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Targeting miR-223 in neutrophils enhances the clearance of *Staphylococcus aureus* in infected wounds

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 April 2018

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript.

As you will see from the reports below, both referees are positive and support publication of the article in EMBO Molecular Medicine pending appropriate revisions. Addressing the reviewers concerns in full will be necessary for further considering the manuscript in our journal. Particular attention should be given to spelling and grammatical errors. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.
Please also contact us as soon as possible if similar work is published elsewhere. If other work is published, we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.
***** Reviewer's comments *****

Referee #1 (Remarks for Author):

In this paper the authors show, by various approaches, that miR-223 has an active role in wound repair, in particular in Staphylococcus aureus infected wounds. Using both a knock-out model for miR-223 (miR-223Y/-) and antisense oligodeoxynucleotides (AS ODN), the authors are able to decrease the time of healing, either by treating the wounds with miR-223Y/--derived neutrophils or by administering a gel containing AS ODN. They also show that miR-223 directly binds to IL6 and that the expression of miR-223 is regulated by C/EBPα. Overall, the authors demonstrate that miR-223 could be seen as a potential therapeutic target, especially in the case severe chronic S. aureus-infected skin wounds.
This is a comprehensive and straightforward paper with a considerable amount of work. The experiments and the strategy to decipher the role of miR-223 in healing of infected wounds are appropriate and well designed. Of note, this paper is technically of outstanding quality, in particular regarding the purification system to isolate miRNAs and the approach to design the AS ODN. Most of the experiments support the conclusions and the potential therapeutic possibilities. The scheme included in the last figure is well appreciated and helpful for the overall interpretation of the data. Nevertheless, some issues should be addressed to strengthen the paper.

What was the rational for choosing the subset of miRs displayed in figure 1A and B? What about the other identified top-candidates?

Information about the antibody to detect neutrophils by IHC should be given (Figure 2).

Although the authors have estimated the re-epithelialization in miR223y/- mice, information about the granulation tissue might be provided.

In Figure 2E and 5A, the selected images of wound closure are not representative of the results displayed in the related graph.

The paragraph regarding the regulation of acute inflammatory responses at wound sites deserves clarifications. Indeed, the experiments showing in vivo imaging of EGFP-expressing neutrophils (Fig. 3A and B) in WT and miR223y/- mice are over interpreted. The differences between the 2 conditions are minimal, probably resulting from a low number of animals. Based on these data, the conclusion "both delayed onset but subsequent impaired resolution of the acute wound inflammatory responses in miR223y/- mice" might be revised. Moreover, it does not fit with the MPO experiments showing a peak of neutrophil activity at day 1 (Fig. 3C-E) whereas the amount of neutrophils is maximal at day 3 (Fig. 3A and B).

The experiment investigating the role of miR-223 on IL6 expression at wound site cannot be ascribed exclusively to neutrophils since the analyses are performed on the entire wound site (Fig. 4). A representative picture of IL6 immunostaining in the miR223y/- mice (Fig. 4A) should be displayed.

In Figure 6 and the related methods, the sequence of the LNA-modified AS ODN does not match the aligned miR-223 AS ODN sequence below. Please check.

The authors should explain the relevance of using both PGN and miR-223 AS ODN (Fig. 7C).

Abbreviations such as mmu and hsa should be defined.

The paper should be revised for typing and grammatical errors, in the text and figures (e.g. figure 4G).

Referee #2 (Comments on Novelty/Model System for Author):

De Kerckhove et al. identified Ago-2-bound miRNAs in mouse skin wounds, which included- among others - miR-223. This miRNA was upregulated during the early inflammatory phase of wound healing and is expressed mainly by neutrophils. Functional studies revealed that miR-233Y/- mice have impaired healing of sterile wounds, but enhanced healing of S. aureus infected wounds, most likely due to stronger activation of neutrophils and enhanced production of IL-6 by these cells.

The potential therapeutic relevance of these results was demonstrated by knock-down of miR-233 at the wound site and by application of miR-223Y/- neutrophils, which resulted in enhanced healing of S. aureus infected wounds.

A role of miR-223 in inflammation and infection control had previously been demonstrated by others, and IL-6 had previously been identified as a miR-223 target. Therefore, these aspects are not completely novel. However, a role of miR-223 in wound healing has not been demonstrated. In particular, the role of this miRNA in healing of infected wounds is novel and interesting and of potential medical importance. However, there are also a few problems with the manuscript, which are summarized below.
1.) The authors should provide more information on the miR-223Y/- mice in Materials and Methods. In particular, it should be mentioned that these hemizygous mice are completely deficient in miR-223 (at least according to the original publication).
2.) Since the manuscript focuses on miR-223, the authors should show expression of this miRNA during the whole time course of wound healing. There may be a second peak of miR-223 expression and this would be important for the interpretation of the wound healing data in the mutant mice.
3.) It should be clarified that the early increase in miR-223 in skin wounds results from the infiltration of neutrophils and is most likely not a result of a real upregulation in immune cells.
4.) Fig. 2A: The authors should mention which antibody they used for the detection of neutrophils - Ly6G? In addition, they should mention in the legend that the area indicated with a reactange is shown at high magnification below.
5.) Fig. 2D: The information in this figure is limited, since there is no comparison with other cells at the wound site, in particular fibroblasts and keratinocytes. Given the delayed reepithelialization in the mutant mice, it is particularly important to determine if this is a cell autonomous effect that results from expression of miR-223 in keratinocytes or a secondary effect resulting from enhanced inflammation (more likely).
6.) Fig. 2D-H: The authors should confirm that miR-223 is indeed not expressed in the mutant mice. Wound healing is a combination of reepithelialization and wound contraction - is contraction also affected in the mutant mice?
7.) Fig. 3B is not convincing - there is only a statistically significant difference at the 3h time point and at the 3d time point - this needs to be formulated more carefully. The different functionality of the neutrophils may be more important than this minor difference in number. I am also not convinced that there is impaired resolution, since no difference was seen at day 7.
8.) Fig. S3A: Please show representative stainings.
9.) The authors should show Il-6 mRNA levels in non-stimulated and activated neutrophils of wt and miR-223 mutant mice - this would further support the regulation of IL-6 by miR-223. The qPCR shown in Fig. 4B only shows that Il-6 expression is enhanced in total wounds of miR-223 mutant mice, which may be secondary to the enhanced numbers of neutrophils (and not a real regulation by the miRNA).
10.) The upregulation of IL-6 is unlikely to explain the impaired healing in miR-223 mutant mice (IL-6 knockout mice have impaired wound healing; Lin et al., 2003; IL-6 promotes wound healing in glucocorticoid-treated mice; Gallucci et al., 2001). Therefore, the mechanism underlying the impaired healing in the miR-223 mutant mice under sterile conditions remains unclear. This should at least be discussed.
11.) Fig. 5A and G: In addition to the macroscopic analysis, the authors should show H/E-stained sections from 7-day and 14-day wounds (and ideally use them to determine if there is an effect on reepithelialization and contraction). Given the rather high error bars, analysis of these histological parameters would clearly strengthen the data. At least one would like to get an idea about the histological features of the healing and healed wounds.
12.) Fig. 5G,H: Is it possible to determine how long the neutrophils used for treatment remain in the wound tissue? The cells could be labeled for this purpose.
13.) Fig. 6: The authors should verify that miR-223 is indeed downregulated by the ODNs at the wound site and check if IL-6 is upregulated.
14.) The paragraph describing the results shown in Fig.7 includes various errors in spelling and grammar and is therefore difficult to read. Most importantly, the results do not allow the conclusion that miR-223 is regulated by C/EBPa in response to S. aureus. To text this possibility, the authors would have to overexpress C/EBPa and determine if the PGN-mediated decline in miR-223 is rescued. Therefore, the scheme shown in Fig. 6G is not fully supported by the data.

Referee #2 (Remarks for Author):

This is an interesting manuscript and the data are generally convincing. However, additional experiments and some rewriting are required for publication in EMM.

1st Revision - authors' response 13 July 2018

Reviewer comments:
Reviewer: 1
Referee #1 (Remarks for Author):

Comment 1: *In this paper the authors show, by various approaches, that miR-223 has an active role in wound repair, in particular in Staphylococcus aureus infected wounds. Using both a knock-out model for miR-223 (miR-223Y/-) and antisense oligodeoxynucleotides (AS ODN), the authors are able to decrease the time of healing, either by treating the wounds with miR-223Y/--derived neutrophils or by administering a gel containing AS ODN. They also show that miR-223 directly binds to IL6 and that the expression of miR-223 is regulated by C/EBPα. Overall, the authors demonstrate that miR-223 could be seen as a potential therapeutic target, especially in the case severe chronic S. aureus-infected skin wounds.*
This is a comprehensive and straightforward paper with a considerable amount of work. The experiments and the strategy to decipher the role of miR-223 in healing of infected wounds are appropriate and well designed. Of note, this paper is technically of outstanding quality, in particular regarding the purification system to isolate miRNAs and the approach to design the AS ODN. Most of the experiments support the conclusions and the potential therapeutic possibilities. The scheme included in the last figure is well appreciated and helpful for the overall interpretation of the data. Nevertheless, some issues should be addressed to strengthen the paper.

What was the rational for choosing the subset of miRs displayed in figure 1A and B? What about the other identified top-candidates?
Response: First, we screened candidates for inflammatory-related miRNAs using the results from next generation sequencing (NGS) and found nine candidates for inflammation-related miRNAs that peaked on day (d) 1 after injury (Appendix Table S2.). Next, we rechecked NGS data using qPCR and confirmed the expression of the top 8 candidate miRs (*miR-147, miR-223, miR-129-3p, miR-139-5p, miR-21*, miR-340-5p, miR-142-3p*, and *miR-142-5p*) was significantly increased compared with intact skin, indicating that these miRs might be candidates for inflammation-related genes. We could not confirm the expression of *miR-486* (fold change 4.51), suggesting that the cutoff value was >4.5 in our NGS results. We next tested for the expression of our 8 candidates in *PU.1*$^{-/-}$ mice that lack an inflammatory response in skin wound sites versus WT sibs. This approach allowed us to definitively confirm that *miR-223, miR-142-3p, miR-142-5p,* and *miR-139-5p* are inflammation-related miRNAs in skin wound healing because these molecules were not expressed at wound sites in *PU.1*$^{-/-}$ mice.

As suggested, we have summarized the other identified top candidates in Appendix Table S3. One of these, miR, *miR-21,* was more highly expressed during skin wound healing compared with the other miRs and this expression was markedly increased on d 3 (4.70), 7 (8.64), and 14 (5.77) compared with intact skin, suggesting *miR-21* might be involved in skin wound healing. Indeed, the function of *miR-21* in skin wound healing has been well studied (Han Z et al, *J Cell Biochem,* 2017, PMID: 28374893) (Pastar I et al, *J Biol Chem,* 2012, PMID: 22773832) (Wang T et al, *Am J Pathol,* 2012, PMID: 23159215) (Yang X et al, *Int J Biol Sci*, 2011, PMID: 21647251). We are currently investigating other candidate skin wound healing-related miRs using the NGS results.

Comment 2: *Information about the antibody to detect neutrophils by IHC should be given (Figure 2).*
Response: In accord with the comments by you and another reviewer, we have now added information for the neutrophil antibody to the Fig 3A legend (page 50, line 21) and Appendix Table S5.

Comment 3: *Although the authors have estimated the re-epithelialization in miR223y/- mice, information about the granulation tissue might be provided.*

Response: In accord with the comments by you and another reviewer, we investigated the area of granulation tissues at d 7 and 14 in aseptic wound sites (Fig EV1A-EV1C). We found that aseptic wound sites in *miR-223*$^{Y/-}$ mice were significantly increased compared with WT mice. The use of histological analysis allowed us a better understanding compared with gross appearance. The gross appearance of wound closure at d 14 in the wound sites of *miR-223*$^{Y/-}$ mice was not altered compared with WT mice. However, wound contraction might be related to the area of granulation tissue. To investigate wound contraction we performed immunohistochemistry (IHC) for α-smooth muscle actin (αSMA), a marker of contracting myofibroblasts, according to our previous report (Mori et al, J Cell Sci, 2006, PMID: 17158921). Expression of αSMA at aseptic wound sites in *miR-223*$^{Y/-}$ mice at d 7 were markedly decreased compared with WT mice. We have modified the text accordingly (page 9, line 12 to 20) and Fig EV1.

Comment 4: *In Figure 2E and 5A, the selected images of wound closure are not representative of the results displayed in the related graph.*
Response: As mentioned, we have modified Fig 3E (previously Fig 2E) and Fig 6A (previously Fig 5A).

Comment 5: *The paragraph regarding the regulation of acute inflammatory responses at wound sites deserves clarifications. Indeed, the experiments showing in vivo imaging of EGFP-expressing neutrophils (Fig. 3A and B) in WT and miR223y/- mice are over interpreted. The differences between the 2 conditions are minimal, probably resulting from a low number of animals. Based on these data, the conclusion "both delayed onset but subsequent impaired resolution of the acute wound inflammatory responses in miR223y/- mice" might be revised. Moreover, it does not fit with the MPO experiments showing a peak of neutrophil activity at day 1 (Fig. 3C-E) whereas the amount of neutrophils is maximal at day 3 (Fig. 3A and B).*
Response: We agree with your comments and have revised the paragraph regarding the onset and resolution of inflammatory responses in WT and *miR-223*$^{Y/-}$ mice according to your advice. In Fig 4A and 4B (previously Fig 3A and 3B), we wanted to show the neutrophil influx into the wound site over time using EGFP green fluorescent labeled neutrophils. Kim and colleagues (J Invest Dermatol, 2008), reported that neutrophil influx after skin wounding in *lys*-EGFP mice increased most rapidly over the initial 12 h and reached a maximum between d 1 and d 3. It then decreased precipitously at d 5 (Fig 2 and 3 in Kim et al., J Invest Dermatol, 2008). Our results are similar to theirs; at 12 h in WT mice, the neutrophil influx had started to increase and at d 3 the influx had peaked. In contrast, the rate of influx in *miR-223*$^{Y/-}$ mice appeared somewhat slower (3 h) although it also peaked at d 3. By d 3, the influx rate of *miR-223*$^{Y/-}$ mice had surpassed that of WT mice, and the neutrophil influx became excessive. We also modified the representative results of *in vivo* fluorescent images of EGFP-expressing neutrophils in skin wound sites as reflected in the related graph (Fig 4A).

Regarding MPO (Fig 4C-4E), we measured MPO to show the change in neutrophil function in *miR-223*$^{Y/-}$ mice *in vivo*. Because mature neutrophils do not produce new MPO, and only activated neutrophils activate MPO, we could assess neutrophil responses to wounding stimuli. Klebanoff (J Leukoc Biol. 2005), reported that MPO synthesis in neutrophil development starts in the promyelocyte stage and ends as enclosed azurophil granules at the beginning of the myelocyte stage; thus, mature neutrophils no longer produce MPO (Klebanoff SJ, J Leukoc Biol. 2005). MPO imaging using an inflammation probe measured the MPO activity produced by activated neutrophils; therefore, the peak of this image at d 1 shows the response to the wounding stimulus. At d 1, the wounding stimulus induces neutrophils to release MPO according to phagocyte function, such that MPO reaches a peak at d 1. At d 3, as the wounding stimulus decreases and MPO is no longer needed, the amount of MPO decreases. Fig 4E indicates that the amount of MPO measured by ELISA decreased relative to the time post-wounding stimulus.

We have also examined *in vitro* reactive oxygen species (ROS) production in neutrophils (Fig 4F and 4G). We performed live cell imaging analysis using confocal microscopy to dissect time-dependent neutrophil activation. ROS production in peripheral blood neutrophils (PBNs) derived from *miR-223*$^{Y/-}$ mice was slowly activated and interestingly, at 60 min *miR-223*$^{Y/-}$ PBNs exhibited increased ROS production compared with WT PBNs.

Collectively, our *in vivo* and *in vitro* analyses indicate that *miR-223* regulates the acute inflammatory response at wound sites and subsequently affects macrophage infiltration at wound sites. We have modified the text accordingly (page 10, line 1 to page 12, line 1).

Comment 6: *The experiment investigating the role of miR-223 on IL6 expression at wound site cannot be ascribed exclusively to neutrophils since the analyses are performed on the entire wound*

site (Fig. 4). A representative picture of IL6 immunostaining in the miR223y/- mice (Fig. 4A) should be displayed.
Response: As you suggest IL-6 might be expressed by various skin wound-related cells such as neutrophils, epidermal keratinocytes, macrophages, Langerhans' cells, and fibroblasts (Paquet P et al, Int Arch Allergy Immunol, 1996, PMID: 8634514) (Sato Y et al, Int J Legal Med, 2000, PMID: 10876984) (Gallucci RM et al, FASEB J, 2000, PMID: 11099471). Our *PU.1*$^{-/-}$ study suggests that *miR-223* is not expressed in keratinocytes and fibroblasts at wound sites during the early wound response; therefore, we speculated that keratinocytes and fibroblasts probably do not affect IL-6 expression related to the deletion of *miR-223*. Furthermore, keratinocytes and fibroblasts did not migrate to wound sites in the early acute inflammatory phase (d 1); for these reasons we focused on IL-6 expression in neutrophils. We have demonstrated that *miR-223* is predominantly expressed by neutrophils in the acute inflammatory phase using IHC and *in situ* hybridization (Fig 3A-3C), suggesting that it is largely neutrophils that exhibit altered IL-6 expression.

As you suggested, we have now performed IHC for IL-6 using double immunofluorescence staining of d 1 wound sites in *miR-223*$^{Y/-}$ and WT mice. We confirmed that the expression of IL-6 (red color) in wound-infiltrated neutrophils (green color) in *miR-223*$^{Y/-}$ mice were markedly increased compared with WT mice. We modified the text accordingly (page 12, line 11 to 13) and changed Fig 5A (previously Fig 4A).

Comment 7: *In Figure 6 and the related methods, the sequence of the LNA-modified AS ODN does not match the aligned miR-223 AS ODN sequence below. Please check.*
Response: As mentioned, we modified the sequence of the LNA-modified AS ODN to match the aligned *miR-223* AS ODN (Fig 7A).

Comment 8: *The authors should explain the relevance of using both PGN and miR-223 AS ODN (Fig. 7C).*
Response: We wanted to show that *miR-223* expression in *miR-223* AS ODN-treated dHL60 cells was significantly downregulated after PGN stimulation even though *miR-223* was knocked down similar to the control experiments using normal dHL60 cells. We now explain the relevance of using both PGN and *miR-223* AS ODN more carefully in the text (page 17, line 16 to 17).

Comment 9: *Abbreviations such as mmu and hsa should be defined.*
Response: Thank you, we now have defined *mmu* and *hsa* in the text (page 51, line 2 and 6).

Comment 10: *The paper should be revised for typing and grammatical errors, in the text and figures (e.g. figure 4G).*
Response: As suggested, typing and grammatical errors in the text and all Figures have now been checked by an English editing company.

Reviewer comments:
Reviewer: 2
Referee #2 (Comments on Novelty/Model System for Author):

De Kerckhove et al. identified Ago-2-bound miRNAs in mouse skin wounds, which included- among others - miR-223. This miRNA was upregulated during the early inflammatory phase of wound healing and is expressed mainly by neutrophils. Functional studies revealed that miR-233Y/- mice have impaired healing of sterile wounds, but enhanced healing of S. aureus infected wounds, most likely due to stronger activation of neutrophils and enhanced production of IL-6 by these cells. The potential therapeutic relevance of these results was demonstrated by knock-down of miR-233 at the wound site and by application of miR-223Y/- neutrophils, which resulted in enhanced healing of S. aureus infected wounds.

A role of miR-223 in inflammation and infection control had previously been demonstrated by others, and IL-6 had previously been identified as a miR-223 target. Therefore, these aspects are not completely novel. However, a role of miR-223 in wound healing has not been demonstrated. In particular, the role of this miRNA in healing of infected wounds is novel and interesting and of potential medical importance. However, there are also a few problems with the manuscript, which are summarized below.

Comment 1: *1.) The authors should provide more information on the miR-223Y/- mice in Materials and Methods. In particular, it should be mentioned that these hemizygous mice are completely deficient in miR-223 (at least according to the original publication).*
Response: As suggested, we have added information regarding the *miR-223* locus to the Materials and Methods (page 23, line 4 to 7).

Comment 2: *2.) Since the manuscript focuses on miR-223, the authors should show expression of this miRNA during the whole time course of wound healing. There may be a second peak of miR-223 expression and this would be important for the interpretation of the wound healing data in the mutant mice.*
Response: We have now analyzed the expression of *miR-223* at wound sites in WT mice on days (d) 1, 3, 7, 10, and 14 after injury and in intact skin, and find that the expression of *miR-223* peaked at d 1 and was decreased by d 7 thereafter. The expression levels of *miR-223* at d 10 and 14 in wound sites of WT mice were very low, similar to that in intact skin (undetectable levels), because *miR-223* is expressed by inflammatory cells (i.e. neutrophils, macrophages) (Appendix Fig S2).

Comment 3: *3.) It should be clarified that the early increase in miR-223 in skin wounds results from the infiltration of neutrophils and is most likely not a result of a real upregulation in immune cells.*
Response: It was reported that neutrophils mainly migrate to aseptic murine skin wound sites on d 1: the acute inflammation phase during skin wound healing, and therefore it is difficult to detect other immune cells (macrophages, lymphocytes) at this timepoint. Macrophages appeared in wound sites (Fig EV2) at d 3 and we found that *miR-223* was expressed in wound infiltrated macrophages at d 3 after injury (Fig 3D). Taken together, we suspect that the expression of *miR-223* in aseptic skin wound sites at the early timepoint (d 1 after injury) was predominantly from wound-infiltrated neutrophils, because macrophages had not migrated at d 1 after injury. On days 3 and 7, neutrophils and macrophages are present in wound sites, so that *miR-223* might be expressed by both cell types. We are currently investigating the function of *miR-223* in macrophages in skin wound healing.

Comment 4: *4.) Fig. 2A: The authors should mention which antibody they used for the detection of neutrophils - Ly6G? In addition, they should mention in the legend that the area indicated with a reactange is shown at high magnification below.*
Response: As suggested by you and another reviewer, we have added information regarding the neutrophil antibody (Ly6-G and Ly6-C) and the rectangle in the Fig 3A and 3B (previously Fig 2A and 2B) legend (page 50, line 21 to 22) (page 51, line 4 to 5) and Appendix Table S5.

Comment 5: *5.) Fig. 2D: The information in this figure is limited, since there is no comparison with other cells at the wound site, in particular fibroblasts and keratinocytes. Given the delayed reepithelialization in the mutant mice, it is particularly important to determine if this is a cell autonomous effect that results from expression of miR-223 in keratinocytes or a secondary effect resulting from enhanced inflammation (more likely).*
Response: We think that *miR-223* is only expressed by inflammatory cells and not by fibroblasts and keratinocytes, because *miR-223* was not expressed by *PU.1*$^{-/-}$ mice that had no inflammatory responses at skin wound sites because they lack neutrophils, macrophages, and lymphocytes (Fig 2B) (page 4, line 15 to 17). We confirmed that wound-infiltrated neutrophils predominantly express *miR-223* at d 1 after injury and not keratinocytes by using *in situ* hybridization (ISH) (Fig 3A and 3B). With regard to the delayed re-epithelialization of *miR-223*$^{Y/-}$ mice, we, like you, think that this is a consequence of increased acute inflammatory responses (secondary effect).

Comment 6: *6.) Fig. 2D-H: The authors should confirm that miR-223 is indeed not expressed in the mutant mice. Wound healing is a combination of reepithelialization and wound contraction - is contraction also affected in the mutant mice?*
Response: We confirmed that the expression of *miR-223* was not expressed at wound sites in *miR-223*$^{Y/-}$ mice at d 1, 3, and 7 after injury (undetectable expression level of *miR-223* in *miR-223*$^{Y/-}$ mice) (see next page Fig 1 for reviewer only).

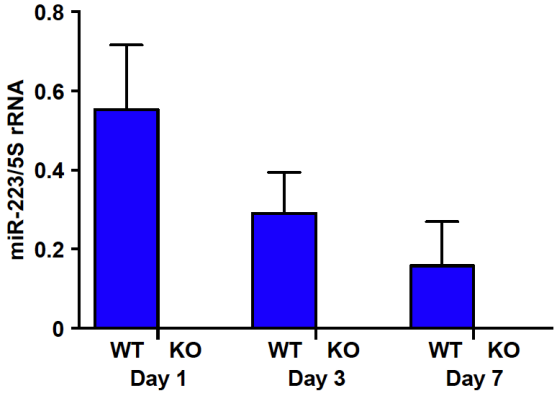

Figure 1 (reviewer only). Expression of *miR-223* in skin wound healing of WT and *miR-223*$^{Y/-}$ mice
Expression of *miR-223* in murine skin wound healing measured by qPCR relative to 5S rRNA (n = 4 - 6). Data information. All values represent the mean ± SD.

As suggested by you and another reviewer, we investigated the expression of α-smooth muscle actin (αSMA), a marker of myofibroblast wound contraction, at skin wound sites. We found that the expression of αSMA was markedly decreased at d 7 in aseptic wound sites of *miR-223*$^{Y/-}$ mice. We have modified the text accordingly (page 9, line 12 to 20) and Fig EV1D and EV1E.

Comment 7: *7.) Fig. 3B is not convincing - there is only a statistically significant difference at the 3h time point and at the 3d time point - this needs to be formulated more carefully. The different functionality of the neutrophils may be more important than this minor difference in number. I am also not convinced that there is impaired resolution, since no difference was seen at day 7.*
Response: We agree with your comments and have revised the paragraph regarding the onset and resolution of inflammatory responses in WT and *miR-223*$^{Y/-}$ mice according to your advice. In Fig 4A and 4B (previously Fig 3A and 3B), we wanted to show the neutrophil influx into the wound site over time using EGFP green fluorescent labeled neutrophils. Kim and colleagues (J Invest Dermatol, 2008), reported that neutrophil influx after skin wounding in *lys*-EGFP mice increased most rapidly over the initial 12 h and reached a maximum between d 1 and d 3. It then decreased precipitously at d 5 (Fig 2 and 3 in Kim et al., J Invest Dermatol, 2008). Our results are similar to theirs; at 12 h in WT mice, the neutrophil influx begins to increase and at d 3 the influx peaks. In contrast, the rate of influx in *miR-223*$^{Y/-}$ mice appeared somewhat slower (3 h) although it also peaked at d 3. By d 3, the influx rate of *miR-223*$^{Y/-}$ mice had surpassed that of WT mice, and the neutrophil influx became excessive. We also modified the representative results of *in vivo* fluorescent images of EGFP-expressing neutrophils in skin wound sites as reflected in the related graph (Fig 4A).

Comment 8: *8.) Fig. S3A: Please show representative stainings.*
Response: We have added representative images of IHC for F4/80 (Fig EV2A, previously Fig S3A).

Comment 9: *9.) The authors should show Il-6 mRNA levels in non-stimulated and activated neutrophils of wt and miR-223 mutant mice - this would further support the regulation of IL-6 by miR-223. The qPCR shown in Fig. 4B only shows that Il-6 expression is enhanced in total wounds of miR-223 mutant mice, which may be secondary to the enhanced numbers of neutrophils (and not a real regulation by the miRNA).*
Response: We found no significant difference in numbers of neutrophils at d 1 in the wound sites of WT and *miR-223*$^{Y/-}$ mice using *in vivo* imaging analysis (Fig 4A and 4B). Our ISH study demonstrated that *miR-223* was only expressed by neutrophils in wound sites at d 1 (Fig 3A and 3B). Taken together, we suspect the cause of increased *Il6* expression at 1 d in the wound sites of *miR-223*$^{Y/-}$ mice might be associated with the regulation of *miR-223* in neutrophils.

Comment 10: *10.) The upregulation of IL-6 is unlikely to explain the impaired healing in miR-223 mutant mice (IL-6 knockout mice have impaired wound healing; Lin et al., 2003; IL-6 promotes wound healing in glucocorticoid-treated mice; Gallucci et al., 2001). Therefore, the mechanism*

underlying the impaired healing in the miR-223 mutant mice under sterile conditions remains unclear. This should at least be discussed.
Response: As you suggest we have no more fully, discussed the function of IL-6 in skin wound healing and inflammatory responses in the text. We understand that IL-6 is required for skin wound healing based on earlier IL-6 KO study (Gallucci et al, FASEB J, 2000; Lin et al, J Leukoc Biol, 2003). However, it is also the case that excess IL-6 causes inflammatory diseases, leading to the use of IL-6 receptor antibody (Tocilizumab) as a therapeutic agent against Castleman disease and rheumatic diseases in the clinic (Yoshizaki et al, Hematol Oncol Clin North Am, 2018, PMID: 29157617) (Rubbert-Roth et al, Rheumatol Ther, 2018, PMID: 29502236). Therefore, it is important to control the amount of IL-6 at inflamed sites. We have modified the text accordingly (page 20, line 20 to page 21, line 5).

Comment 11: *11.) Fig. 5A and G: In addition to the macroscopic analysis, the authors should show H/E-stained sections from 7-day and 14-day wounds (and ideally use them to determine if there is an effect on reepithelialization and contraction). Given the rather high error bars, analysis of these histological parameters would clearly strengthen the data. At least one would like to get an idea about the histological features of the healing and healed wounds.*
Response: Thank you for this suggestion. We have performed histological analysis on *S. aureus*-infected, neutrophil-transplanted, and *miR-223* AS ODN-treated skin wound sites. Re-epithelialization in *S. aureus*-infected wound sites of *miR-223*$^{y/-}$ mice showed enhanced re-epithelialization at d 3 and 7 (Fig EV3C).

We found that total wound area and pathological post-infectious necrotic lesion at d 7 and area of scar sites at d 14 in *S. aureus*-infected, neutrophil-transplanted, and *miR-223* AS ODN-treated skin wound sites were significantly decreased, accompany by changing αSMA expression in granulation tissues (Fig 7H-7J) (Fig EV3) (Appendix Fig S4C-S4E). We see no αSMA expression cells in pathological postinfectious necrotic lesion. We have modified the text accordingly (page 13, line 15 to 20) (page 14, line 16 to 20) (page 16, line 10 to 18).

Comment 12: *12.) Fig. 5G,H: Is it possible to determine how long the neutrophils used for treatment remain in the wound tissue? The cells could be labeled for this purpose.*
Response: We have observed how long transplanted neutrophils remain in the *S. aureus*-infected skin wound sites using WT EGFP-expressing neutrophils purified from the bone marrow of lys-EGFP mice using magnetic beads (see the Materials and Methods). Large numbers of EGFP-expressing neutrophils were retained in *S. aureus*-infected skin wound sites at d 1 (Appendix Fig S4A). However, there were very low numbers of EGFP-expressing neutrophils in *S. aureus*-infected skin wound sites remaining at d 3, and none at d 7. These results indicate that transplanted neutrophils can remain in skin wound sites up to 3 d after transplantation.

Comment 13: *13.) Fig. 6: The authors should verify that miR-223 is indeed downregulated by the ODNs at the wound site and check if IL-6 is upregulated.*
Response: We verified the effect of *miR-223* AS ODN using the *S. aureus*-infected skin wound healing model. The expression of *miR-223* at *miR-223* AS ODN-treated skin wound sites was significantly reduced compared with controls at 6 h and 1 d after injury (Fig 7D).

We also investigated the expression of *Il6* using qPCR and found it to be significantly increased at d 1 in *miR-223* AS ODN-treated *S. aureus*-infected skin wound sites compared with controls (Fig 7E). We have modified the text accordingly (page 15, line 18 to page 16 line 7) and Fig 7C to 7E.

Comment 14: *14.) The paragraph describing the results shown in Fig.7 includes various errors in spelling and grammar and is therefore difficult to read. Most importantly, the results do not allow the conclusion that miR-223 is regulated by C/EBPa in response to S. aureus. To text this possibility, the authors would have to overexpress C/EBPa and determine if the PGN-mediated decline in miR-223 is rescued. Therefore, the scheme shown in Fig. 6G is not fully supported by the data.*
Response: We have now obtained a full-length human C/EBPα overexpression vector (Clone ID: OHu20497C, GenScript, Piscataway, NJ) and transfected it into differentiated HL-60 (dHL-60) cells but, unfortunately, we could not establish C/EBPα overexpression dHL-60 cells (see below Fig 2 for reviewer only).

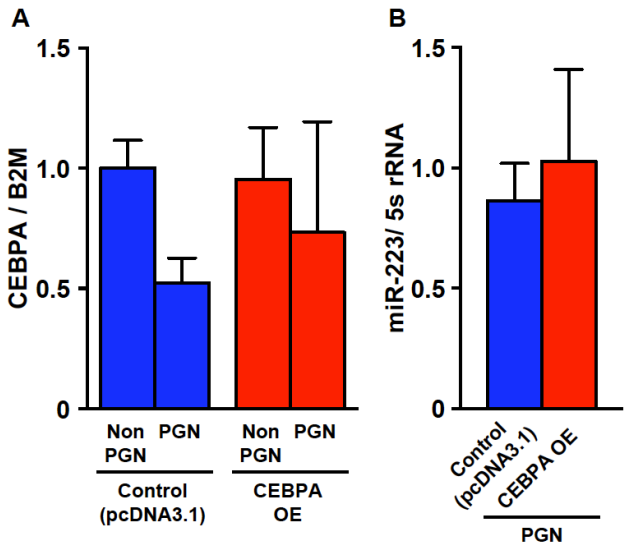

Figure 2 (reviewer only). Expression of *CEBPA* and miR-223 measured by qPCR
Expression of *CEBPA* (A) and *miR-223* (B) in control vector (pcDNA3.1)-transfected and full length human C/EBPα over expression (OE) vector-transfected dHL-60 after stimulation with PGN for 6 h measured by qPCR relative to B2M (n = 6). All values represent the mean ± SD.

Generally, it is difficult to transfect mature immune cells with plasmids (pcDNA3.1; 5428 bp, C/EBPα overexpression vector; 6523 bp) but not ODN (18-mer) because of the low efficiency. And we were also concerned that overexpression of C/EBPα in dHL60 cells might have detrimental effects on mature neutrophil nature because C/EBPα regulates myeloid differentiation. Indeed, c/ebpa$^{-/-}$ mice exhibited a lack of mature neutrophils (Zhang DE et al, Proc Natl Acad Sci U S A. 1997, PMID: 9012825). Overexpression of C/EBPα in non-differentiated HL60 cells triggered them to become mature granulocytes (Radomska HS et al, Mol Cell Biol, 1998, PMID: 9632814). The expression level of C/EBPα was constant in the bone marrow–derived cells and decreased in mature neutrophils (Bjerregaard MD et al, Blood, 2003, PMID: 12560239).

As an alternative strategy and to verify Fig 8H (previously Fig 6G), we performed a ChIP assay to investigate C/EBPα binding to the *miR-223* promoter site. The binding activity of C/EBPα was significantly decreased at 6 h after PGN stimulation compared with non-stimulated dHL-60 cells (Fig 8F and 8G) (Fig EV5B and EV5C). Unfortunately, we could not verify the full-length human C/EBPα overexpression vector; however, we conclude that the expression of *miR-223* in neutrophils might be controlled by C/EBPα after PGN stimulation. We have modified the text accordingly (page 18, line 7 to 11) and Fig 8F and 8G, Fig EV5B and EV5C.

Referee #2 (Remarks for Author):

This is an interesting manuscript and the data are generally convincing. However, additional experiments and some rewriting are required for publication in EMM.

2nd Editorial Decision 18 July 2018

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Please include the additional control requested by referee 1. Please also address referees' comments in writing.

Please submit your revised manuscript within two weeks.

I look forward to reading a new revised version of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

The authors have greatly improved the paper, in particular by including new results about the granulation tissue formation and epithelialization processes. However these results should be summarized and discussed in the Discussion.
Likewise the results related to PGN and IL-6 should be better discussed. Again the relevance of using both PGN and miR-223 AS ODN is lacking.

The authors have used a rabbit polyclonal α-smooth muscle actin antibody from ABCAM. In the datasheet of the provider this antibody recognizes at least one additional unspecific band at 75 kDa by western blotting. The results should be confirmed by using the worldwide recognized mouse monoclonal α-smooth muscle actin antibody (clone 1A4) that should be used as a biotinylated antibody (direct immunostaining to overcome the species issue).

The fact that α-smooth muscle actin is a marker of contractile myofibroblasts has not been first demonstrated by this group of research. Therefore appropriate references should be quoted (e.g Gabbiani's publications).

Referee #2 (Remarks for Author):

The authors have performed new experiments to address my comments and they have significantly revised the manuscript. These changes have further improved the quality of the manuscript. This is a very important study and the work is of high technical quality.

2nd Revision - authors' response 28 July 2018

Reviewer comments:
Referee #1 (Remarks for Author):
Comment 1: *The authors have greatly improved the paper, in particular by including new results about the granulation tissue formation and epithelialization processes. However these results should be summarized and discussed in the Discussion.*
Response: Thank you for your interesting comments. With regard to the alteration of re-epithelialization and granulation tissue formation in each model, we think that this is a consequence of increased acute inflammatory responses (secondary effect), because *miR-223* could be not expressed in wound-infiltrated fibroblasts and keratinocytes. We have modified the text accordingly (p21, lines 19 to p22, line 4)

Comment 2: *Likewise the results related to PGN and IL-6 should be better discussed.*
Response: It was reported that murine neutrophils produced IL-6 after PGN stimulation (Strassheim D et al, J Immunol, 2005, PMID: 15944314). We have modified the text accordingly (p22, lines 7 to 8).

Comment 3: *Again the relevance of using both PGN and miR-223 AS ODN is lacking.*
Response: Even if *miR-223* expression is strongly suppressed by *miR-223* AS ODN, when PGN stimulation (*S. aureus* recognition) is received, positive feedback occurs; thus, through increased IL-6 production, infection control becomes more effective. We have modified the text accordingly (p17, lines 18 to 21).

Comment 4: *The authors have used a rabbit polyclonal α-smooth muscle actin antibody from ABCAM. In the datasheet of the provider this antibody recognizes at least one additional unspecific band at 75 kDa by western blotting. The results should be confirmed by using the worldwide recognized mouse monoclonal α-smooth muscle actin antibody (clone 1A4) that should be used as a biotinylated antibody (direct immunostaining to overcome the species issue).*
The fact that α-smooth muscle actin is a marker of contractile myofibroblasts has not been first demonstrated by this group of research. Therefore appropriate references should be quoted (e.g Gabbiani's publications).

Response: We observed an additional nonspecific band at 75 kDa in 3T3 cell lysates by western blotting (ab5694, Abcam). In contrast, no nonspecific band was observed for murine heart tissue homogenate, suggesting that the nonspecific band in the murine sample was only detected in the cancer cell line lysate; therefore, this antibody could be useful to detect αSMA in normal murine tissues. Additionally, this rabbit-derived αSMA antibody is utilized worldwide in immunohistochemistry for murine samples to an extent similar to mouse monoclonal αSMA antibody (clone 1A4). Recently, Plikus and colleagues showed that murine skin wound-infiltrated myofibroblasts were identified using the same antibody (Plikus et al, Science, 2017, PMID: 28059714, see Fig. 2A) (we confirmed antibody information via supplemental information in this paper and by direct confirmation from Drs. Plikus and Guerrero-Juarez). Moreover, we performed western blotting and confirmed a highly specific band that corresponded with αSMA protein (see below Fig. 1 for reviewer only). Therefore, we are confident that this anti-αSMA antibody (ab5694, Abcam) can be used for the immunohistochemistry of murine skin wound slices. We understand mouse monoclonal αSMA antibody (clone 1A4) is utilized for pathologic diagnosis of human tissue in soft tissue tumors such as leiomyoma and so on. Similarly, as our study is of murine wound tissues, we preferred to select rabbit-derived antibody rather than mouse-derived antibody.

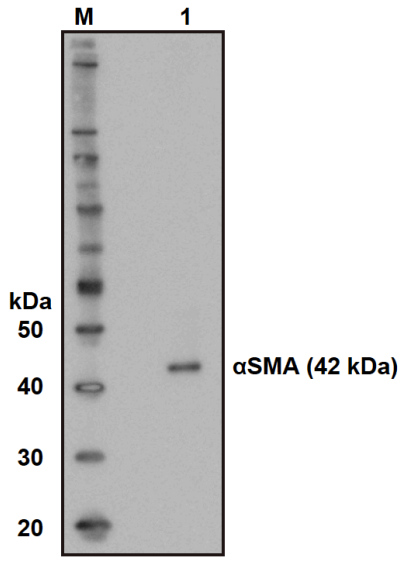

Figure 1 (reviewer only). Expression of αSMA protein in day 7 murine skin wounds of WT mice.
We performed western blotting using polyclonal rabbit αSMA antibody (1:3000) (ab5694, Abcam) with overnight incubation at 4°C and a blocking time of 2 hours at room temperature (PVDF Blocking Reagent, TOYOBO). Then the secondary antibody (anti-Rabbit IgG HRP-linked whole antibody, GE Healthcare) (1:10,000) was incubated for 1 hour at room temperature. Protein bands were visualized by chemiluminescence (ImmunoStar LD, Fujifilm Wako Pure Chemical Corp.), and LAS3000 mini (exposure time: 1 second) (Fujifilm). The predicted band size of αSMA protein is 42 kDa. M, marker; lane 1, day 7 murine skin wound homogenate (10 μg).

With regard to the reference for αSMA, we have replaced the reference (p9, line 16).

Referee #2 (Remarks for Author):
The authors have performed new experiments to address my comments and they have significantly revised the manuscript. These changes have further improved the quality of the manuscript. This is a very important study and the work is of high technical quality.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ryoichi Mori
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2018-09024

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Preliminary experiments were performed when possible to determine the requirements for sample size, taking into account the resources available.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Preliminary experiments were performed when possible to determine the requirements for sample size, taking into account the resources available and ethical, reductionist animal use.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Mice were housed under SPF conditions to avoid any stress and bias according to animal welfare guidelines. Mice were maintained for at least one week in the new environment (biosafety level 2) to let them adapt to any stress and bias before they were used and analyzed as an infection model.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomized prior to treatment to allow random sampling.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The investigators were not blinded during the determination of the novel object preference nor for analysis of the data. However, we used mice that were selected by a third party to minimize subjective bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The investigators were not blinded.
5. For every figure, are statistical tests justified as appropriate?	Yes. The appropriate statistical tests have been described for every figure in the manuscript.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	GraphPad Prism Software 6.0 was used to determine statistical significance by unpaired t-tests and one and two way ANOVA .
Is there an estimate of variation within each group of data?	Yes. Variation estimates have been included for all graphs.
Is the variance similar between the groups that are being statistically compared?	Yes. We determined whether the variance was similar between the different groups.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes. We described the details of antibodies (i.e. catalog number) in the Appendix. All antibodies in this study are commercially available.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The HL-60 cell line (RBRC-RCB0041) was provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan, with no mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

Ω

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All experiments were conducted according to the provisions of the Ethics Review Committee for Animal Experimentation at Nagasaki University. Mice were kept in a barrier facility (temperature; 22-25ºC, 12 h light/dark cycle) under specific pathogen free condition. Mice were fed ad libitum with Charles River-LPF diet (360 kcal/100g; 13% fat calories, 26% protein calories, and 61% carbohydrate calorie, [Oriental Yeast, Tokyo, Japan]). miR-223Y/- mice were generated as described previously (Johnnidis et al, 2008). The miR-223 locus is located on the X chromosome and is transcribed independently of any known genes, so that miR-223Y/- hemizygous male mice are completely deficient in mature miR-223 expression. WT male mice (6–12 weeks) and miR-223Y/- male mice (B6.Cg-Ptprca Mir223tm1Fcam/J, 6–12 weeks [The Jackson Laboratory, Bar Harbor, ME, USA]) were anaesthetized and 2 or 4 full-thickness excisional wounds (4-mm biopsy punch; Kai Industries, Gifu, Japan) were aseptically made to the shaved dorsal skin. Generation of PU.1-/- mice was described previously (McKercher et al, 1996). One day-old pups received anesthetic and full-thickness 1-cm incisional wounds were performed on the dorsal skin; then the wounds were harvested. Faust et al., generated lys-EGFP mice as described previously (Faust et al, 2000). To generate male lys-EGFP expressing miR-223Y/- mice, male miR-223Y/- mice were crossed with female lys-EGFP mice to produce lys-EGFP heterozygous-expressing male miR-223Y/- and female miR-223+/- mice. A second cross generated male lys-EGFP homozygous-expressing miR-223Y/- mice. Mice genotypes were defined by PCR as previously described (Faust et al, 2000; Johnnidis et al, 2008). S. aureus type strain (NBRC 100910) was obtained from the National Institute of Technology and Evaluation (Tokyo, Japan). In the S. aureus-infected group, mice were locally inoculated with S. aureus (1×108 CFU per 10 μL in saline) at pre-skin wound sites followed by making the wound. For wound area analysis, digital images of wound areas were measured by Photoshop CC (Adobe Systems, San Jose, CA, USA) and the mean wound area was calculated from two or four wounds from a single mouse (Mori et al, 2008; Mori et al, 2014; Tanaka et al, 2017).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were conducted according to the provisions of the Ethics Review Committee for Animal Experimentation at Nagasaki University.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All animal experiments were designed and conducted according to Japanese and International guidelines following the 3R rules.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Human skin samples were harvested from Japanese patients at the time of surgery, and diagnosis was confirmed by routine pathological examination (see Appendix Table S4). All experiments were conducted with the approval of the ethics committee of Nagasaki University Graduate School of Biomedical Sciences, and in accordance with the Declaration of Helsinki principles.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All participating patients were informed of the study and had to provide signed written informed consent before enrolment.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Yes.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	miRNA-Seq data reported are available in the DNA Data Bank of Japan (DDBJ, http://trace.ddbj.nig.ac.jp/DRASearch/) under accession no. DRA004094. RNA-Seq data reported are available in the DDBJ under accession no. DRA004092.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA