

**Identification of specific microRNAs in neutrophils of type 2 diabetic mice:
overexpression of *microRNA-129-2-3p* accelerates diabetic wound healing**

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Running title: miRNAs play a key role in diabetic-derived neutrophils

Abbreviations used in this paper:

BM, bone marrow

miRNA, microRNA

Casp6, caspase 6

Ccr11, chemokine (C-C motif) receptor 1-like 1

Ccr2, chemokine (C-C motif) receptor 2

Casp8, caspase 8

Dedd2, death effector domain-containing DNA binding protein 2

Db, diabetic mouse

Non-db, non-diabetic mouse

ABSTRACT

Neutrophils are involved in the first stage of acute inflammation. Following injury, they are mobilized and recruited to the injured tissue. In diabetes, wound healing is delayed and aberrant, leading to excessive recruitment and retention of neutrophils that fail to promote angiogenesis and prolong inflammation. However, the exact pathological mechanisms of diabetic-derived neutrophils in chronic inflammation remain unclear. Here, microRNA (miRNA) profiling of neutrophils from bone marrow in type 2 diabetic mice was performed using a microarray. miRNAs regulate the post-transcriptional expression of target mRNAs and are important in countering inflammation-related diseases. Our study revealed that miRNAs exhibited differential expression in diabetic-derived neutrophils compared with non-diabetic-derived neutrophils, especially *miR-129* family members. *miR-129-2-3p* directly regulated the translation of *Casp6* and *Ccr2*, which are involved in inflammatory responses and apoptosis. Furthermore, *miR-129-2-3p* overexpression at the wound site of type 2 diabetic mice accelerated wound healing. These results suggest possible involvement of *miR-129-2-3p* in diabetic-derived neutrophil dysfunction and that retention kinetics of neutrophils and chronic inflammation may be initiated via *miR-129-2-3p*-regulated genes. This study characterized changes in global miRNA expression in diabetic-derived neutrophils and systematically identified critical target genes involved in certain biological processes related to the pathology of diabetic wound healing.

Keywords: microRNA, diabetic-derived neutrophil, inflammation-related gene

INTRODUCTION

Diabetes can delay the healing of wounds and cause complications such as foot ulcers (1). Effective tissue repair requires the recruitment of immune cells from bone marrow (BM) to injured sites. In chronic wounds, the continuous influx of neutrophils and macrophages to the wound site can be maintained by stimuli such as tissue hypoxia, bacterial components, foreign bodies, and fragments of necrotic tissue (2). Chronic inflammation is predominantly characterized by excessive and prolonged infiltration of neutrophils and macrophages (3) which is frequently found in diabetic foot ulcers (4).

Skin tissue repair consists of three phases: inflammation, proliferation/migration, and maturation/resolution. Previously, we showed that the inflammatory phase is aberrant in diabetes, and the numbers of myeloid cells, including monocytes, granulocytes, and precursors, in cutaneous wounds were shown to be significantly raised on day 2 after wounding (D2W), D7W, and D10W in diabetic mice (Db) compared with those in control mice (Non-db); moreover, the recruitment and/or accumulation kinetics of these cells were altered (5). In many bacterial and autoimmune inflammatory diseases, one of the most important mechanisms of neutrophil accumulation is a delay in apoptosis due to the excessive production of neutrophil survival factors (6). As a result, inflammation is prolonged, followed by tissue damage, a feature associated with chronic inflammation in various diseases. Intrinsic factors have been shown to play an important role in aberrant myeloid cell behavior (7). Accordingly, the pathogenesis of chronic inflammation in diabetic foot ulcers may be due to intrinsic defects of diabetic-derived neutrophils. To promote diabetic skin wound healing, the mechanism behind such chronic inflammation should therefore be elucidated.

MicroRNAs (miRNAs) are small non-coding RNAs of approximately 21 to 25 nucleotides in length; they regulate post-transcriptional expression through binding to the 3' untranslated region (3'-UTR) of target mRNAs (8, 9). Reports have described that miRNAs have an important function in several diseases (10) and wound healing, and they have been shown to comprehensively regulate a number of important biological processes within the cell (11, 12). Specifically, miRNAs play key roles in diseases such as diabetes and cancer, and chronic wounds, and are associated with cell migration, proliferation, invasion, and apoptosis. *miR-126* overexpression was shown to rescue the diabetes-induced impairment of phagocytosis of apoptotic cardiomyocytes (13). In addition, *Let-7b* was revealed to inhibit keratinocyte migration in cutaneous wound healing (14). Moreover, reports have described that the topical application of miR-132 mimic mixed with pluronic F-127 gel in chronic wounds promoted re-epithelialization (15), and that miR-27b rescued impaired bone marrow-derived angiogenic cell function and improved wound healing in type 2 diabetic mice (16). miR-191 modulates cellular migration and angiogenesis to delay the tissue repair process (17). We also reported that miR-142 is required for the clearance of *Staphylococcus aureus* at skin wound sites (18). Against this background, functional analysis of miRNAs in the complex process of wound healing could confer great benefits for manipulation in the clinic, but the exact molecular mechanisms involved in diabetic skin wound healing leading to chronic inflammation remain largely unknown.

We hypothesized that miRNAs might be involved in the functional regulation of diabetic-derived neutrophils in chronic inflammation. To clarify the molecular mechanism of inflammatory control in diabetic-derived neutrophils, we screened for changes of miRNA expression in diabetic-derived neutrophils using microarrays. Next,

we evaluated the expression of specific miRNA and its target genes in diabetic-derived neutrophils and/or skin wounds. Finally, we examined the involvement of this miRNA in diabetic skin wound healing.

MATERIALS AND METHODS

Mouse wounding model

The Animal Care Committee of Nagasaki University approved the protocol for this study (approval number: 1407101159). BKS.Cg-*Dock7^m* *+/+* *Lep^r^{db}/J* (*Lep^r^{db/db}* and *Lep^r^{db/+}*) mice (5 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan). They were housed under a 12/12-h light/dark cycle (light on: 07:00, off: 19:00) at constant temperature and humidity and allowed free access to food and water. Male mice were used between 8 and 12 weeks of age and were age-matched to controls. To eliminate the effect of hormonal action related to sexual maturation on skin wound healing, we used only male mice. Full-thickness excisional dorsal wounds (4 mm) were made by a biopsy punch. Wounds were harvested, including a 2-mm margin of skin.

Mature miRNA purification for microarray analysis

Bone marrow (BM) was flushed from femurs and tibiae. Neutrophils from the pooled BM of three db or three non-db mice were isolated using a neutrophil isolation kit (Miltenyi Biotec Inc., Bergisch Gladbach, Germany), and miRNA was purified from neutrophils using a microRNA isolation kit, Mouse Ago2 (Wako, Osaka, Japan) for microarray analysis.

Microarray analysis

Microarray analysis was performed on a total of eight pools (four pools of three db BM samples and four pools of three non-db BM samples) using SurePrint G3 Mouse miRNA microarray, in accordance with the manufacturer's instructions (Agilent Technologies, Tokyo, Japan). Bioinformatic analyses were performed using GeneSpring v13 (Agilent Technologies). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE100577.

Isolation of neutrophils, macrophages, T cells, and B cells

Neutrophils from BM of six non-db mice were isolated using a neutrophil isolation kit (Miltenyi Biotec Inc.). Macrophages, T cells, and B cells from BM of six non-db mice were isolated with a Microbead Kit (Miltenyi Biotec Inc.), in accordance with the manufacturer's instructions. Cells were incubated with anti-CD11b Ab, anti-CD5 Ab, and anti-CD19 Ab to isolate macrophages, T cells, and B cells, respectively.

RNA isolation for real-time quantitative PCR

Neutrophils from BM and skin wounds of six db and six non-db mice were isolated using a neutrophil isolation kit and Anti-Ly-6G Microbead kit (Miltenyi Biotec). Wound skin from D2W and D3W was harvested by a biopsy punch (6 mm). It was then dissolved in QIAzol Lysis Reagent (QIAGEN, Germantown, MD, USA). Total RNA, including miRNA, was extracted using miRNeasy Mini kit and RNeasy Mini Kit (QIAGEN), in accordance with the manufacturer's instructions. Total RNA was quantified using NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific,

Waltham, MA, USA). RNA samples were stored at -80°C until use.

cDNA synthesis for mRNA and microRNA, and quantitative real-time PCR

Total RNA (850 ng) was utilized as a template and complementary DNA (cDNA) was synthesized using Prime-Script® RT Reagent Kit for mRNA expression analysis (Takara Bio, Kusatsu, Japan), in accordance with the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed in a 10- μL reaction system using SYBR Premix Ex Taq (Takara Bio) and a Thermal Cycler Dice Real Time System (Takara Bio). The contents of the amplification mix and thermal cycling conditions were set in accordance with the manufacturer's instructions. Primers [*Caspase 6 (Casp6)*, *Chemokine (C-C motif) receptor 1-like 1 (Ccr1l1)*, *Chemokine (C-C motif) receptor 2 (Ccr2)*, *Caspase 8 (Casp8)*, *Death effector domain-containing DNA binding protein 2 (Dedd2)*, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), *CCR2*, *DEDD2* and *GAPDH*] were purchased from Takara Bio Inc. TaqMan® Gene Expression Assay (Thermo Fisher Scientific) for *CASP6* was performed in accordance with the manufacturer's instructions.

Total RNA (10 ng) was utilized as a template and cDNA synthesis and qRT-PCR were performed using miRCURY LNA™ Universal RT microRNA PCR and LNA™ PCR primer set for miRNA expression analysis (EXIQON, Vedbaek, Denmark). Primers (*mmu-* (*hsa-*) *miR-129-2-3p* and *5S rRNA*) were purchased from EXIQON. The relative quantification of mRNA transcripts and miRNA was performed using the $\Delta\Delta\text{Ct}$ method (19).

Synthesis of DNA, miRNA mimic, and mutation

Putative target genes of *miR-129-2-3p* were predicted using GeneSpring (Agilent Technologies). DNA synthesis of *Casp6*, *Ccr2* and *Dedd2* was performed by Hokkaido System Science Co., Ltd. (Sapporo, Japan). Luciferase reporter plasmids were constructed to confirm the regulation of target genes by *miR-129-2-3p*. *miR-129-2-3p* mimic (chemically synthesized double-stranded mature *miR-129-2-3p*) and mutation as a negative control were chemically synthesized by GeneDesign, Inc. (Ibaraki, Osaka, Japan).

Cell culture and reagents

3T3 cells were cultured for luciferase reporter assay in Dulbecco's modified Eagle's medium (DMEM) (Wako) with high glucose, L-glutamine, 10% FBS, and 1% penicillin–streptomycin. These cells were then harvested, seeded onto a 96-well plate at about 3.0×10^4 cells per well in DMEM (Wako) with 10% FBS without 1% penicillin–streptomycin, and cultured for 24 h. Subsequently, these cells were washed with Opti-MEM (Thermo Fisher Scientific), supplemented with 100 μ L of Opti-MEM in each well, and incubated at 37 °C prior to transfection.

Transfection and luciferase reporter assay

The 3'-UTRs of *miR-129-2-3p* targets were predicted using TargetScan (http://www.targetscan.org/vert_71/) and microT-CDS in DIANA TOOLS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index).

Vectors were constructed with pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corp., Madison, WI, USA), in accordance with the manufacturer's instructions. Primers consisting of the 3'-UTRs of predicted *miR-129-2-3p* target

sequences and appropriate restriction sites were synthesized, annealed, and cloned downstream of the firefly luciferase reporter (*luc2*) gene in pmirGLO. Sequences were as follows:

Casp6 sense 5'-aacTGTGGACGTGGTGGGAAGGGCTAt-3'

Casp6 antisense 5'-ctagaTAGCCCTTCCACCACGTCCAACAgttt-3'

Ccr2 sense 5'-aacAGTGATTAGACTAAAAATAATAAGGGCTt-3'

Ccr2 antisense 5'-ctagaAGCCCTTATTATTTTTAGTCTAATCACTgttt-3'

Dedd2 sense 5'-aacCTGCCCCACACTTTAGCCTAAGGGCTAt-3'

Dedd2 antisense 5'-ctagaTAGCCCTTAGGCTAAAGTGTGTGGGGCAGgttt-3'.

Upper- and lower-case letters indicate the 3'-UTR and restriction sites (PmeI and XbaI), respectively.

Sequences of *miR-129-2-3p* mimic and mutation of seed sequence as a negative control were as follows:

miR-129-2-3p mimic 5'-AAGCCCUUACCCCAAAAAGCAU-3'

miR-129-2-3p mutation 5'-AAUCCCUUACCCCAAAAAGCAU-3'.

3T3 cells (3.0×10^4 cells/100 μ l) were cotransfected with the *miR-129-2-3p* mimic or mutation and a reporter plasmid containing the 3'-UTR of *Casp6*, *Ccr2*, and *Dedd2*. The *miR-129-2-3p* mimic and mutation were added at a final concentration of 40 nM with Lipofectamine3000 (Invitrogen). At 48 h after transfection, luciferase activity was assessed using a Dual-Glo Luciferase Assay System (Promega Corp.), in accordance with the manufacturer's instructions.

Induction of neutrophil differentiation

The HL-60 human promyelocyte cell line (RBRC-RCB0041) was provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. The HL-60 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific) with 10% FBS, and 1% penicillin–streptomycin. Neutrophil differentiation was induced by exposing HL-60 cells to 1.3% DMSO (Wako) for 5 days. At 3 days after exposure to 1.3% DMSO, HL-60 cells were transfected with *miR-129-2-3p* mimic or mutation at a final concentration of 70 nM using Lipofectamine3000 (Thermo Fisher Scientific) in Opti-MEM (Thermo Fisher Scientific) with 1.3% DMSO. Two days after transfection, cells and conditioned media were harvested and applied to target gene expression analysis.

Immunohistochemistry

Harvested tissues (D0W–D3W) were fixed in 4% paraformaldehyde (PFA) overnight and embedded in paraffin. All specimens were cut into 4- μ m-thick sections. IHC was performed with anti-Ly-6G (α -Ly-6G, neutrophil marker) as a primary antibody in accordance with manufacturer's protocol (Abcam, Cambridge, UK). Rat anti-mouse Ly-6G was purchased from Novus Biologicals (Littleton, CO, USA). Samples were incubated with Histofine® Simple Stain TM Mouse MAX PO (Rat) (Nichirei Bioscience Inc., Tokyo, Japan) for 1 h at room temperature. The Histofine® DAB-3S kit (Nichirei Bioscience Inc.) was used as a color developer. Hematoxylin was used as a nuclear counterstain. Observations were made via Aperio AT Turbo (Leica Microsystems, Tokyo, Japan).

Double-label fluorescent IHC (fIHC) was performed with CASP6 (Abcam [Table 1], Dilution: 1:500) or CCR2 (Abcam: ab203128, Dilution: 1:100), and anti-Ly-6G

(α -Ly-6G, neutrophil marker, Dilution: 1:200) as primary antibodies in accordance with IHC and immunofluorescence (IF) protocols (Abcam). Goat anti-rabbit IgG (H+L), Alexa Fluor® 488 conjugated (α -rabbit 488), and Alexa Fluor® 594 conjugated (α -rat 594) secondary antibodies (Dilution: 1:500) were purchased from Life Technologies. Observations were made via confocal microscopy (C2+ system; Nikon Corp., Tokyo, Japan). NIS-Elements C software version 4.13 (Nikon Corp.) and IMARIS 7.6.5 (BITPLANE, Zurich, Switzerland) was used for data analysis.

Morphometric analysis of neutrophils

For each tissue image of 3–5 wounds in wound area for IHC, binarization was performed. Ratios of the neutrophil-positive area relative to the wound area were calculated.

***In situ* hybridization**

In situ hybridization (ISH) was performed using microRNA ISH buffer set and miRCURY LNA Detection 5'- and 3'-DIG labeled probes (QIAGEN), in accordance with the manufacturer's instructions. In brief, 4% PFA perfusion-fixed tissues were embedded in paraffin. Six-micrometer-thick sections were deparaffinized and incubated with Proteinase K solution (DAKO, Glostrup, Denmark) for 10 min at 37°C. After washing in PBS, sections were dehydrated. Hybridization was performed using 20 nM miRNA probe in microRNA ISH buffer (QIAGEN) at 50°C for 3 h. Sections were rinsed in 5× SSC at 50°C for 5 min, twice with 1× SSC at 50°C for 5 min, twice with 0.2× SSC at 50°C for 5 min, and with 0.2× SSC at room temperature for 5 min. Sections were treated with blocking solution (Nacalai Tesque Inc., Kyoto, Japan) for 15 min at

room temperature and were then incubated with anti-DIG Ab (1:800) (Roche Diagnostics GmbH, Mannheim, Germany) in blocking solution (Nacalai Tesque Inc.) overnight at 4°C. Sections were developed using NTB/BCIP (Roche Diagnostics GmbH) at 30°C. Observations were made via Aperio AT Turbo (Leica Microsystems) and confocal microscopy (C2+ system). NIS-Elements C software version 4.13 was used for data analysis.

Total protein extraction and western immunoblot analysis

Skin tissue was homogenized using a TissueLyzer II (QIAGEN). T-PER Reagent (Thermo Fisher Scientific), consisting of proteinase and dephosphorylation inhibitor (Thermo Fisher Scientific), was then added. Debris was removed from the supernatant using an Ultrafree-MC 0.45-mm filter (Merck Millipore, Darmstadt, Germany). Filtered protein samples were quantified using a Direct Detect Spectrometer (Merck Millipore), separated on 4% to 12% NuPAGE Novex Bis-Tris gels (Thermo Fisher Scientific), transferred to polyvinylidene difluoride (PVDF) membranes, and blotted in accordance with standard protocols (antibody details are listed in Table 1). Protein bands were visualized using ImmunoStar® LD (Wako), and band intensity was calculated using Multi Gauge version 3.X (Fujifilm, Tokyo, Japan).

Skin wound healing studies using *miR-129-2-3p* mimic and mutation

For *in vivo* experiments, *miR-129-2-3p* mimic or mutation as a negative control [10 µmol/L in 50 µL of 30% Pluronic F-127 gel (Sigma Aldrich, St. Louis, MO)] was topically applied immediately after wounding. Thereafter, the proportion of wound area on each day after wounding relative to the initial wound area was measured using

Adobe Photoshop CC.

Statistical analysis

Data are shown as means \pm SD. The statistical significance of differences between means was assessed by Mann–Whitney *U* test, one-way ANOVA, followed by Tukey’s multiple comparison test and two-way ANOVA, followed by Bonferroni post-tests to compare replicate means (GraphPad Software, San Diego, CA, USA). A *p*-value < 0.05 was considered significant.

RESULTS

miRNA expression is altered in diabetic-derived neutrophils

Microarray analysis showed that the expression levels of 22 miRNAs in diabetic-derived neutrophils were more than double those in non-diabetic-derived neutrophils (Supplementary Fig. 1A), while those of 80 miRNAs were decreased to less than half in fold change analysis (Supplementary Fig. 1B), although statistical analysis was not performed on this.

In turn, we performed a moderated *t*-test (cut-off <0.05) and Storey with bootstrapping for microarray data using GeneSpring. Microarray analysis showed that the expression levels of 10 miRNAs in diabetic-derived neutrophils were significantly decreased compared with those in non-diabetic-derived neutrophils (Fig. 1A, Table 2). We focused here on *miR-129-2-3p* because the microarray data indicated that the signal values of db in miRNAs were too low, with the exception of *miR-129-2-3p* (Fig. 1B), and that *miR-129-2-3p* in diabetic-derived neutrophils was downregulated to less than one-third of the level in non-diabetic-derived neutrophils (Table 2).

qRT-PCR using the SYBR Green I assay showed that the expression of *miR-129-2-3p* in diabetic-derived neutrophils was significantly decreased [expression level (mean \pm SD): Non-db: 6.3 ± 4.4 , Db: 0.70 ± 0.35 , $p=0.0079$] (Fig. 1C).

***miR-129-2-3p* is mainly expressed in neutrophils**

To examine the cellular expression of *miR-129-2-3p*, we isolated neutrophils, macrophages, B cells, and T cells from BM and spleen in non-db using Microbead Kit, and examined the expression of *miR-129-2-3p* using qRT-PCR. Its expression was significantly increased in neutrophils compared with that in other cells in BM (Fig. 1D), and increased in neutrophils and macrophages compared with the levels in B cells and T cells in the spleen (Supplementary Fig. 2). Accordingly, this suggests that *miR-129-2-3p* is related to inflammation, especially early inflammation.

mRNAs are predicted to be target genes of differentially expressed miRNAs in microarray

More than 800 mRNAs were predicted to be targets of the miRNAs shown in Table 2 that were significantly differentially expressed in diabetic-derived neutrophils (Supplementary Fig 3). We thus performed Gene Ontology (GO) and pathway analyses using GeneSpring to survey them. The results showed that candidate target mRNAs for *miR-129-2-3p* were associated with many biological processes and pathways, including inflammatory response, apoptosis, chemotaxis, phagocytosis, endocytosis, and chemokine signaling. Accordingly, a number of biological processes may be defective in diabetic-derived neutrophils (Table 3).

Predicted target mRNAs show an inverse correlation with *miR-129-2-3p* expression

GO and pathway analyses showed that *caspase 6 (Casp6)*, *chemokine (C-C motif) receptor 1-like 1 (Ccr1l1)*, and *chemokine (C-C motif) receptor 2 (Ccr2)* are associated with inflammatory responses, and *caspase 8 (Casp8)* and *death effector domain-containing DNA binding protein 2 (Dedd2)* are also involved in apoptosis. These genes were expressed at high levels in diabetic-derived neutrophils compared with their levels in non-diabetic-derived neutrophils, as confirmed by qRT-PCR [expression levels (mean \pm SD): *Casp6*: Non-db: 0.76 ± 0.13 , Db: 0.97 ± 0.079 , $p=0.0042$; *Ccr1l1*: Non-db: 0.33 ± 0.22 , Db: 1.0 ± 0.23 , $p=0.0023$; *Ccr2*: Non-db: 0.37 ± 0.061 , Db: 1.4 ± 0.33 , $p=0.0022$; *Casp8*: Non-db: 0.88 ± 0.074 , Db: 1.2 ± 0.16 , $p=0.0077$; and *Dedd2*: Non-db: 0.60 ± 0.13 , Db: 0.89 ± 0.086 , $p=0.0043$] (Fig. 2A–E). These results support the prediction that these mRNAs are targets of *miR-129-2-3p*, as the expression of these genes was significantly increased, whereas the expression of *miR-129-2-3p* was significantly decreased, in diabetic-derived neutrophils.

miR-129-2-3p* directly regulates *Casp6*, *Ccr2*, and *Dedd2* translation *in vitro

To verify that the mRNAs that we identified were targets of *miR-129-2-3p*, we tested each in a luciferase reporter assay. *miR-129-2-3p* is predicted to bind with high affinity to *Casp6*, *Ccr2*, and *Dedd2* 3'-UTRs (Fig. 3A). In this assay, a decrease in luciferase activity indicates the binding of the miRNA mimic to the 3'-UTR of the target sequence. Luciferase reporter assays showed that the *miR-129-2-3p* mimic could effectively inhibit the expression of *Casp6* (Control: 1.0 ± 0.15 , mimic: 0.61 ± 0.13 , $p=0.0022$), *Ccr2* (Control: 1.0 ± 0.083 , mimic: 0.73 ± 0.10 , $p=0.029$), and *Dedd2* (Control: $1.0 \pm$

0.13, mimic: 0.69 ± 0.089 , $p=0.0079$) (Fig. 3B-D); thus, we concluded that *miR-129-2-3p* directly regulates the expression of *Casp6*, *Ccr2*, and *Dedd2* *in vitro*.

***miR-129-2-3p* directly regulates *CASP6* and *DEDD2* translation in HL-60 cells**

To determine whether *CASP6*, *CCR2*, and *DEDD2* can be direct targets of *miR-129-2-3p*, we used HL-60 cells, which are human neutrophil-like cells. qRT-PCR showed that the expression of *CASP6* and *DEDD2* in HL-60 cells transfected with *miR-129-2-3p* mimic was significantly decreased compared with that in those transfected with mutant *miR-129-2-3p* [expression levels (mean \pm SD): *CASP6*: mutant: 1.0 ± 0.23 , mimic: 0.71 ± 0.050 , $p=0.029$; *DEDD2*: mutant: 1.1 ± 0.30 , mimic: 0.57 ± 0.12 , $p=0.017$] (Fig. 3E-F), although the expression of *CCR2* could not be detected in HL-60 cell. These results suggested that these target genes might be directly regulated by *miR-129-2-3p* in HL-60 cells. Therefore, further investigation of these genes is necessary using human diabetic wound samples in order to apply the obtained findings to diabetes in humans.

Wound neutrophils are increased in db D2W, and *miR-129-2-3p* is predominantly expressed in wound neutrophils

To investigate the proportion of neutrophils among cells present at the early stage of inflammation (D1W-D2W), we performed IHC at wound sites in db and non-db mice. IHC for neutrophils showed a stronger signal in db D2W compared with non-db (Fig. 4A). Moreover, we calculated ratios of neutrophil-positive area relative to the wound area at D1W and D2W. The results showed that there were significantly more neutrophils present in D2W of db [signal level (mean \pm SD): Non-db: 4.5 ± 0.97 , Db:

9.01 ± 1.3, $p=0.029$] (Fig. 4B).

To determine which cells express *miR-129-2-3p* during the early stage of inflammation, we performed ISH in D1W of non-db. ISH showed that *miR-129-2-3p* was predominantly expressed in wound-infiltrating neutrophils in D1W (Fig. 4C).

***miR-129-2-3p* is insufficiently activated in diabetic-derived neutrophils**

To elucidate whether *miR-129-2-3p* and its target genes are involved in prolonged inflammation and delayed wound healing, we examined the expression of these genes at the skin wound site in D2W.

The expression of *miR-129-2-3p* did not show a significant difference between non-db and db at D2W (data not shown), upon isolating the neutrophils from non-db and db wound skin at day 2 after wounding using Anti-Ly-6G Microbead kit (Miltenyi Biotec) and examining the expression of *miR-129-2-3p* in equal numbers of neutrophils from the two groups. The expression of *miR-129-2-3p* in neutrophils from db D2W was significantly decreased compared with that in non-db [expression level (mean ± SD): Non-db: 7.0 ± 2.1, Db: 2.2 ± 0.69, $p=0.016$] (Fig. 4D). The expression of the *miR-129-2-3p* target gene, *Casp6* in db D2W was significantly increased compared with that in non-db [expression level (mean ± SD): Non-db: 2.1 ± 0.24, Db: 2.6 ± 0.082, $p=0.0029$] (Fig. 4E). Similarly, the expression of *Ccr2* in db D2W was significantly increased compared with the level in non-db [expression level (mean ± SD): Non-db: 4.4 ± 0.55, Db: 5.7 ± 0.34, $p=0.0025$] (Fig. 4F). In contrast, the expression of *Dedd2* in db D2W was significantly decreased compared with the level in non-db [expression level (mean ± SD): Non-db: 0.85 ± 0.11, Db: 0.39 ± 0.12, $p=0.0022$] (Fig. 4G). The data clearly show that, in neutrophils, *Dedd2* is a target of *miR-129-2-3p*; however, it may be

that, at this time point during wound healing, some other factors such as other miRNAs inhibit *Dedd2*, and that this mechanism is even more effective in diabetic wounds. Additionally, the expression of *Casp8* in db D2W was significantly increased compared with the level in non-db (Supplementary Fig 4A).

The expression of cleaved CASP6 in D2W did not show significant change between non-db and db although the number of neutrophils at D2W in db was significantly higher than in non-db (Fig. 4H). This result suggests that apoptosis of cells present in the wound site might be delayed.

To determine whether neutrophils express CASP6 and CCR2 during the early stage of inflammation, we performed fIHC in db D2W. fIHC showed that CASP6 and CCR2 was predominantly expressed in neutrophils (Supplementary Fig 4B).

Based on the results of IHC (Fig. 4A–B) and our previous report (5), the expression of *miR-129-2-3p* showed a significant decrease in db, although the number of neutrophils at D2W in db was significantly higher than in non-db. In addition, *Casp6* and *Ccr2* tended to be overexpressed in db D2W, and *miR-129-2-3p*, CASP6 and CCR2 was predominantly expressed in wound-infiltrating neutrophils (Fig. 4C, Supplementary Fig 4B). These results suggested that *miR-129-2-3p* was insufficiently activated in diabetic-derived neutrophils in D2W.

Overexpression of *miR-129-2-3p* in skin wound site of type 2 diabetic mice accelerates wound healing

We previously reported the usefulness of AS ODN using pluronic F-127 gel in skin wound (20). Therefore, it is useful to use a gel to verify the role of molecules in wound healing.

First, to clarify the pathophysiological role of *miR-129-2-3p* in skin wound healing, we made a wound in the dorsal skin of db and topically applied *miR-129-2-3p* mimic or mutant negative control mixed with pluronic F-127 gel immediately after wounding. Wound closure was significantly accelerated in *miR-129-2-3p* mimic-treated compared with *miR-129-2-3p* mutant control-treated wounds from D7W to D21W in db mice [Wound area (%) (mean \pm SD): day 7: mutation: 130.5 ± 18.2 , mimic: 101.4 ± 18.1 , $p < 0.05$, day 10: mutation: 132.2 ± 38.3 , mimic: 96.6 ± 16.3 , $p < 0.01$, day 14: mutation: 115.2 ± 25.9 , mimic: 70.4 ± 29.0 , $p < 0.001$, day 21: mutation: 36.8 ± 14.4 , mimic: 11.0 ± 9.5 , $p < 0.05$] (Fig. 5A and 5B), although there was no apparent effect in non-db mice [Wound area (%) (mean \pm SD): day 7: Non-db: 38.2 ± 25.7 , Db: 130.5 ± 18.2 , $p < 0.001$, day 10: Non-db: 10.1 ± 1.9 , Db: 132.2 ± 38.3 , $p < 0.001$, day 14: Non-db: 2.0 ± 0.86 , Db: 115.2 ± 25.9 , $p < 0.001$].

Next, to investigate the proportion of neutrophils among cells present at D3W, we performed IHC at the wound site in day 3 from application of mimic or mutation immediately after wounding of db. IHC for neutrophils showed a positive signal in *miR-129-2-3p* mutant control-treated wounds (Fig. 5C). Moreover, we examined the relative neutrophil-positive area in D3W and found a reduced signal in *miR-129-2-3p* mimic-treated D3W compared with that in *miR-129-2-3p* mutant control-treated [signal level (mean \pm SD): mutation: 8.9 ± 2.8 , mimic: 5.1 ± 1.1 , $p=0.057$] (Fig. 5D).

Finally, to confirm the specificity of the *miR-129-2-3p* mimic, the expression of *Casp6* and *Ccr2* was examined at the wound site in day 3 from application of mimic or mutant control immediately after wounding in db mice. The results showed that the expression of *Casp6* and *Ccr2* were significantly decreased in mimic-treated D3W [expression level (mean \pm SD): *Casp6*: mutation: 1.1 ± 0.12 , mimic: 0.85 ± 0.17 ,

$p=0.016$; *Ccr2*: mutation: 1.3 ± 0.25 , mimic: 0.96 ± 0.22 , $p=0.029$] (Fig. 5E-F).

Taken together, these results strongly suggest that the *miR-129-2-3p* mimic was effective at regulating gene expression in diabetic-derived neutrophils and could potentially rescue biological processes such as apoptosis in diabetic-derived neutrophils in the wound healing process. This would result in an improvement in delayed wound healing (Fig. 6), although this does not completely rule out the possibility that *miR-129-2-3p* may also impact the behavior of other cells *in vivo*, and that this may also contribute to enhanced healing.

DISCUSSION

Wound healing is a complex process that comprises inflammatory, proliferative, and remodeling phases. Bone marrow-derived cells (BMDCs) migrate to and participate in the homeostasis of skin tissue. After cutaneous injury, a heterogeneous population of BMDCs are recruited to the site of injury and contribute directly to the repair process by differentiating into various types of skin cells, such as fibroblasts, keratinocytes, and endothelial cells (21-22). They can also indirectly modulate repair and regeneration by producing cytokines and growth factors that promote re-epithelialization, neovascularization, and wound closure at the site of injury (3). In diabetic patients and animal models of diabetes, BMDCs, including neutrophils, contribute to an impaired healing/chronic wound environment by prolonging the inflammatory response and/or failing to promote the regenerative phase of wound healing (23-25). Neutrophils are the first immune cells recruited to the injured site in acute wound inflammation; they constitute up to 50% of the cells during the early phase of inflammation (5) and prevent microbe invasion through the process of phagocytosis (26). We previously showed that

the recruitment and/or retention kinetics of a heterogeneous population of BMDCs, including neutrophils, in diabetic cutaneous wounds are aberrant, leading to prolonged inflammation (5). These cells constitute the first subset of leukocytes to localize to injured tissue and may influence the entire localized inflammatory response.

miRNAs have been reported to be involved in both innate and adaptive immune responses (10); however, their role and regulation in neutrophils in the diabetic environment have remained unknown. To shed light on their possible role in dysfunction of diabetic-derived neutrophils, we examined miRNA expression and function in diabetic-derived neutrophils. Interestingly, the results showed that the expression of miRNAs involved in the inflammatory response changed in diabetic-derived neutrophils. Regarding *miR-223*, the expression of which was increased in diabetic-derived neutrophils in our study, it was reported to be associated with cell proliferation, apoptosis, migration, and invasion in gastric cancer (27). We further elucidated the function of *miR-223* in skin wound healing by analyzing *miR-223* knockout mice (28). Similarly, regarding *miR-31*, it was highly expressed during the transition from the inflammatory to the proliferative phase in vivo human skin wound healing model, and the overexpression of *miR-31* promoted cell proliferation and migration in human primary keratinocytes (29). Regarding *miR-149*, the expression of which was decreased, its target genes were shown to be involved in cell proliferation and apoptosis in patients suffering from acute injuries of the skin (30). These miRNAs might thus be involved in cell proliferation, migration, and apoptosis in diabetic-derived neutrophils. A recent paper shows that one of the factors associated with delayed wound healing in type 2 diabetic mice is Dnmt1-dependent dysregulation of hematopoietic stem cell (HSC) differentiation towards macrophages, and that the expression of Dnmt1

is regulated by miRNAs (31). In our study, the expression of miRNAs and mRNAs was significantly altered in diabetic-derived neutrophils, and this was associated with impaired wound healing. Alterations in gene expression in diabetic-derived neutrophils might be predetermined at the level of HSCs as described above, and epigenetic modifications in HSCs may be induced by type 2 diabetes mellitus.

In this study, microarray and qRT-PCR showed that the expression of *miR-129-2-3p* was downregulated in diabetic-derived neutrophils. Other recent studies have shown that *miR-129-2* is regulated epigenetically by DNA methylation (32). Analysis of CHIP data of the regulatory region in putative intron 1 of the gene (~4000 bp upstream of the sequence encoding the mature miRNA) showed that this region is bound by many transcription factors, including Pu.1 and Cebp transcription factors, both of which are underexpressed in diabetic-derived Gr-1⁺CD11b⁺ myeloid cells (33), which include neutrophils (Supplementary Fig. 4A–B). Thus, it is possible that the decrease in these transcription factors in diabetic-derived neutrophils contributes to the decreased expression of *miR-129-2-3p*. There are also reports that *miR-129* family members are associated with proliferation and apoptosis in some types of cancer such as esophageal carcinoma and breast cancer (34–35). Moreover, Wang *et al.* reported that the topical administration of miR-129 agomir in diabetic animals promoted diabetic wound healing (36). GO and pathway analyses in this study also indicated that the predicted target genes of *miR-129-2-3p* are involved in a number of biological processes, including the inflammatory response, neutrophil chemotaxis, phagocytosis, and the execution phase of apoptosis, and are associated with multiple pathways such as cell differentiation, Toll-like receptor signaling, chemokine signaling, IL-6 signaling, and the inflammatory response pathway. We thus hypothesized that *miR-129-2-3p* in particular might be

involved in the functional regulation of diabetic-derived neutrophils in chronic inflammatory processes.

Neutrophils are constantly produced in large numbers in BM, and by definition the same numbers of cells must die or migrate away within a defined time period for homeostasis to be maintained (6). Several studies have also suggested that the caspase family plays an important role in both spontaneous and Fas receptor-mediated apoptosis in neutrophils (37-39). The activation of death receptors with Fas ligand is involved in the activation of *Casp8*, which is actually a component of the death-induced signaling complex (DISC) and activates downstream signaling. The activation of *Casp8* has been noted in neutrophils and the inactivation of this protease was shown to delay apoptosis (37). In this study, the expression of *Casp8* was significantly increased in db skin wound on D2W. In addition, the expression of *Casp6*, which is a downstream executioner caspase, also increased in db skin wound on D2W. These results suggest that apoptosis of neutrophils in db skin wound sites on D2W might be facilitated and/or inhibited by the activation of *Casp8* and/or the direct regulation of *Casp6* (40). *Ccr2* is a chemokine receptor expressed in monocytes and lymphocytes, but not in neutrophils. However, its expression changes under acute inflammation or in response to specific inflammatory stimuli in wounds. In mice with severe sepsis, *Ccr2* is expressed in neutrophils (41-45), and wound recruitment is controlled by *Ccr2* (46, 47). Accordingly, *Ccr2* expression on diabetic-derived neutrophils may be critical for chronic inflammation. In this study, the expression of *Ccr2* was also significantly increased in D2W. Thus, diabetic-derived neutrophils from BM may be excessively recruited to wound sites on D2W.

Previously, we showed that the numbers of myeloid cells, including neutrophils, in cutaneous wounds were significantly increased on day 2 after wounding, and the recruitment and/or accumulation kinetics of these cells were altered (5). In this study, the expression levels of *Casp6* and *Ccr2* in D2W of db increased compared with those in non-db. Moreover, our results showed that *miR-129-2-3p* directly regulated *Casp6* and *Ccr2* translation. Our *in vivo* analysis showed that skin wound healing in db was significantly accelerated from day 7. These results strongly suggest that the recruitment and accumulation kinetics of diabetic-derived neutrophils might be improved by overexpression of *miR-129-2-3p*, resulting in improved wound healing.

In conclusion, miRNAs are differentially expressed in diabetic-derived neutrophils compared with their levels in non-diabetic-derived neutrophils, particularly *miR-129-2-3p*. Our results suggest that *miR-129-2-3p* directly regulates *Casp6* and *Ccr2* translation, and is involved in inflammatory responses, apoptosis, chemotaxis, phagocytosis, and endocytosis. These findings further suggest that the deregulation of *miR-129-2-3p* contributes to the dysfunction of diabetic-derived neutrophils. The retention kinetics of neutrophils and chronic inflammation may be initiated via *miR-129-2-3p*-regulated genes such as *Casp6* and *Ccr2*. Accordingly, we suggest that *miR-129-2-3p* might be involved in the cellular kinetics and functional regulation of wound-recruited neutrophils and as such may prove to be a useful target for manipulation in a clinical context.

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Author contributions are as follows: T.U., R.M., K.A.M. and K.I. conceived the experiments; T.U., T.Y., T.M., and Y.A. conducted the experiments; and T.U. and R.M. analyzed the results. All authors reviewed the manuscript. T.U. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

Table 1 List of Antibodies

Primary antibody (Manufacturer)	Species	Dilution	Blocking (Manufacturer)	Secondary antibody (Manufacturer)	Dilution
CASP6 (Cell Signaling Technology: 9762)	Rabbit	1:1000 (WB)	PVDF Blocking Reagent (TOYOBO)	Anti-Rabbit IgG HRP-linked whole antibody (GE Healthcare)	1:25,000
GAPDH (Abcam: ab9485)	Rabbit	1:1000 (WB)	PVDF Blocking Reagent (TOYOBO)	Anti-Rabbit IgG HRP-linked whole antibody (GE Healthcare)	1:25,000

WB: Western immunoblotting

Table 2 MicroRNAs expressing with moderated t-test (cut-off<0.05) and Storey with bootstrapping in diabetic-derived neutrophils compared with non-diabetic-derived neutrophils

Gene symbol	Fold difference (Db/Non-db)	Regulation	<i>p</i> value
<i>mmu-miR-129-1-3p</i>	-19.658678	down	0.00000004
<i>mmu-miR-129-2-3p</i>	-3.2003143	down	0.00022570
<i>mmu-miR-129-5p</i>	-91.52625	down	0.00010058
<i>mmu-miR-20b-3p</i>	-9.698807	down	0.00000449
<i>mmu-miR-466c-5p</i>	-23.062893	down	0.00000005
<i>mmu-miR-669a-5p</i>	-22.38164	down	0.00000007
<i>mmu-miR-678</i>	-15.44842	down	0.00127955
<i>mmu-miR-6916-5p</i>	-38.222507	down	0.00000010
<i>mmu-miR-6997-5p</i>	-29.356022	down	0.00000081
<i>mmu-miR-770-3p</i>	-15.649054	down	0.00122081

Table 3 GO analysis for genes predicted to be targets of miR129-2-3p

Gene symbol	Gene name
Inflammatory response	
<i>Hyal3</i>	Hyaluronoglucosaminidase 3
<i>Casp6</i>	Caspase 6
<i>Ccr11l</i>	Chemokine (C-C motif) receptor 1-like 1
<i>Ccr2</i>	Chemokine (C-C motif) receptor 2
<i>Havcr2</i>	Hepatitis A virus cellular receptor 2
<i>Myd88</i>	Myeloid differentiation primary response gene 88
<i>Cxcl5</i>	Chemokine (C-X-C motif) ligand 5
<i>Il23r</i>	Interleukin 23 receptor
<i>Tlr5</i>	Toll-like receptor 5
<i>Ccl24</i>	Chemokine (C-C motif) ligand 24
<i>Pycard</i>	PYD and CARD domain containing
<i>Vimp</i>	VCP-interacting membrane protein
<i>Lgals9</i>	Lectin, galactose binding, soluble 9
<i>Ppara</i>	Peroxisome proliferator activated receptor alpha
<i>Metrn1</i>	Meteorin, glial cell differentiation regulator-like
<i>Stat5a</i>	Signal transducer and activator of transcription 5A
Execution phase of apoptosis	
<i>Casp8</i>	Caspase 8
<i>Taok1</i>	TAO kinase 1

Dedd2 Death effector domain-containing DNA binding protein 2

Neutrophil chemotaxis

Ccl24 Chemokine (C-C motif) ligand 24

Vav3 Vav 3 oncogene

Endocytosis

Ildr1 Immunoglobulin-like domain containing receptor 1

Arrb1 Arrestin, beta 1

Ache Acetylcholinesterase

Grn Granulin

Timd2 T cell immunoglobulin and mucin domain containing 2

Tmprss13 Transmembrane protease, serine 13

Micall1 Microtubule associated monooxygenase, calponin
and LIM domain containing-like 1

Cltb Clathrin, light polypeptide (Lcb)

Add1 Adducin 1 (alpha)

Cdh13 Cadherin 13

Cnn2 Calponin 2

Pycard PYD and CARD domain containing

Phagocytosis

Ccr2 Chemokine (C-C motif) receptor 2

Lepr Leptin receptor

Trem14 Triggering receptor expressed on myeloid cells-like 4

Megf10 Multiple EGF-like-domains 10

FIGURE LEGENDS

Figure 1 A. Microarray analysis. A total of 10 miRNAs showed a level in diabetic-derived neutrophils that was less than half that in non-diabetic-derived neutrophils. B. Signal value with 90th percentile shift normalization of microarray for each miRNA. The data were log₁₀-transformed. *mmu-let-7i-5p* and *mmu-miR-484* are control miRNAs. Graphs show mean \pm SD, for which no statistical analysis was performed. C. Relative expression of *miR-129-2-3p* in neutrophils isolated from BM. *miR-129-2-3p* was downregulated in diabetic-derived neutrophils, as determined by qRT-PCR. D. Relative expression of *miR-129-2-3p* in neutrophils, macrophages, B cells, and T cells isolated from BM. *miR-129-2-3p* mainly expressed in neutrophils. Graphs show mean \pm SD (n=5). The statistical significance of differences between means in Fig. 1C was assessed by Mann–Whitney *U* test. The statistical significance of differences between means in Fig. 1D was assessed by one-way ANOVA, followed by Tukey’s multiple comparison test. ***P* < 0.01; ****P* < 0.001.

Figure 2 A-E. Relative expression of *Casp6*, *Ccr11l*, *Ccr2*, *Casp8*, and *Dedd2* in neutrophils isolated from BM. *Casp6*, *Ccr11l*, *Ccr2*, *Casp8*, and *Dedd2* were expressed at high levels in Db. Graphs show mean \pm SD (n=5–7). The statistical significance of differences between means was assessed by Mann–Whitney *U* test. ***P* < 0.01.

Figure 3 A. Alignment of *miR-129-2-3p* seed sequences and the corresponding seed sequences of *Casp6*, *Ccr2*, and *Dedd2* mRNA. *miR-129-2-3p* is predicted to bind with high affinity to *Casp6*, *Ccr2*, and *Dedd2* 3'-UTRs. B–D. A luciferase reporter vector encoding the 3'-UTRs was cotransfected with *miR-129-2-3p* mimic or mutation into

3T3 cells. A decrease in luciferase activity indicates binding of the miRNA mimic to the 3'-UTR of the target sequence. E–F. Relative expression of *CASP6* and *DEDD2* in HL-60 cells transfected with mutation or mimic. Graphs show mean \pm SD (n=4-6). The statistical significance of differences between means was assessed by Mann–Whitney *U* test. **P* < 0.05; ***P* < 0.01.

Figure 4 A. Representative images of neutrophil IHC in skin wounds at D2W. Arrowhead indicates wound margin. *Scale bar* = 400 μ m (upper) and 50 μ m (lower). B. Ratios of neutrophil-positive area relative to the wound area at D1W and D2W in each skin wound (3-4 wounds), and the average value of each positive area was used for comparative analysis. C. ISH of *miR-129-2-3p* showing that wound-infiltrated neutrophils were predominantly present in the wound sites of non-db at D1W. *Scale bar* = 300 μ m (upper) and 100 μ m (lower). Arrowhead, wound margin. D. Relative expression of *miR-129-2-3p* in equal numbers of neutrophils from non-db and db D2W (each n=5). E–G. Relative expression of *Casp6*, *Ccr2*, and *Dedd2* in skin wounds on D2W. Graphs show mean \pm SD (n=5–11). H. Relative expression of cleaved CASP6 to total CASP6 in skin wounds on D2W. Graphs show mean \pm SD (4 wounds). The statistical significance of differences between means was assessed by Mann–Whitney *U* test. **P* < 0.05; ***P* < 0.01.

Figure 5 A. Representative images of the gross appearance of db excisional wounds with *miR-129-2-3p* mimic, mutation as a negative control and mutation in non-db. B. Proportion of wound area on each day after wounding (3, 7, 10, 14, and 21 days) relative to the initial wound area. Wound area was measured using Adobe Photoshop

CC. Graphs show mean \pm SD (mutation in non-db: n=4, mutation and mimic in db: n=9). The statistical significance of differences between means was assessed by two-way ANOVA, followed by Bonferroni post-tests to compare replicate means. C. Representative images of neutrophil IHC in skin wounds at D3W with *miR-129-2-3p* mimic or mutation. Arrowhead, wound margin. *Scale bar* = 400 μ m. D. Ratios of neutrophil-positive area relative to the wound area at D3W with mimic or mutation in each skin wound (3-4 wounds), and the average values of each positive area were used for comparative analysis. E-F. Relative expression of *Casp6* and *Ccr2* in skin wounds at D3W with *miR-129-2-3p* mimic or mutation. Graphs show mean \pm SD (4-8 wounds). The statistical significance of differences between means was assessed by Mann-Whitney *U* test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Figure 6 Model summarizing the interplay among *miR-129-2-3p*, *Casp6*, and *Ccr2* in diabetic-derived neutrophils from bone marrow to wound site.

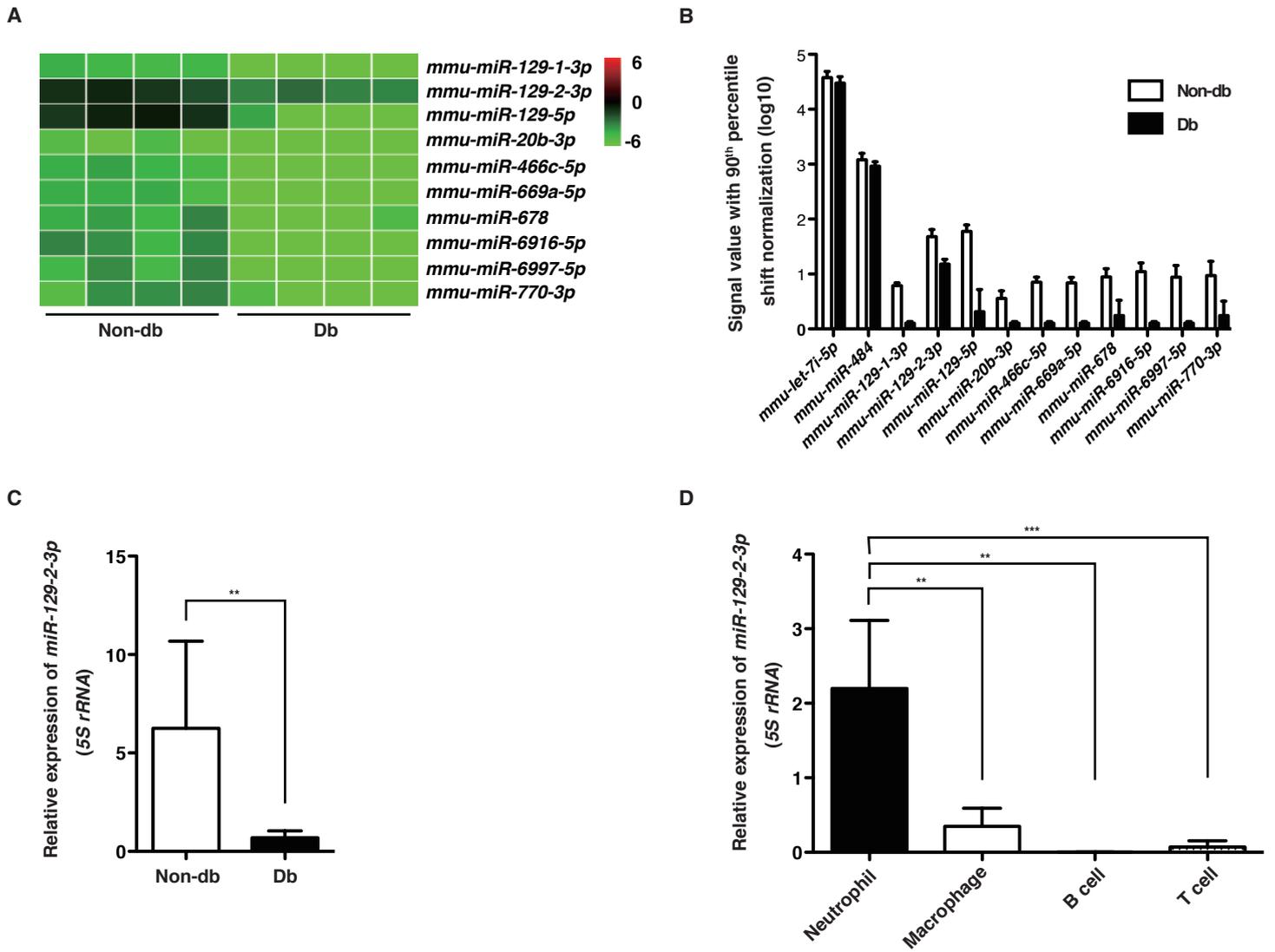


Figure 1

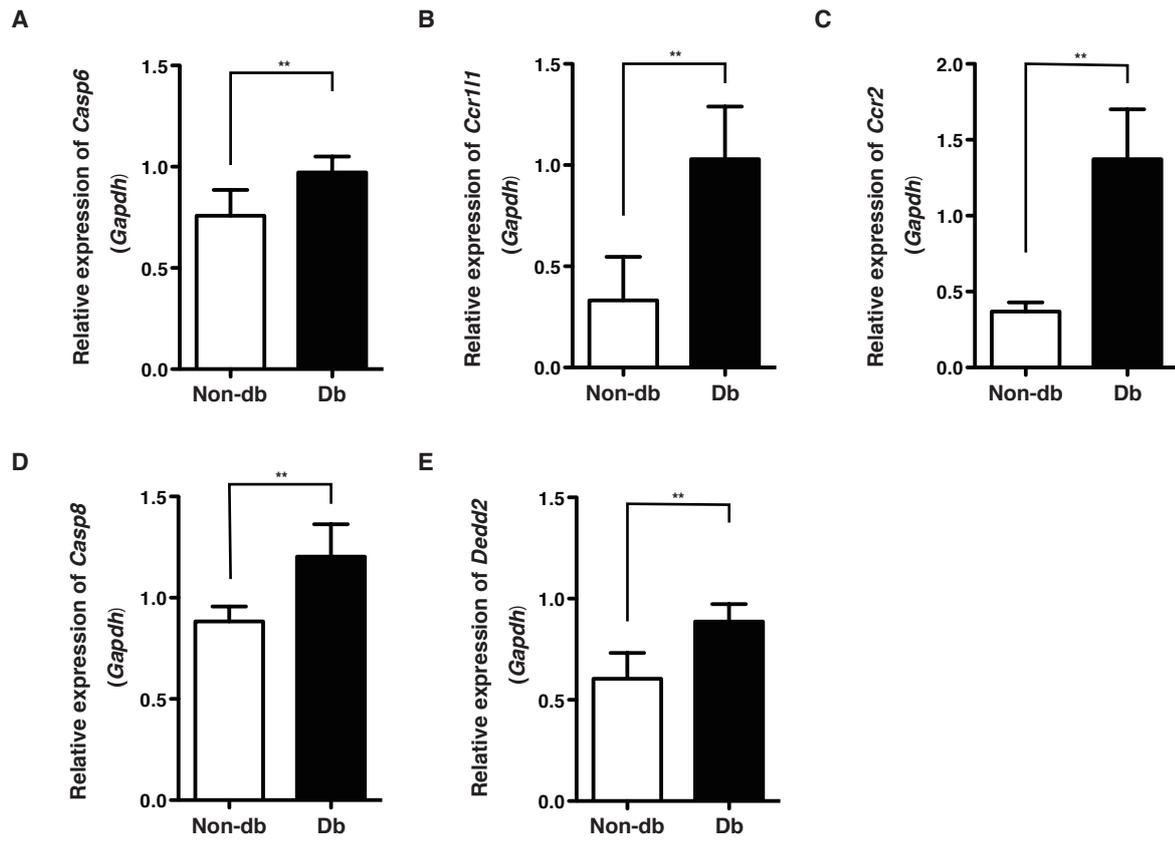
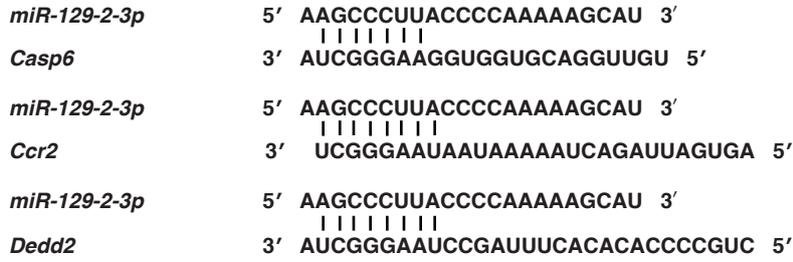
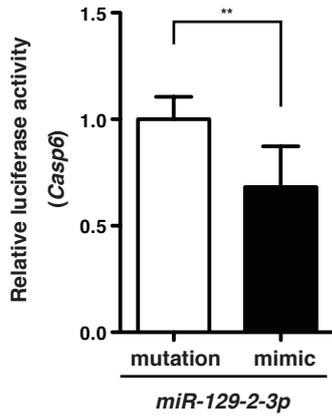


Figure 2

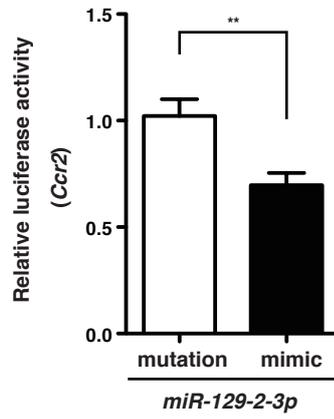
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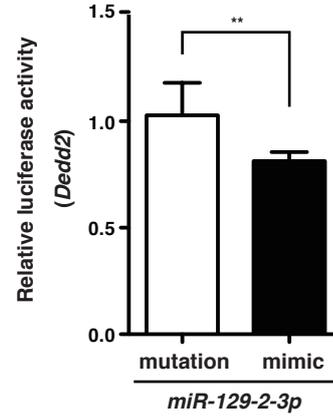
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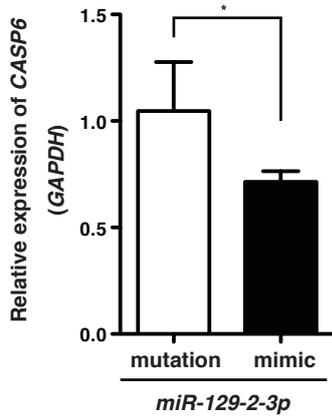
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D



E



F

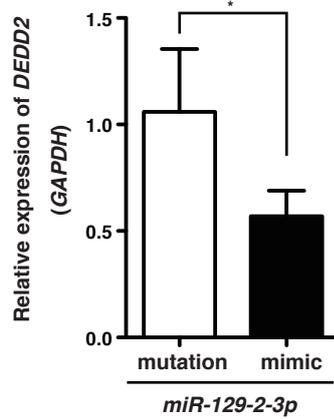


Figure 3

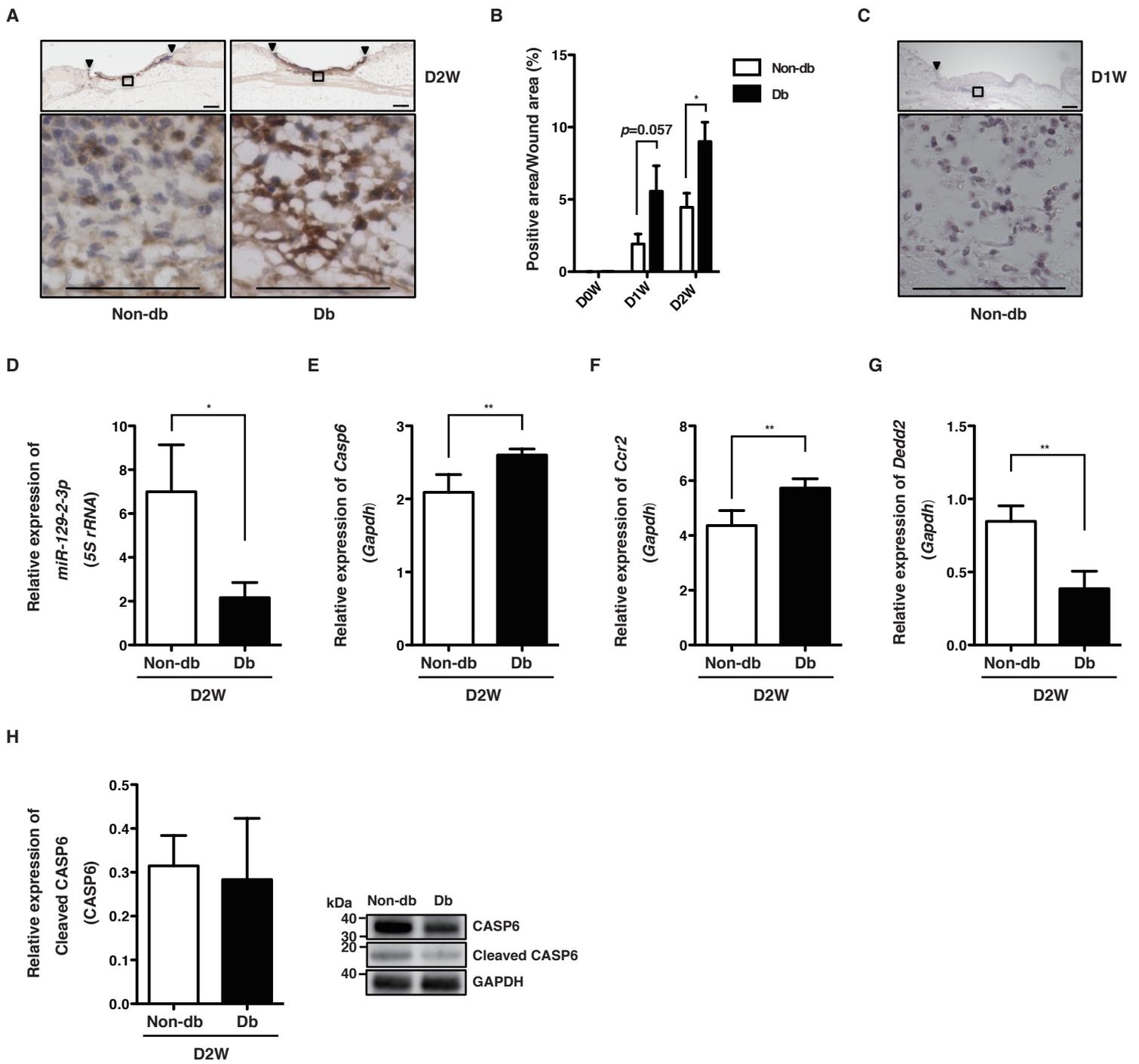


Figure 4

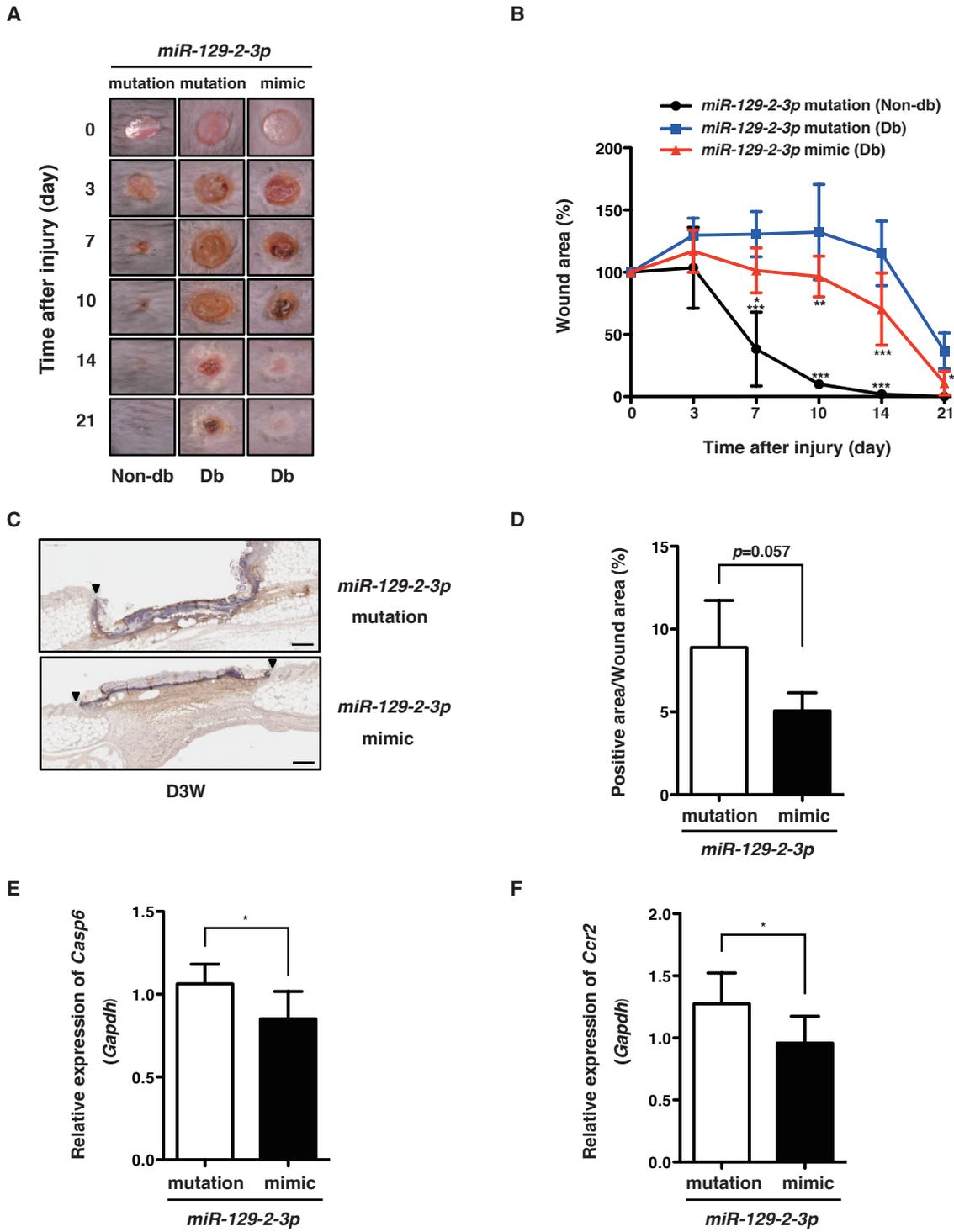


Figure 5

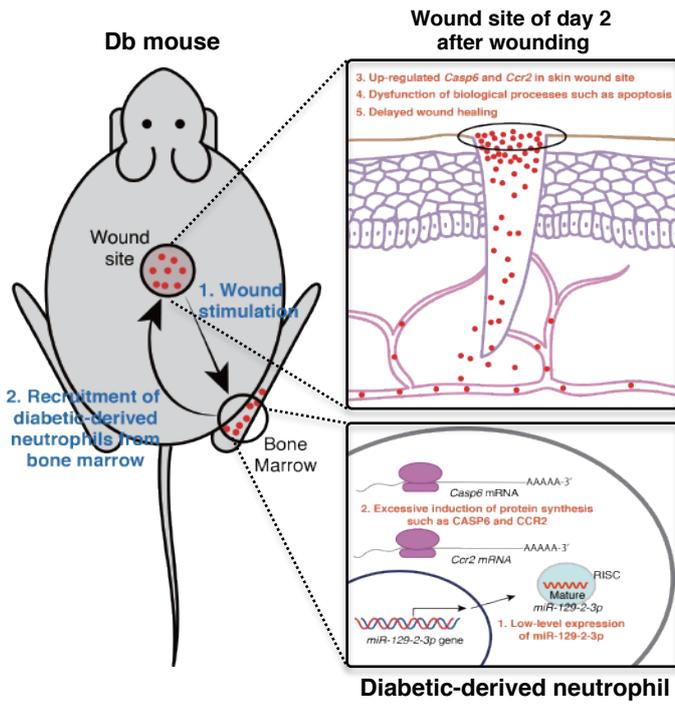


Figure 6