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<th>MiR-142 is required for Staphylococcus aureus clearance at skin wound sites via small GTPase-mediated regulation of the neutrophil actin cytoskeleton</th>
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<td>Citation</td>
<td>Nagasaki University (長崎大学) 博士 医学 博士論文 (2017-03-21)</td>
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<tr>
<td>Issue Date</td>
<td>2017-03-21</td>
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<td>URL</td>
<td><a href="http://hdl.handle.net/10069/37164">http://hdl.handle.net/10069/37164</a></td>
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MicroRNAs (miRNAs) are small noncoding RNAs that regulate protein translation by binding to complementary target mRNAs. We previously identified two mature members of the miR-142 family, miR-142-5p and miR-142-3p, as inflammation-related miRNAs with potential roles in wound healing. Here, we demonstrated that these two miRNAs are prominently expressed in wound-infiltrated neutrophils and macrophages and play central roles in wound healing. We generated miR-142−/− mice using the exchangeable gene-trap method and showed that healing of Staphylococcus aureus-infected skin wounds was significantly delayed in miR-142−/− mice compared with that in wild-type mice. MiR-142−/− mice exhibited abnormal abscess formation at S. aureus-infected skin wound sites and were also more susceptible to horizontal transmission of wound infections. MiR-142−/− neutrophils showed altered phagocytosis as a consequence of chemotactic behavior, including enhanced F-actin assembly, disturbed cell polarity, and increased cell motility. We showed that these changes were linked to cytoskeletal regulation, and that expression of the small GTPases was markedly increased in miR-142−/− neutrophils. Collectively, our data demonstrate that the miR-142 family is indispensable for protection against S. aureus infection and its clearance at wound sites. MiR-142-3p and miR-142-5p play nonredundant roles in actin cytoskeleton regulation by controlling small GTPase translation in neutrophils at wound sites.


INTRODUCTION

Skin wound healing can be considered to consist of three phases: inflammation, proliferation/migration, and maturation/resolution. During the inflammatory phase, neutrophils are the first to migrate to the breach in the skin barrier to protect against microbes. Subsequently, during the proliferation/migration phase, macrophages are drawn to the wound where they secrete growth factors, cytokines, chemokines, and phagocyte-spent neutrophils, and other cell and matrix debris. In parallel, leading-edge epithelial cells are activated to re-cover the denuded wound surface, and local endothelial cells contribute to the spraying of new blood vessels within the contractile granulation tissue. Finally, in the wound maturation/resolution phase, immune cells and contractile myofibroblasts resolve and/or die by apoptosis, and excess extracellular matrix is degraded by proteinase activity (Eming et al., 2014).

Staphylococcus aureus is an indigenous cutaneous bacterium frequently linked to the exacerbation of chronic skin wounds. Patients with diabetes and obese or immunosuppressed individuals are particularly at risk of nonhealing wounds accompanied by abnormal inflammatory responses, and associated with S. aureus overgrowth at such wound sites (Jenkins et al., 2016).

MicroRNAs (miRNAs) are key indirect regulators of protein translation, with each miRNA being able to target a broad range of up to hundreds of mRNAs (Baek et al., 2008; Selbach et al., 2008). It is becoming clear that miRNAs play critical roles in numerous physiological processes via their capacity to globally regulate the levels of large numbers of proteins within a cell, and hence their

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RESULTS AND DISCUSSION

MiR-142 contributes to the clearance of *S. aureus* at skin wound sites

We found that WT mice show comparatively high expression of miR-142 family members in lung, spleen, colon, bone marrow, and thymus, suggesting that they play critical roles in immune defense (Supplementary Figure S1 online). To clarify the role of miR-142 family members in skin wound healing, we examined their expression in wound-associated cells. We made wounds on the dorsal skin of WT mice, excised the wound tissue 1 and 3 days later, and isolated wound-infiltrated Ly-6G<sup>−</sup> neutrophils and CD11b<sup>+</sup> cells, which by immunoaffinity selection appeared to be up to 88% positive for the murine macrophage marker F4/80 (Austyn and Gordon, 1981). Quantitative PCR of the isolated cells confirmed that the wound-infiltrated neutrophils and macrophages indeed expressed miR-142-3p and miR-142-5p (Figure 1a). To examine the biological function of these miRNAs in skin wound healing, we generated whole-body miR-142<sup>−/−</sup> mice using an exchangeable gene-trap clone: Ayu21-KBW111 (http://egtc.jp/action/access/clone_detail? id=21-KBW111) (Araki et al., 2014) (Supplementary Figure S2 online). Our miR-142<sup>−/−</sup> mice had a phenotype similar to that of another miR-142<sup>−/−</sup> mouse strain, and exhibited splenomegaly, altered lymphocyte and red blood cell counts, and altered ratios of immune cells in the spleen (Supplementary Figure S3 and Supplementary Table S1 online) (Chapnik et al., 2014; Kramer et al., 2015).

Our initial studies of aseptic healing after streptozotocin-induced type 1 diabetes showed no significant difference between WT and miR-142<sup>−/−</sup> mice (Supplementary Figure S4 online). To determine whether the miR-142 family is involved in wound repair and in the clearance of *S. aureus* infection in skin wounds, we made 4-mm excisional wounds in the dorsal skin of WT and miR-142<sup>−/−</sup> mice (Figure 1b), inoculated the wounds with *S. aureus* (1 × 10<sup>8</sup> colony-forming units [CFU]/10 μl), and monitored wound healing over the next several days (Figure 1c and d). In WT mice, the *S. aureus*-infected wound had not healed by 7 days after injury, but showed signs of healing between 7 and 14 days, with 90% of all wounds fully closed by 14 days. Healing of infected wounds was considerably worsened in miR-142<sup>−/−</sup> mice with wounds only just beginning to heal after 10 days and only 69% of wounds healed at 14 days. Histologic analysis allowed us to quantify the extent of re-epithelialization at various points during repair. Re-epithelialization at *S. aureus*-infected wound sites of miR-142<sup>−/−</sup> mice (0.62 ± 0.42 mm) was markedly slower than for *S. aureus*-infected wound sites of WT mice (1.0 ± 0.30 mm) (Figure 1e–g), but by 21 days, *S. aureus*-infected wound sites of both WT and miR-142<sup>−/−</sup> mice were fully closed. We assessed the quantity of live *S. aureus* at the wound sites and found that 6.2-fold more bacteria were present in the wounds of miR-142<sup>−/−</sup> mice (3.6 × 10<sup>6</sup> CFU/ml) compared with those of WT mice (5.8 × 10<sup>5</sup> CFU/ml) at 3 days after injury (Figure 1h), suggesting that neutrophil clearance of infection might be the mechanistic link to poor wound closure.

In general, bacterial infections are driven by close contact (horizontal infection) (Fritz et al., 2012). We therefore next investigated the role of miR-142 in the defense against naturally transmitted *S. aureus* skin infection. We established a horizontal infection model in which WT and miR-142<sup>−/−</sup> mice with aseptic skin wounds were housed in the same cage as a WT mouse with an *S. aureus*-infected skin wound (Figure 1i). We confirmed that wound-inoculated *S. aureus* bioparticles of the host mouse had been transmitted to two aseptic mice after 1 day (Figure 1j). We next assessed live *S. aureus* colonization and found significantly more (13.6-fold) *S. aureus* at the wound sites of miR-142<sup>−/−</sup> mice (7.1 × 10<sup>5</sup> ± 7.0 × 10<sup>5</sup> CFU/ml) than at those of WT mice (5.2 × 10<sup>4</sup> ± 4.0 × 10<sup>4</sup> CFU/ml) after 3 days (Figure 1k). We could not detect *S. aureus* colonization in the intact skin of WT and miR-142<sup>−/−</sup> mice. Collectively, our data show that miR-142 participates in protection against *S. aureus* infection at skin wound sites.

MiR-142<sup>−/−</sup> mice show altered abscess formation and delayed immune cell phagocytosis at *S. aureus*-infected skin wound sites

Neutrophils are the first cells to migrate to skin wound sites. At the cell level, miR-142<sup>−/−</sup> neutrophils showed impairments of both chemotactic and phagocytic behavior, due to abnormal expression of Rac and Rho family of small GTPases and consequently disturbed F-actin assembly. Collectively, our data suggest a mechanistic link between miR-142 family regulation of small GTPase levels and activity, and that these miRNAs function in the protection against *S. aureus* infection and its clearance at wound sites.
wounds is 1.65-fold lower in infected miR-142^-/-:lys-EGFP mice than in WT:lys-EGFP mice (Figure 2a and b).

A hallmark of S. aureus infection is abscess formation at the skin wound site, which involves neutrophils “wallowing off” the site of infection to enable effective bacterial clearance (Kobayashi et al., 2015; Molne et al., 2000). Histologic analysis of cross sections of WT wound tissues 3 days after injury showed abscesses with clearly recognizable borders under the dermis (Figure 2c). However, there was no clear abscess border in the wounds from miR-142^-/- mice, but

Figure 1. MiR-142 is necessary for healing of S. aureus-infected skin wounds. (a) Expression of miR-142 family members in wound-infiltrated neutrophils and macrophages, measured by qPCR (n = 3). (b) Schematic diagram of the murine skin wound healing model. Excisional wounds (4 mm thickness) were made in the shaved dorsal skin of adult male mice, and S. aureus was inoculated (1 x 10^6 CFU/10 μl) directly into the wound sites after injury. (c) Representative images of the gross appearance of S. aureus-infected excisional wounds in WT and miR-142^-/- mice. (d) Time course showing the proportion of wounds remaining open compared with the initial wound area (WT, n = 10; miR-142^-/-, n = 14). (e and f) H&E staining of re-epithelialization in WT (e) and miR-142^-/- mice (f) (wound margin [arrowheads] and the leading edge of epithelia [arrows]). (g) Measurement of epithelial tongue 10 days after injury (n = 12). (h) Quantity of colonizing S. aureus 3 days after injury, showing significantly higher levels at the wound sites of miR-142^-/- mice (n = 6) compared with WT mice (n = 8). (i) Illustration of the horizontal infection study. WT and miR-142^-/- mice with aseptic skin wounds were placed in the same cage as the host WT mouse bearing an S. aureus-infected wound. (j) Inoculated S. aureus (red) was naturally transmitted to other wound sites through direct contact. (k) Quantity of colonizing S. aureus was significantly higher at the wound sites of miR-142^-/- than at those of WT mice 3 days after cohousing with the S. aureus-infected host mouse (n = 7). ND, not detected. All data are expressed as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 by two-way analysis of variance (d), unpaired t-test (g) with Welch’s test (h, k), CFU, colony-forming units; H&E, hematoxylin and eosin; qPCR, Quantitative PCR; SD, standard deviation; WT, wild type.
Figure 2. 

**MiR-142 is necessary for abscess formation and phagocytosis at the sites of *S. aureus*-infected skin wounds.** (a) Representative in vivo fluorescence microscopy images of EGFP-expressing neutrophils at *S. aureus*-infected skin wound sites. Activity fluorescent imaging on a color scale overlaid on a gray scale image of wound sites. (b) Recruitment of neutrophils at *S. aureus*-infected skin wound sites, as measured by in vivo fluorescence. Values are the mean radiant efficiency (µW/cm²/µW/cm²) ± SD (aseptic model, n = 6; infection model, n = 8). (c) H&E staining shows altered abscess formation and cytomorphology of neutrophils at the wound sites of *MiR-142*−/− mice 3 days after injury. The dotted line indicates the abscess interface. High-magnification view of the white asterisks indicates the recruitment of neutrophils (open arrowheads). Closed arrowheads indicate wound margin. (d) Images of Gram staining were obtained using serial sections. High-magnification view of the white asterisks indicates *S. aureus* (arrows). Images shown are representative of eight independent experiments.
the mechanisms of neutrophil chemotaxis, we incubated bone marrow-derived neutrophils in the presence of a local source of the chemotactic peptide N-formylmethionyl-leucyl-phenylalanine (fMLP) and imaged their migratory tracks by differential interference contrast imaging microscopy. fMLP-stimulated miR-142−/− neutrophils exhibited multiple leading edges and a hyper-stretched morphology (reminiscent of that seen in vivo in miR-142−/− mice, as mentioned above), as they migrated toward the attractant (Figure 4a, Supplementary Movies S1 and S2 online). To structurally and
functionally characterize the neutrophils during chemotaxis, we examined the F-actin network in cells stained with fluorescent phalloidin using structured illumination microscopy, a form of super-resolution fluorescence microscopy (Cox, 2015). Before stimulation, WT and miR-142/−/− neutrophils had identical morphologies (Figure 4b); however, on fMLP stimulation, miR-142/−/− neutrophils exhibited markedly increased F-actin content within their lamellipodia (Figure 4c), as well as clear uropod retraction defects (Supplementary Figure S5 online). We visualized chemotaxis of WT:lys-EGFP and miR-142/−/−:lys-EGFP neutrophils by confocal microscopy and confirmed that fMLP-stimulated
miR-142−/−:lys-EGFP neutrophils behaved similarly to miR-142−/− neutrophils (Figure 4d, Supplementary Movies S3 and S4 online). Time-lapse videos of 2-hour duration revealed that miR-142−/−:lys-EGFP neutrophils moved toward a high concentration of fMLP at a higher velocity than WT:lys-EGFP neutrophils (Figure 4e and f). The fMLP-stimulated movement of miR-142−/−:lys-EGFP neutrophils was also significantly more sustained than that of WT:lys-EGFP neutrophils (WT 82.5 ± 4.7 minutes [43 cells from 3 mice]; miR-142−/− 105.0 ± 4.2 minutes [47 cells from 3 mice], P = 0.0006; Supplementary Movies S3 and S4). However, miR-142−/−:lys-EGFP neutrophils tended to lose their polarity more rapidly than WT cells, leading to a haphazard migratory route (Figure 4g), even though preferential migration directionality in miR-142−/−:lys-EGFP neutrophils (22 per 47 cells with a higher frequency of migration in the direction of 0° ± 45°) was not significantly different from that in WT:lys-EGFP neutrophils (21 per 46 cells with a higher frequency of migration in the direction of 0° ± 45°) (Figure 4h). In general, neutrophils migrate toward a wound or site of infection and then remain static while secreting biomolecules at the site of inflammation. We speculate that miR-142−/− neutrophils may have defective “stop signaling,” which normally allows them to shift from the migratory phase to the cytokine secretory phase, resulting in a greater susceptibility of miR-142−/− mice to infection at wound sites. Taken together, our data indicate that miR-142 in neutrophils contributes to the maintenance of polarity and the shift from the migratory to the static, bactericidal state.

Expression of small GTPases is regulated by miR-142 in neutrophils

The small GTPases Rho, Rac, and Cdc42 are necessary for various aspects of leukocyte migration induced by

Figure 5. MiR-142 family regulates translation of small GTPases during chemotaxis. (a) Immunoblot analysis of Rac1, RhoA, and Cdc42 expression in neutrophils 30 minutes after fMLP stimulation. (b) Densitometric analysis of Rac1, RhoA, and Cdc42 expression (n = 4). (c and d) Representative SIM images showing the expression and localization of Rac1 and RhoA in neutrophils 30 minutes after fMLP stimulation of chemotaxis. A marked difference is observed between WT and miR-142−/− neutrophils. Scale bar = 10 μm. (e) MiR-142-3p and miR-142-5p bind with high affinity to Rac1 and RhoA 3′-UTRs, respectively, but not to the Cdc42 3′-UTR. A luciferase reporter vector encoding the 3′-UTRs was cotransfected with miR-142 mimics into 3T3 cells. A decrease in luciferase activity indicates binding of the miRNA mimic to the 3′-UTR of the target sequence. (f) Proposed model for the regulation of cytoskeleton organization in neutrophils by the miR-142 family. MiR-142-3p and miR-142-5p control lamellipodia and stress fiber/focal adhesion via Rac1 and RhoA protein synthesis. Rupture of miR-142 function leads to abnormal morphology and chemotaxis. All data are expressed as the mean ± SD. *P < 0.05 and **P < 0.01 by the unpaired t-test (b, e). fMLP, N-formylmethionyl-leucyl-phenylalanine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miRNA, microRNA; SD, standard deviation; SIM, structured illumination microscopy; 3′-UTRs, 3′-untranslated region; WT, wild type.
chemokine cues during the innate immune response (Bokoch, 2005), and mouse genome informatics (see Supplementary Materials and Methods online) predicts that all three of these molecular switches may be candidate target genes for miR-142 (Supplementary Figure S6 online). Indeed, miR-142-3p knockdown enhances the migration of human CD4⁺ T cells and modulates actin cytoskeleton regulation through Rac1 and Rock2, which are miR-142-3p target genes (Liu et al., 2014). Our related previous in vivo imaging studies of small GTPases in Drosophila embryo wound inflammation showed that Rac, Cdc42, and Rho signaling contributes to immune cell lamellipodial activity, migratory polarity, and uropod retraction, respectively, as these cells migrate to wound sites (Stramer et al., 2005). Here, we show that the expression of Rho and Rac, but not Cdc42, was significantly increased and their localization was altered in fMLP-stimulated miR-142⁻/⁻ neutrophils, compared with those in WT neutrophils, as measured by immunoblotting and immunocytochemistry (Figure 5a–d).

To investigate the direct mechanism behind this, we tested whether the miR-142 family can bind to mammalian Cdc42, Rac1, and RhoA target 3’-untranslated region (3’-UTR) mRNAs. Specifically, we cotransfected the murine fibroblast cell line with miR-142-3p or miR-142-5p mimics with a firefly luciferase vector encoding the 3’-UTRs of the predicted mRNA target sites (Supplementary Figure S6). Analysis of luciferase activity showed that the miR-142-3p and miR-142-5p mimics bound to Rac1 and RhoA 3’-UTRs, respectively (Figure 5e). These results suggest that miR-142-3p and miR-142-5p interact with Rac1 and RhoA mRNA 3’-UTRs, respectively, which may in part influence neutrophil chemotaxis (Figure 5f).

Chapnik et al. (2014) reported that megakaryocytes from miR-142⁻/⁻ mice exhibit disturbed actin cytoskeletal dynamics owing to changes in the expression levels of several cytoskeletal regulatory genes, such as cofillin-2 (Cfl2), Rho GTPase activating protein 35 (Arhgap35), and Wiskott-Aldrich syndrome-like (Wasl), all of which are target genes for miR-142-3p. Taken together with our results, this suggests that the miR-142 family may play a role in regulating neutrophil migration by modulating Rac and Rho expression levels and the consequent regulation of the actin cytoskeleton, which is clearly pivotal for efficient neutrophil migration.

We found that the miR-142 family may regulate the expression of small GTPases and thus orchestrate neutrophil motility during chemotaxis, but it is known that the small GTPase family also plays a central role in phagocytosis. For instance, Fc receptor-mediated phagocytosis, which depends on binding to the Fc portion of antibodies, is mediated by Cdc42 and Rac, and the internalization of complement-opsonized particles is dependent on Rho (Caron and Hall, 1998). Our findings in this study reveal that miR-142 may be involved in neutrophil migration through effects on small GTPase expression, and we speculate that it may also control engulfment efficiency. Macrophage, lymphocyte, and cytokine production are important for bacterial killing (Hume et al., 2006). We are currently investigating the molecular mechanism linking miR-142 and other biological functions (i.e., cytokine production) in detail.

Several hereditary diseases that involve genes critical for neutrophil function or production have been identified, some of which are associated with immunodeficiency and severe susceptibility to bacterial infection; for example, using a zebrafish model of wound repair and systemic infection, we revealed that immunodeficiency-related Wiskott-Aldrich syndrome protein (WASP), which is known to coordinate actin polymerization, plays an essential role in leukocyte wound recruitment and S. aureus clearance (Jones et al., 2013). A number of studies have shown that Rac isoforms are crucially important for neutrophil functions in mice, such as chemotaxis, bacterial killing, and nicotinamide adenine dinucleotide phosphate oxidase pathway activation (Koh et al., 2005; Pick, 2014), and genetic defects in small GTPase family members also affect neutrophil function in humans; for example, neutrophils derived from individuals with a point mutation in Rac2 exhibit decreased chemotactic polarization, azurophilic granule secretion, and superoxide anion production (Williams et al., 2000). Although miR-142 deficiencies have not yet been identified in humans, our findings implicate miR-142 family members in disorders related to neutrophil function and immunodeficiency.

In conclusion, we have revealed that the miR-142 family regulates actin cytoskeleton dynamics in neutrophils by controlling small GTPase translation, and showed that it plays a central role in the protection against S. aureus infection at skin wound sites. Our findings suggest that these miRNAs might be involved in multiple regulatory steps that enable the killing of opportunistic pathogens at wound sites.

MATERIALS AND METHODS

Skin wounding

All experiments were conducted according to the provisions of the Ethics Review Committee for Animal Experimentation at Nagasaki University. The wound model was established as described previously (Mori et al., 2014). In brief, full-thickness excisional wounds (4-mm biopsy punch; Kai Industries, Seki, Japan) were made in the dorsal skin (after shaving under anesthesia) of 6- to 12-week-old mice (Figure 1b). Wound tissues were harvested using a 6-mm biopsy punch (Kai Industries). The gross appearance of wound closure was recorded using a digital camera. Wound areas were calculated using Photoshop CS4 (Adobe Systems, San Jose, CA).

Artificial and horizontal S. aureus infection at skin wound sites

S. aureus type strain (NBRC 100910) was obtained from the National Institute of Technology and Evaluation (Tokyo, Japan). Mice were inoculated with S. aureus (1 x 10⁸ CFU/10 μl) at the skin wound sites. The presence of S. aureus at the wound sites was quantified by two methods. S. aureus gene-specific PCR was performed with a Quick Primer S. aureus kit (Takara Bio, Kusatsu, Japan) and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster, CA). Alternatively, bacteria were quantified by counting CFU after culture.

For the horizontal S. aureus infection study, two WT mice and one miR-142⁻/⁻ mouse were placed overnight in a cage (17 cm in length, 30 cm in width, and 13 cm in height) to check that they did not fight each other. The next day, skin wounds were made on all three mice and the wound site of one WT mouse was inoculated with Alexa Fluor 594-conjugated S. aureus BioParticles (10 μl of 20 mg/ml; Life Technologies, Carlsbad, CA) or S. aureus
(1 × 10^8 CFU/10 μl). The second WT mouse and the miR-142^{−/−} mouse were placed in the same cage as the host mouse for 3 days. The amount of S. aureus at the wound sites was then quantified by CFU counting.

Analysis of phagocytosis at wound sites

pHrodo Red S. aureus Bioparticles (10 μl of 10 mg/ml; Life Technologies) were inoculated into the skin wounds. After excision, tissues were fixed in 4% paraformaldehyde, embedded in O.C.T. Compound, and frozen. Sections (60 μm thick) were counterstained with DAPI. Phagosomes were visualized by z-stack confocal fluorescence microscopy (C2+ system; Nikon Corporation, Tokyo, Japan) equipped with Plan Apo VC20x (0.75 NA), and the images were processed using IMARIS software (Bitplane, Zurich, Switzerland).

Analysis of phagocytosis in vitro

Ly-6G^{+} neutrophils were isolated from bone marrow by MACS Separation (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The phagocytosis assay was performed with pHrodo Red S. aureus Bioparticles. In brief, 30 μl of pHrodo Red S. aureus Bioparticles (1 mg/ml) were mixed with neutrophils suspended in Dulbecco’s modified Eagle’s medium without phenol red (1 × 10^7 cells/200 μl) and placed in a glass-bottomed dish. Cells were incubated in an atmosphere of 5% CO_2 at 37°C in a stage top incubator (Tokai Hit, Fujinomiya, Japan). Cells were examined by confocal microscopy (C2+ system; Nikon Corporation) equipped with Plan Apo VC20x (0.75 NA). Images were acquired every minute and fluorescent phagosome-positive cells were counted every 10 minutes.

Statistical analysis

Data are presented as the mean ± standard deviation. Differences between means were analyzed with GraphPad Prism 6 software (GraphPad Software, San Diego, CA).

SUPPLEMENTARY MATERIAL AND METHODS

The generation of miR-142^{+/−} mice and miR-142^{−/−}:lys-EGFP mice; isolation of tissue neutrophils, macrophages, T lymphocytes, and B lymphocytes; establishment of the streptozotocin-induced diabetes model; and methods for miRNA isolation and quantitative PCR analysis, histology, transmission electron microscopy, chemotaxis assay, immunocytochemistry, total protein extraction and immunoblotting, and assay for miRNA binding to the 3'-UTR of mRNA are described in the Supplementary Materials and Methods.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Drs Thomas Graf (Centre for Genomic Regulation, Barcelona, Spain), Shintaro Hashimoto, and Masaki Honda (Kumamoto University, Kumamoto, Japan) for providing lys-EGFP mice. We are also grateful for comments on the experiments and manuscript from Dr Eun Seong Hwang (University of Seoul, Seoul, South Korea), for assistance with the transmission electron microscopy analysis from Takashi Suematsu (Department of Electron Microscopy, Nagasaki University). This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Research Activity Start-up, 20890258; Grants-in-Aid for Young Scientists A, 21689049 and 24689069; Grant-in-Aid for Scientific Research B, 16H05493; Challenging Exploratory Research, 23650448 and 25560055 to RM and 26670773 to HY; Grants-in-Aid for Young Scientists B, 26615103 and 16K20361 to KT); Takeda Science Foundation (RM); the Uehara Memorial Foundation (RM); the Nakatomi Foundation (RM); the Wellcome Trust (Senior Investigator Award 097791MA, PM); and the Royal Society (International Joint Projects, PM and RM).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.11.018.

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