asian) 1-8 were enrolled at SHDMU (Supplementary Table S2). One 3mm-surgical wound was created on the lower back region of each healthy volunteer, wound-edge tissue was excised using 6mm biopsy punch 7 days after injury (Figure 1a). Human skin samples used for establishment of *ex vivo* wound model (donor 9-12 listed in the Supplementary Table S2, Caucasian) were obtained from reconstructive skin surgery at the Department of Reconstructive Plastic Surgery, Karolinska University Hospital Solna (KUHS, Stockholm, Sweden). 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.
**Laser capture microdissection**

Formalin-fixed, paraffin-embedded (FFPE) tissue samples were cut to 10 µm tissue sections and stained with hematoxylin. Laser capture microdissection was performed with Leica LMD7000 (Leica, Bernried, Germany). RNA from microdissected tissue was purified using the miRNAeasy FFPE Kit (Qiagen, Hilde, Germany).

**Human ex vivo wound model**

Human skin samples (n = 4) were obtained from routine abdominal reduction surgeries. The wounds were made using 2 mm biopsy punch on the epidermal side of the skin, excised using 6 mm biopsy punch and subsequently transferred to a cell culture plate containing DMEM plus 10% FBS and antibiotics (penicillin 50 U/l and streptomycin 50 mg/ml; ThermoFisher Scientific, Carlsbad, CA). 0.1 nmol mirVana™ hsa-miR-132-3p mimic (MC10166) and mirVana™ miRNA mimic negative control #1 (4464058) (ThermoFisher Scientific) were packed in transfection reagent Max Suppressor™ In Vivo RNA-LANCEr II (Bioo Scientific, Austin, TX), and then dissolved in 30% pluronic F-127 gel (Sigma-Aldrich, St Louis, MO). This mixture was topically applied onto the wounds immediately after injury. Wound samples were collected 3 and 5 days after injury for gene expression and histological analysis.

**Statistics**

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 two-way repeated-measures ANOVA. Correlation of the expression of
different genes was made using Pearson’s correlation test on log-transformed data. \( P \)-value < 0.05 was considered to be statistically significant.

**CONFLICTS OF INTEREST**

The authors state no conflict of interest.

**ACKNOWLEDGEMENTS**

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### Table 1. Information of patients with diabetic foot ulcer

<table>
<thead>
<tr>
<th>Number, n</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (range)</td>
<td>64 ± 12.21 (41 - 91)</td>
</tr>
<tr>
<td>Gender male, n (%)</td>
<td>22 (75.86%)</td>
</tr>
<tr>
<td>Wound duration, months (range)</td>
<td>4 ± 9.19 (2 - 48)</td>
</tr>
<tr>
<td>Wound size, cm² (range)</td>
<td>9 ± 9.40 (2 - 40)</td>
</tr>
<tr>
<td>Severity (Wagner grade ≥ 3), n (%)</td>
<td>23 (79.31%)</td>
</tr>
<tr>
<td>Complications / other diagnosis, besides T2DM and DFU n (%)</td>
<td>PAD 16 (55.17%)</td>
</tr>
<tr>
<td></td>
<td>DPN 14 (48.28%)</td>
</tr>
<tr>
<td></td>
<td>HTN 13 (44.83%)</td>
</tr>
<tr>
<td></td>
<td>CVD 7 (24.14%)</td>
</tr>
<tr>
<td></td>
<td>DN 2 (6.90%)</td>
</tr>
</tbody>
</table>

Data represent the median ± SD (range or percentages of all patients). CVD: cardiovascular disease, DN: diabetic nephropathy, DPN: diabetic peripheral neuropathy, HTN: hypertension, PAD: peripheral arterial disease (lower limbs), T2DM: type 2 diabetes mellitus.
FIGURES LEGENDS

Figure 1. MiR-132 expression in normal and diabetic wounds. (a) Wound-edge biopsies were collected from day-7 wound of healthy donors (n = 8) and DFUs (n = 29). MiR-132 expression in skin and wounds of healthy donors and DFUs was analyzed by qRT-PCR (b) and in situ hybridization (c). (d) MiR-132 expression was analyzed in skin and day-6 wounds of 10 wild-type (WT) and 9 db/db mice. *P < 0.05, **P < 0.01 and ***P < 0.001 by Wilcoxon Matched Pairs Signed Rank Test or Mann-Whitney U Test. Scale bar: 100µm

Figure 2. Wound treatment with miR-132 mimics. (a) MiR-132 or control mimics (0.5, 1 or 2 nmol/wound) or PBS were injected into the wound-edges of wild-type mice (2 wounds/mouse, 3 mice/group) after wounding and biopsies were collected 6 days later. (b) MiR-132 level was analyzed by qRT-PCR. (c) MiR-132 or control mimics (0.5 nmol/wound) were injected into the wound-edges of db/db mice (2 wounds/mouse, 6 mice/group) after wounding. (d) MiR-132 in the wounds and inner organs collected 6 days later was analyzed by qRT-PCR. (e) QRT-PCR analysis of miR-132 in the wound-edge epidermis and dermis from db/db mice, which were separated by laser capture microdissection (LCM). Arrows demarcate the wound-edges. Scale bar: 100µm **P < 0.01, ****P < 0.0001 by Mann-Whitney U test.

Figure 3. MiR-132 promotes wound healing in diabetic mice. (a) Representative images of wounds on days 0, 2, 4, and 6 after wounding. (b) Wound closure was quantified and presented as healing rate = 100% - the percentage of the initial wound area size (n = 12 wounds/ group). (c) Representative pictures of H&E stained wound tissues. Dashed lines mark the newly formed epithelial tongue. Scale bars: 500 µm. The length (d) and area (e) of
epithelial tongue were quantified in the control (n = 10) and the miR-132-treated wounds (n = 8). (f) Immunofluorescence staining of Ki-67 in wounds 6 days after injury. White arrows demarcate the wound edges; dashed lines mark the epidermal/dermal boundary, Scale bars: 50 µm. (g) The number of Ki-67-positive cells was counted (n = 3/group). (h) Cxcl1 expression was analyzed in wounds by qRT-PCR. (i) Immunostaining of Gr-1 in the wounds 6 days after injury. Scale bars: 50 µm. (j) The number of Gr-1-positive cells was counted (n = 8/group). (k) Immunohistochemistry of Krt5 in the wounds 6 days after injury (n = 4/group). Red arrows indicate wound-edges, green arrow heads point hair follicle adjacent to the wound-edges. Scale bars: green 100 µm, black 50 µm. *P < 0.05, **P < 0.01 and *** P < 0.001 by repeated measures Two-way ANOVA (b) or Student’s t test (d, e, g, h, j).

Figure 4. Transcriptome analysis of the wounds treated with miR-132 mimics. (a) Genes in microarray were ranked by fold change (miR-132/Ctrl). GSEA evaluated enrichment for the predicted miR-132 targets. NES, normalized enrichment score. (b) QRT-PCR analysis of Hb-egf in the wounds treated with miR-132 or control mimics. Correlation of Hb-egf with miR-132 level in the treated mice wounds, Spearman-correlation on log-transformed values. (c) The top10 gene ontology biological process and KEGG pathway terms for genes regulated by miR-132. P-values were determined by Fisher’s exact test. GSEA evaluated enrichment within the microarray data for the genes involved in the NF-κB signaling pathway (d), among which the genes were significantly regulated by miR-132 (fold change ≥ 1.5 or ≤ -1.5, P < 0.05) were illustrated in heatmap (e).

Figure 5. MiR-132 promotes re-epithelialization. (a) Human ex vivo skin wounds (n = 4 donors) were topically treated with miR-132 mimics or control oligos. (b) QRT-PCR analysis of miR-132 in day-3 wounds. (c) Representative pictures of H&E staining of day-5 wounds.
Black arrows: initial wound edges; green arrows: newly formed epidermis. Scale bars: 200µm.

(d) Re-epithelialization was quantified as healing rate = 100% - the percentage of the initial wound size. (e) Immunostaining of Ki-67 in day-5 wounds. Sections were counterstained with DAPI. Scale bar: 50 µm. (f) The number of Ki-67+ cells was counted (n = 4/group). *P < 0.05 and **P < 0.01 by Student’s t test.