Glycoprotein Nonmelanoma Clone B Regulates the Crosstalk between Macrophages and Mesenchymal Stem Cells toward Wound Repair

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The process of wound repair requires the coordinated participation of multiple types of cells, which are sequentially recruited during the healing process. In response to tissue injury, both macrophages and mesenchymal stem cells (MSCs) are recruited to the site of injury, where they participate in the repair process. Despite considerable understanding of the role of each cell type in the process of wound repair, the nature of the dynamic interplay between these two cell types and how this interaction influences the process of wound repair are not well understood. Here, using an in vivo model of cutaneous wound healing in mice, we provide evidence that GPNMB is functionally important in promoting the recruitment of MSCs to the site of skin injury, which in turn modulates inflammatory responses by directing the M2 polarization of macrophages in acute wound healing. Furthermore, we show that GPNMB activity is impaired in a diabetic wound environment, which is associated with impaired MSC recruitment that is reversed by the topical administration of recombinant GPNMB protein to the wounds of diabetic mice. Our study provides important insight into the crosstalk between macrophages and endogenous MSCs toward wound repair.

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INTRODUCTION

Wound repair is a complex biological process coordinated by dynamic interplay between many different lineages of tissue resident and wound infiltrating cells. Among them, macrophages have been the subject of intensive investigation because of their phenotypic plasticity that exhibits either proinflammatory or tissue reparative activity depending on the environmental stimuli (Brancato and Albina, 2011; Koh and DiPietro, 2011; Snyder et al., 2016). It has been well reported that the tightly regulated switch in macrophage trafficking from an M1-like proinflammatory to an M2-like anti-inflammatory and pro-healing phenotype can ensure the resolution of the inflammatory response and tissue repair (Bystrom et al., 2008; Porcheray et al., 2005; Sica and Mantovani, 2012). As such, the persistence of an unrestrained M1 macrophage population with an incomplete switch to an M2 phenotype was shown to be a common feature of nonhealing wounds such as venous leg ulcers or diabetic wounds (Cucak et al., 2014; Fujisaka et al., 2009; Lumeng et al., 2007).

In addition to the role of macrophages in the process of wound repair, it has been shown that endogenous pools of mesenchymal stem cells (MSCs) are mobilized from their storage niche to the site of injury, where they exert tissue reparative effects through paracrine signaling that leads to immune modulation and secretion of bioactive trophic factors (Sasaki et al., 2008; Seppanen et al., 2013). Despite considerable understanding of the functional role of macrophages and MSCs in the repair of injured tissue, the nature of the dynamic interplay between these two cell types and how this interaction influences the process of tissue repair are not well understood, particularly in the context of the skin wound healing.

We recently reported that the migration and survival of MSCs are substantially influenced by differentially polarized macrophages via a GPNMB-dependent mechanism from in vitro study (Yu et al., 2016). GPNMB is a transmembrane glycoprotein and has shown to be highly expressed in macrophages (Gabriel et al., 2014; Katayama et al., 2015; Li et al., 2010). The extracellular fragments of GPNMB are cleaved on the plasma membrane by ectodomain shedding and secreted into the extracellular milieu where they act as signaling molecules (Hoashi et al., 2010). Increased expression of GPNMB is correlated with attenuation of inflammation and tissue repair in experimental models of tissue injury, including bone injury (Hu et al., 2013), liver injury (Kumagai et al., 2015), kidney injury (Li et al., 2010), amyotrophic lateral sclerosis (Tanaka et al., 2012), cerebral ischemia-reperfusion injury (Nakano et al., 2014), and nonalcoholic fatty liver disease in obesity (Katayama et al., 2015), whereas the loss of GPNMB was shown to be associated with a hyperinflammatory phenotype (Li et al., 2010; Ripoll et al., 2007).

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Abbreviations: Arg-1, arginase-1; BMDM, bone marrow-derived macrophage; IGF, insulin-like growth factor; MACS, magnetically activated cell sorting; MSCs, mesenchymal stem cells; rGPNMB, recombinant GPNMB; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor

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In this study, using an in vivo model of cutaneous wound healing in mice, we examined whether a macrophage-derived GPNMB protein is functionally important in promoting the recruitment of endogenous pools of MSCs to wounds and accelerating wound repair.

RESULTS

Skin wounding elicits increased expression of GPNMB in wound macrophages from wild-type mice

To determine the role of GPNMB in wound healing, we first examined the expression of GPNMB from wounds of C57BL/6 mice. The levels of GPNMB protein in unwounded skin tissue were weakly detected (Figure 1a). After skin wounding, the secretion of the GPNMB protein was significantly increased, reaching the maximal level at day 3 after wounding and then gradually decreasing during the course of wound healing (Figure 1a). We next engaged in a study to identify the source of cells that express GPNMB after skin wounding using immunofluorescence analysis of wounded skin at day 3 after wounding. Immunofluorescence images show that GPNMB-positive cells were preferentially co-localized with F4/80-positive macrophages, whereas the expression levels of GPNMB were very low in other DAPI-stained cells (Figure 1b). To confirm whether macrophages are the major source of cells for producing GPNMB in the wound, the expression levels of GPNMB mRNA were quantified in macrophages isolated from wounds (Figure 1c). The kinetics of GPNMB mRNA expression in wound macrophages exhibits a close correlation with protein levels of GPNMB in the wound. To further confirm whether this phenomenon is also true for human cells, we compared the relative expression of GPNMB mRNA from several types of human cells involved in skin wound healing. The expression of GPNMB mRNA was significantly higher in THP-1 monocyte/macrophages than in other types of cells, including fibroblasts, bone marrow-derived human MSCs, human umbilical vein endothelial cells, and human pericytes (see Supplementary Figure S1 online). This is consistent with prior findings that show macrophages as a major source of cells for secreting GPNMB (Gabriel et al., 2014; Katayama et al., 2015; Li et al., 2010). Taken together, our results support that the expression of GPNMB is induced in response to skin wounding and that macrophages are the major source of cells expressing GPNMB in the wound.

Wound-infiltrated $CD11b^{-}CD45^{-}CD31^{-}Sca\text{-}1^{+}CD29^{+}CD146^{+}$ cells exhibit MSC characteristics of self-renewal and differentiation

To identify endogenous MSCs that infiltrated into the wound, we used three negative markers (CD11b, CD45, and CD31) and three positive markers (Sca-1, CD29, and CD146) (Tang et al., 2009). MSCs are negative for hematopoietic lineage markers of CD45 and CD11b, whereas both Sca-1 and CD29 are considered typical MSC markers. In addition, we used CD31 as a negative marker to exclude endothelial cells and CD146 as a positive marker to differentiate MSCs from fibroblasts in this study (Halfon et al., 2011). To examine if $CD11b^{-}CD45^{-}CD31^{-}Sca\text{-}1^{+}CD29^{+}CD146^{+}$ cells can indeed exhibit characteristics of MSCs, the cells were sorted from wounds of C57BL/6 mice at day 5 after wounding by magnetically activated cell sorting (MACS). After 24 hours of culture, the MACS-sorted cells exhibited the spindle-shape morphology of MSCs (Figure 1d). The colony forming unit-fibroblast assay showed a proliferative capacity of the wound-sorted $CD11b^{-}CD45^{-}CD31^{-}Sca\text{-}1^{+}CD29^{+}CD146^{+}$ cells (Figure 1e). The MSC characteristics of the sorted cells were further confirmed by their tri-mesenchymal lineage differentiation capacity toward osteogenesis, adipogenesis, and chondrogenesis (Figure 1f). These results support that wound infiltrating $CD11b^{-}CD45^{-}CD31^{-}Sca\text{-}1^{+}CD29^{+}CD146^{+}$ cells are indeed MSCs that satisfy criteria of self-renewal and tri-mesenchymal lineage differentiation. After identifying MSCs in the wound, we next quantified the kinetics of MSC recruitment over the course of wound healing using a flow cytometric identification and counting $CD11b^{-}CD45^{-}CD31^{-}Sca\text{-}1^{+}CD29^{+}CD146^{+}$ cells (see Supplementary Figure S2 online). Our data show that skin wounding resulted in a transient increase in the number of MSCs in the wound, which peaked at day 5 after wounding (Figure 1g). Because macrophages appear in the wound within day 2 and reach a peak as early as day 3 after wounding (Mahdavian Delavary et al., 2011, Witte and Barbul, 1997), our data indicate that the trafficking of macrophages may precede the appearance of MSCs in the wound.

The deficiency of GPNMB in macrophages impairs the recruitment of MSCs and prolongs inflammation after skin wounding

We next explored whether the up-regulated expression of GPNMB in wounded skin is functionally important in the recruitment of MSCs to the wound. This was examined by quantifying the extent of MSC recruitment from *Gpnmb*-mutant (*D2J*) and *Gpnmb*-control (*D2J/Gpnmb*$^{+}$) mice. The expression of GPNMB mRNA was negligible in wounds of *D2J* mice (Figure 2a). After skin wounding, *D2J* mice exhibited an attenuated number of MSCs in the wound, approximately 40%, compared with *D2J/Gpnmb*$^{+}$ mice at day 5 after wounding (Figure 2b). The flow cytometric analysis of cells from wounds showed that *D2J* mice exhibited an increased trafficking of M1-like macrophages ($F4/80^{+}CD86^{+}$ cells) and attenuated trafficking of M2-like macrophages ($F4/80^{+}CD206^{+}$ cells) in the wound compared with control mice (Figure 2c and 2d). This was further confirmed by an increase in the expression of the M1 marker inducible nitric oxide synthase (*iNOS*) and decrease of the M2 marker arginase 1 (*Arg-1*) (see Supplementary Figure S3a and b online). In addition, wounds of *D2J* mice were associated with increased accumulation of proinflammatory cytokines including IL-1β and tumor necrosis factor-α (TNF-α) (see Supplementary Figure S3c and d), as well as reduced levels of pro-healing factors insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) (see Supplementary Figure S3e and f). These results indicate that the loss of GPNMB activity in wounds is associated with the reduced recruitment of MSCs to the wound and shift in the wound environment toward an increased trafficking of proinflammatory M1 macrophages.

The administration of GPNMB-expressing macrophages to the wounds of Gpnmb-mutant mice improves the recruitment of MSCs to the wound and accelerates wound closure

Because macrophages were shown to be a major source of cells expressing GPNMB in response to skin wounding

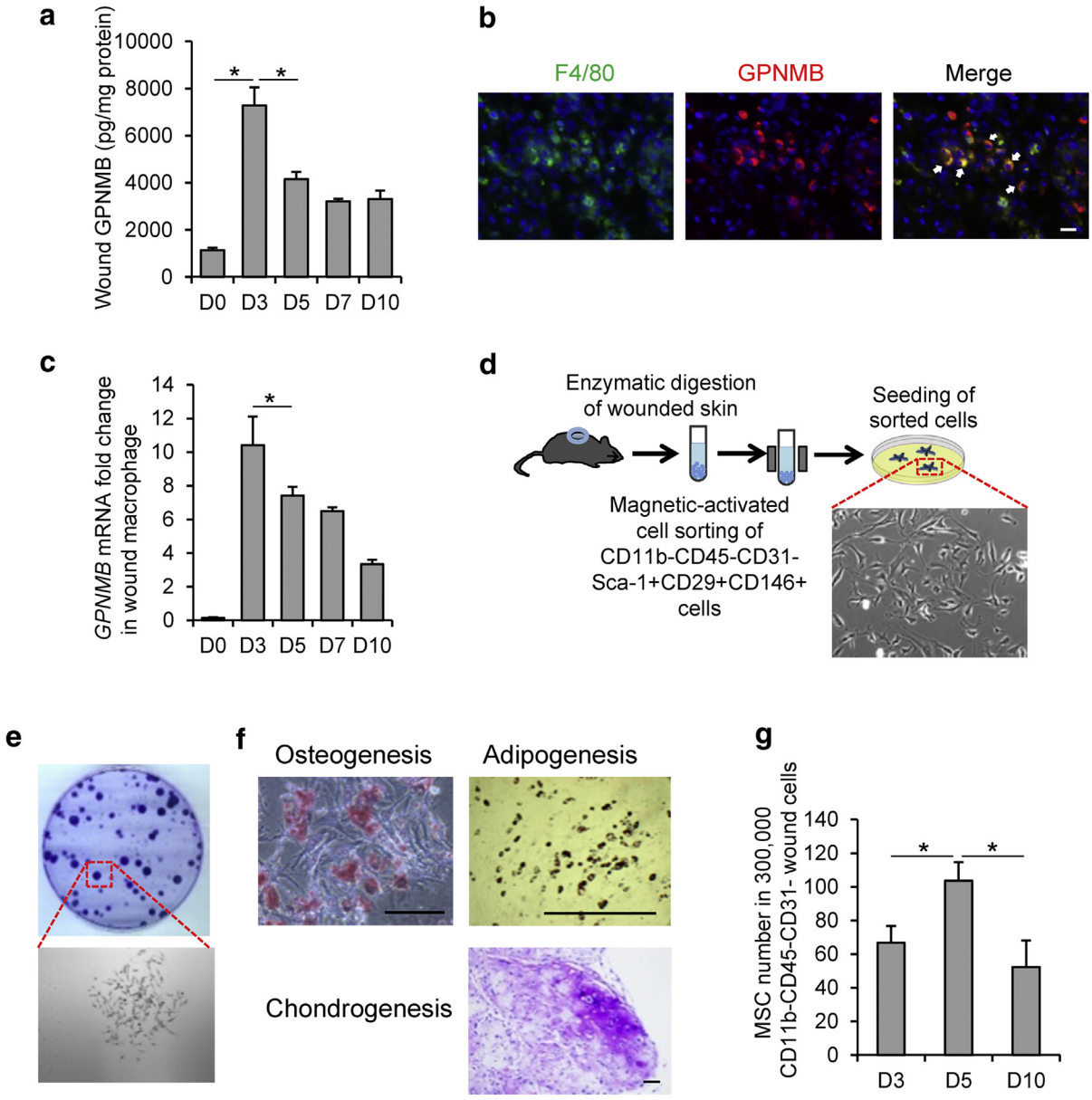

Figure 1. The expression of GPNMB and recruitment of MSCs in wounds after skin wounding from C57BL/6 mice. (**a**) ELISA analysis for quantification of secreted GPNMB protein in wounds. The selected day after wounding is abbreviated as D. (**b**) Immunofluorescent images showing co-localization of GPNMB with macrophages. F4/80 (macrophages) are in green, GPNMB in red, and DAPI (nuclei) in blue. Scale bar = 10 μm. White arrows indicate co-localization of GPNMB with F4/80$^+$ macrophage. (**c**) Quantitative PCR analysis for quantification of GPNMB mRNA in wound macrophages. (**d**) A schematic diagram for sorting of CD11b$^-$CD45$^-$CD31$^-$Sca1$^+$CD29$^+$CD146$^+$ cells from mouse wounds using magnetic-activated cell sorting. The bright-field microscopic image shows the morphology of sorted CD11b$^-$CD45$^-$CD31$^-$Sca1$^+$CD29$^+$CD146$^+$ cells after 24 hours of culture. (**e**) Colony forming unit-fibroblast assay from sorted CD11b$^-$CD45$^-$CD31$^-$Sca1$^+$CD29$^+$CD146$^+$ cells. (**f**) Photomicrographs of mesenchymal tri-lineage differentiation of wound-isolated CD11b$^-$CD45$^-$CD31$^-$Sca1$^+$CD29$^+$CD146$^+$ cells. Osteogenesis: Cells were cultured in osteogenic media for 3 weeks, and the mineralized nodules were evaluated by Alizarin Red staining. Adipogenesis: Cells were cultured in adipogenic media for 2 weeks, and the accumulation of numerous lipid vesicles within the cells was identified by Oil-red-O staining. Chondrogenesis: Cells were cultured in chondrogenic media for 3 weeks, and the micromass pellet was stained with toluidine blue. Scale bar = 100 μm. (**g**) Flow cytometric counting of CD11b$^-$CD45$^-$CD31$^-$Sca1$^+$CD29$^+$CD146$^+$ cells from wounded skin. The number of cells are indicated out of 300,000 CD11b$^-$CD45$^-$CD31$^-$ wound cells at indicated time points after wounding. Data are presented as mean ± standard error of the mean (n = 6). *$P < 0.05$. MSC, mesenchymal stem cell.

(Figure 1), we reasoned that increasing the number GPNMP-expressing macrophages in the wound of *D2J* mice would improve the recruitment of MSCs to the wound. This was assessed by the exogenous administration of GPNMB-positive macrophages isolated from the bone marrow of *D2J/Gpnmb*$^+$ mice to the wound of *D2J* mice and quantifying the extent of MSC recruitment and wound closure. As expected, the administration of GPNMB-positive macrophages resulted in a significantly increased expression of GPNMB in the wounds of *D2J* mice by 4-fold at day 5 after wounding, compared with the administration of bone marrow-derived macrophages (BMDMs) from *D2J* mice (Figure 2e). This

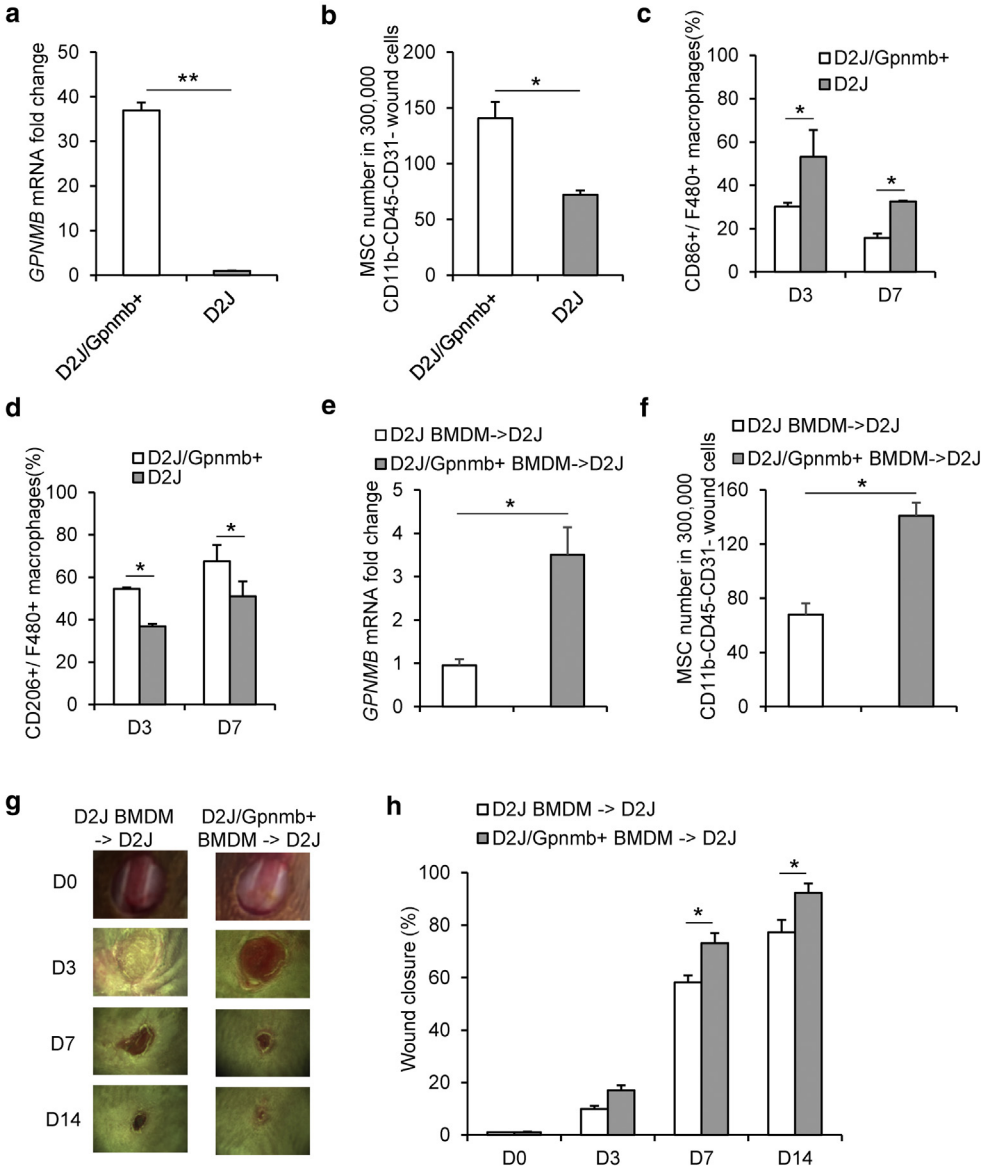

Figure 2. The effect of GPNMB deficiency on the recruitment of MSCs, wound closure, and macrophage polarization in response to skin wounding. (**a**–**d**) Excisional wounds in control *D2J/Gpnmb*$^+$ mice and *Gpnmb*$^-$ mutant (*D2J*) mice were harvested at selected day after wounding. n = 6. (**a**) Expression of *Gpnmb* mRNA from wounds of *D2J/Gpnmb*$^+$ and *D2J* mice. (**b**) Flow cytometric counting of MSCs from wounds of *D2J/Gpnmb*$^+$ and *D2J* mice at 5 days after wounding. (**c, d**) Flow cytometric counting of (**c**) F4/80$^+$CD86$^+$ macrophages and (**d**) F4/80$^+$CD206$^+$ macrophages from wounds of *D2J/Gpnmb*$^+$ and *D2J* mice. (**e**–**h**) The bone marrow-derived macrophages (BMDMs; 1 × 10^6 macrophages) from *D2J/Gpnmb*$^+$ or *D2J* mice were topically administered to the wounds of *D2J* mice, and excisional wounds were harvested at selected day after wounding. n = 4. (**e**) Quantitative PCR analysis for *Gpnmb* mRNA and (**f**) flow cytometric counting of MSCs from wounds of *D2J* mice administered with BMDMs from *D2J/Gpnmb*$^+$ (D2J/Gpnmb$^+$ BMDM → D2J group) or *D2J* mice (D2J BMDM → D2J group), at day 5 after wounding. (**g**) Representative images of wound area and (**h**) quantification of the wound closure in *D2J* mice administered with BMDMs from *D2J/Gpnmb*$^+$ or *D2J* mice. Data are presented as mean ± standard error of the mean. *$P < 0.05$. D, day after wounding. MSC, mesenchymal stem cell.

was associated with the increased trafficking of MSCs to the wound (Figure 2f, and see Supplementary Figure S4 online) and accelerated wound closure in *D2J* mice (Figure 2g and h). Our results suggest that macrophage-derived GPNMBs are functionally important in promoting the recruitment of MSCs to the wound.

Increasing GPNMB activity in the wound promotes the recruitment of MSCs to the wound and improves wound healing in C57BL/6 wild-type and Gpnmb-mutant mice

We next examined whether increasing the availability of GPNMB in the wound promotes the recruitment of MSCs to the wound. This was assessed by topically applying recombinant GPNMB proteins (rGPNMB) to the wound, which mimics the soluble form of the extracellular fragment of the GPNMB protein. We previously showed the capacity of rGPNMB in promoting the migration of MSCs from in vitro study (Yu et al., 2016). The topical treatment of rGPNMB to the wounds of C57BL/6 wild-type mice significantly accelerated wound closure (Figure 3a and 3b) and increased the number of MSCs in the wound by 3-fold at day 5 after wounding (Figure 3c). The improved MSC response with rGPNMB treatment in C57BL/6 mice was accompanied by a decrease in F4/80$^+$CD86$^+$ (M1) macrophages and an increase in F4/80$^+$CD206$^+$ (M2) macrophages in the wound (Figure 3d and 3e). This was further confirmed by gene expression analysis, which displayed a decreased expression of M1 markers (*iNOS, Il-1β, Tnf-α*) (see Supplementary Figure S5a online) and an increased expression of M2 markers (*Arg-1, Igf-1, Vegf*) (see Supplementary Figure S5b) in macrophages isolated from wounds of C57BL/6 mice treated with rGPNMB, compared with saline control group. The increased recruitment of MSCs and M2 macrophages to the wound was associated with concomitant reduction in proinflammatory cytokines IL-1β and TNF-α (Figure 3f and 3g) and increase in prohealing factors IGF-1 and VEGF (Figure 3h and 3i). Consistent with the findings from C57BL/6 mice, the topical treatment of rGPNMB to the wounds of *D2J*

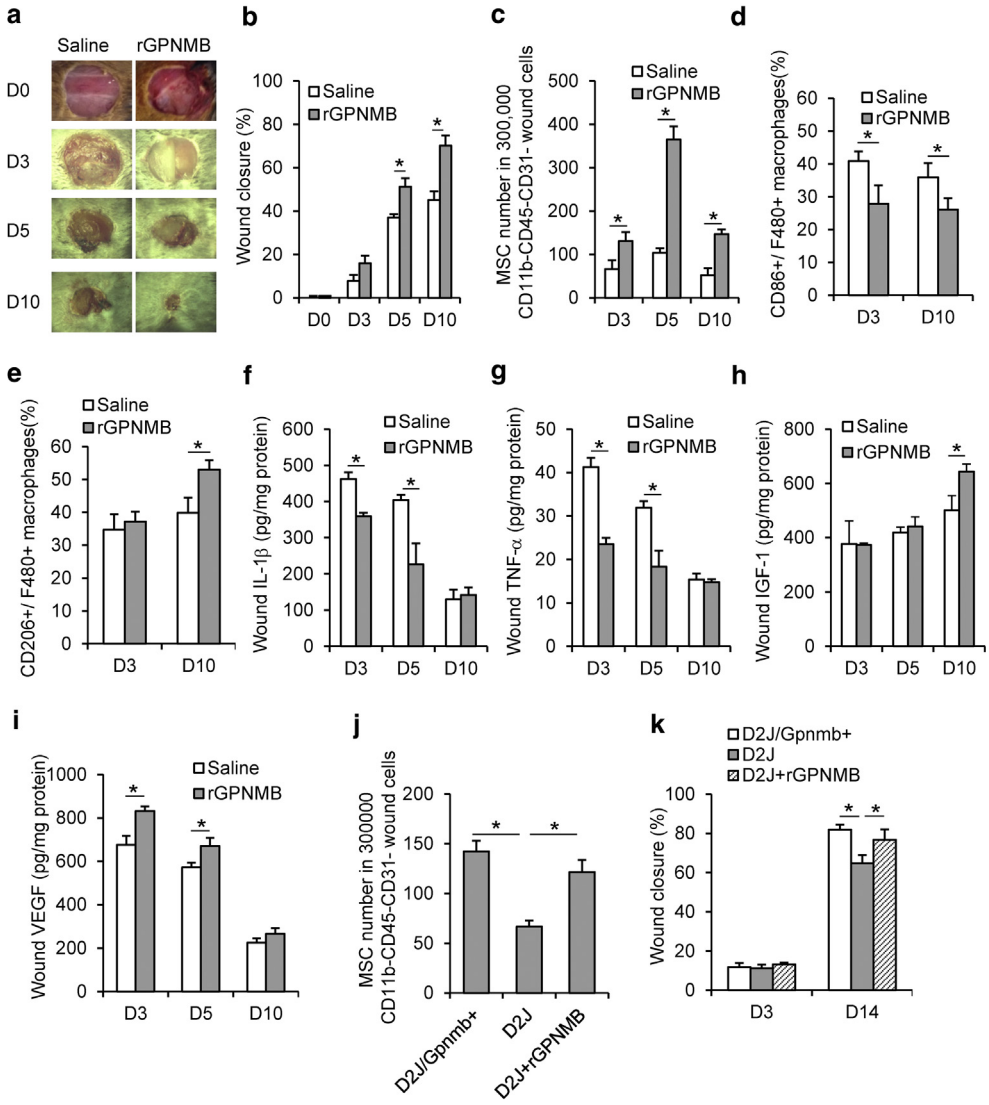

Figure 3. The effect of topical treatment of rGPNMB on the MSC recruitment and wound closure in C57BL/6 and D2J mice. (**a**–**i**) Wounds of C57BL/6 mice were topically treated with saline or rGPNMB (2 μg/wound) immediately after skin wounding at day 0. (**a**) Representative photographic images and (**b**) quantification of the wound closure at selected day after wounding. (**c**) Flow cytometric counting of MSCs from wounds. (**d, e**) Flow cytometric counting of (**d**) M1 ($F4/80^+CD86^+$) and (**e**) M2 ($F4/80^+CD206^+$) macrophages from wounds. (**f, g**) The levels of proinflammatory cytokines (**f**) IL-1β and (**g**) TNF-α in wounds by ELISA. (**h, i**) The levels of pro-healing factors (**h**) IGF-1 and (**i**) VEGF in wounds by ELISA. (**j, k**) Wounds of *D2J/Gpnmb*$^+$ and *D2J* mice were topically treated with saline or rGPNMB (2 μg/wound) immediately after skin wounding at day 0. (**j**) Flow cytometric counting of MSCs from wounds (day 5 after wounding) of *D2J/Gpnmb*$^+$ mice, *D2J* mice, and *D2J* mice topically treated with rGPNMB. (**k**) Quantification of the wound closure at days 3 and 14 after wounding in *D2J/Gpnmb*$^+$ mice, *D2J* mice, and *D2J* mice topically treated with rGPNMB (2 μg/wound). Data are presented as mean ± standard error of the mean (n = 6). $^*P < 0.05$. D, day after wounding. MSC, mesenchymal stem cell; rGPNMB, recombinant GPNMB; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

mice restored MSC recruitment and improved wound healing to the level of *D2J/Gpnmb*$^+$ mice (Figure 3j and k). Taken together, these results indicate that increasing GPNMB activity in the wound promotes prohealing responses, which appears to be mediated by enhanced recruitment of MSCs and trafficking of M2 macrophages to the wound.

Wound-infiltrated MSCs are capable of promoting M2 polarization of macrophages

By observing the increased recruitment of MSCs and the shift of macrophage phenotype toward an M2-like profile in the wound in response to the topical treatment of rGPNMB, we next examined whether wound-infiltrated MSCs are responsible for an enhanced M2 response. For this, MSCs were sorted from wounds of C57BL/6 mice and co-cultured with M0-polarized macrophages isolated from bone marrow of C57BL/6 mice (Figure 4a). The ex vivo co-culture of BMDMs and wound MSCs showed that wound-infiltrated MSCs have the capacity to promote M2 polarization of macrophages, as assessed by increased expression of CD206 in macrophages. The extent of M2 polarization in macrophages was further enhanced for co-cultures with MSCs isolated from wounds of C57BL/6 mice treated with rGPNMB (Figure 4b). However, there was no significant difference in the expression of M1 marker CD86 in the presence of MSCs (Figure 4c). The direct treatment of rGPNMB to the M0-polarized BMDMs alone did not alter the expression of M2 markers, as assessed by the expression of *Arg-1* (see Supplementary Figure S6a online) and CD206 mRNAs (see Supplementary Figure S6b), suggesting that the effect of rGPNMB to promote M2 polarization of macrophages in the wound is mediated by MSCs, not by direct action of rGPNMB on macrophages. Along with our observation that macrophages are a major source of GPNMB secretion in the wound, these results suggest that GPNMB proteins secreted from wound macrophages can promote the recruitment of MSCs to the wound, which may in turn act on macrophages to direct the polarization of macrophages toward the M2 phenotype (Figure 4d).

Diabetic wounds are associated with diminished expression of GPNMB in wound macrophages

By observing the functional role of GPNMB in promoting the recruitment of MSCs in acute wounds of C57BL/6 mice, we next sought to determine whether the diabetic wound

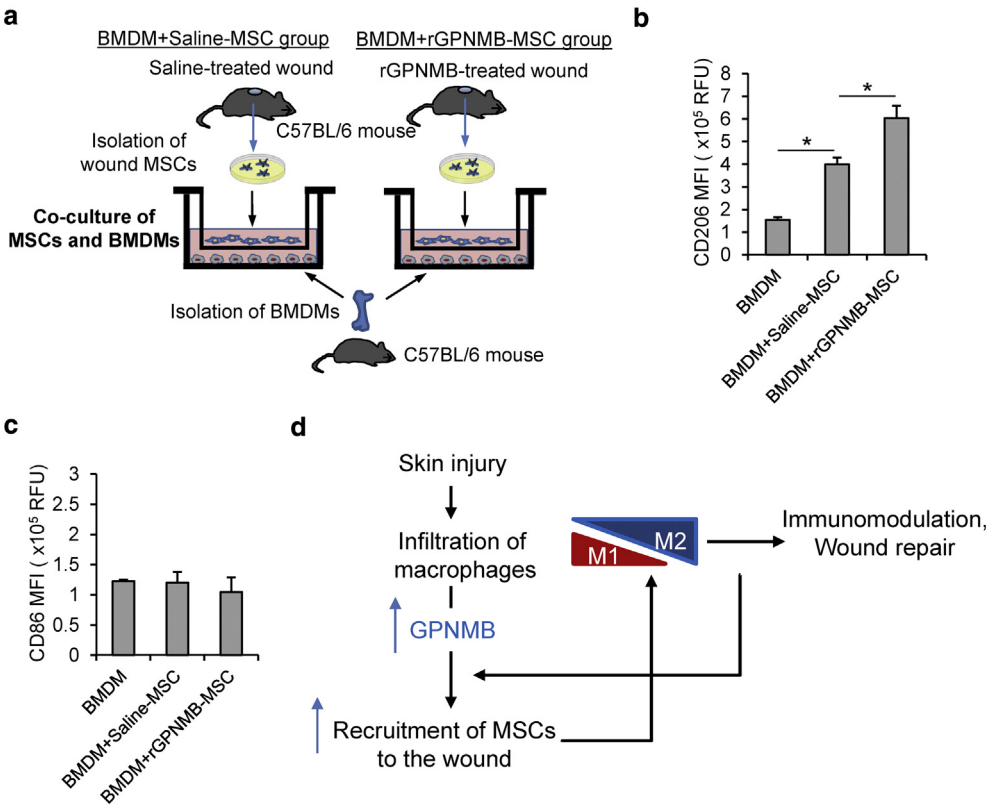

Figure 4. The effect of wound-infiltrated MSCs on the polarization of macrophages. (**a**) A schematic diagram for co-culture of wound-isolated MSCs and bone marrow-derived macrophages (BMDMs) from C57BL/6 mice. Monocytes were isolated from bone marrow of C57BL/6 mice and polarized into M0 macrophages, and MSCs were isolated from wounds of C57BL/6 mice treated with saline or rGPNMB (2 μg/wound), at day 5 after wounding. The BMDMs and wound MSCs were co-cultured for 24 hours, and then the expressions of CD86 and CD206 were assessed from F4/80$^+$ macrophages by flow cytometry. (**b, c**) The mean fluorescence intensity (MFI) of (**b**) CD206 and C(**c**) D86 from F4/80$^+$ macrophages. (**d**) A proposed model for crosstalk between wound-infiltrated macrophages and MSCs for immunomodulation and wound repair. Data are presented as mean ± standard error of the mean (n = 3). *$P < 0.05$. BMDM, bone marrow-derived macrophage; MSC, mesenchymal stem cell; RFU, relative fluorescence unit.

environment is associated with an impaired GPNMB expression after skin wounding. For this, wounded skin was harvested from nondiabetic (*db/*$^+$) and diabetic (*db/db*) mice, and the expression levels of GPNMB mRNA were compared. Wounds of *db/db* mice showed a 4-fold decrease in the expression of GPNMB mRNA at day 3 after wounding, compared with *db/*$^+$ mice (Figure 5a). Subsequent analysis of GPNMB mRNA from wound macrophages showed a significantly attenuated expression of GPNMB mRNA in macrophages from wounds of *db/db* mice compared with *db/*$^+$ mice (Figure 5b). This was further confirmed by immunofluorescent staining of wounded skin from *db/db* mice, which exhibited a diminished expression of GPNMB protein in F4/80-positive wound macrophages compared with *db/*$^+$ mice (Figure 5c). These results support that the diabetic wound environment impairs the capacity of macrophages to express GPNMB in response to skin injury.

Topical treatment of rGPNMB restores MSC recruitment, increases the trafficking of M2 macrophages, and accelerates wound closure in diabetic wounds

Type 2 diabetes has been shown to impair the recruitment of stem and progenitor cells to the site of injury and delay wound healing (Fiorina et al., 2010). In support of the finding, we have observed a decreased recruitment of MSCs to the wounds of *db/db* mice by 3-fold at day 7 after wounding compared with *db/*$^+$ mice (Figure 5d), which was accompanied by delayed wound closure (Figure 5e and 5f). Because the diabetic wound environment substantially attenuated the expression of GPNMB, we next sought to determine whether supplementing the availability of GPNMB protein in diabetic wounds can restore MSC recruitment and improve wound repair. The topical treatment of rGPNMB to the wounds of *db/db* mice could significantly increase the number of MSCs in the wound by 2-fold at day 3 (Figure 5d) and accelerate wound closure (Figure 5e and 5f). Finally, flow cytometric phenotyping of wound-isolated macrophage showed that the topical application of rGPNMB could enhance the trafficking of M2 macrophages while decreasing the number of M1 macrophages in wounds of *db/db* mice (Figure 5g), which were associated with the diminished expression of proinflammatory cytokines including IL-1β and TNF-α (see Supplementary Figure S7a online) and the up-regulated expression of pro-healing factors, including VEGF and IGF-1 in the wound (see Supplementary Figure S7b). Collectively, our results support that increasing the availability of GPNMB to the wounds of *db/db* mice could improve the extent of MSC recruitment, shift the profile of macrophage toward an M2-like phenotype, and accelerate wound closure.

DISCUSSION

In this study, using an in vivo model of cutaneous wound healing in mice, we provide evidence that macrophage-derived GPNMB is functionally important in promoting the recruitment of MSCs to the site of skin injury, which in turn modulates the inflammatory response by directing the polarization of M2 macrophages in acute wounds. We further show that GPNMB activity is attenuated in the wound environment associated with type 2 diabetes and that restoring GPNMB activity could improve the MSC recruitment and pro-healing response in wounds of type 2 diabetic mice.

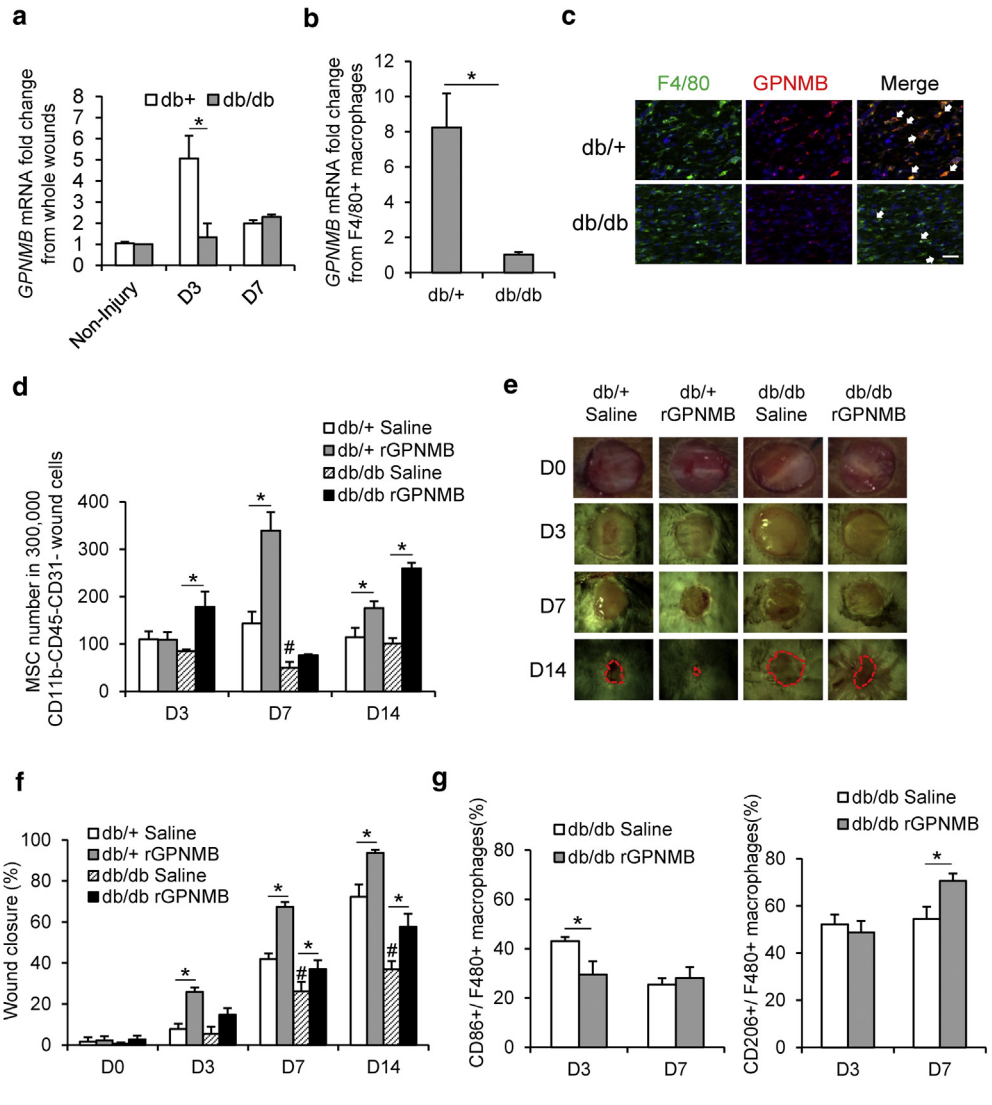

Figure 5. The effect of topical treatment of rGPNMB on the MSC recruitment, wound closure, and macrophage polarization in wounds of diabetic mice. (**a**) Expression of GPNMB mRNA in wounds of nondiabetic (*db/*$^{+}$) and diabetic (*db/db*) mice. (**b**) Expression of GPNMB mRNA in macrophages isolated from wounds of *db/*$^{+}$ and *db/db* mice at day 3 after wounding. (**c**) Immunofluorescent images showing co-localization of GPNMB with macrophages in wounds of *db/*$^{+}$ and *db/db* mice at 3 days after wounding. F4/80 (macrophages) are in green, GPNMB in red, and DAPI (nuclei) in blue. Scale bar = 10 μm. White arrows indicate co-localization of GPNMB with F4/80^{+} macrophage. (**d**) Flow cytometric counting of MSCs from wounds of *db/*$^{+}$ and *db/db* mice topically treated with saline or rGPNMB (2 μg/wound). (**e, f**) Representative photographic images of (**e**) wound area and (**f**) quantification of wound closure after wounding from *db/*$^{+}$ and *db/db* mice topically treated with saline or rGPNMB. Wound edge is indicated with red dotted line. (**g**) Flow cytometric phenotyping of M1-like F4/80^{+}CD86^{+} and M2-like F4/80^{+}CD206^{+} macrophages from wounds of *db/db* mice topically treated with saline or rGPNMB. Data are presented as mean ± standard error of the mean (n = 4–5), $^{*}P < 0.05$ and $^{\#}P < 0.05$ versus db/$^{+}$ saline group. D, day after wounding; MSC, mesenchymal stem cell; rGPNMB, recombinant GPNMB.

Why is the GPNMB activity diminished in diabetic wounds? Although the mechanism for the phenomenon remains to be elucidated, dysregulated trafficking and activation of macrophages in diabetic wounds appears to contribute to attenuated GPNMB activity. Diabetic wounds have been shown to be associated with a significantly reduced expression of M2-related genes and to exhibit upregulated expressions of M1-related genes (Mirza et al., 2015). We have previously shown that the secretion of soluble GPNMB protein can be significantly reduced in M1-polarized macrophages compared with M2-polarized ones (Yu et al., 2016). Furthermore, our current data show that significantly fewer macrophages were recruited to the wounds of *db/db* mice than in control (*db/*$^{+}$) mice, along with an increase in M1/M2 ratio in wound macrophages from *db/db* mice (see Supplementary Figure S8 online). Thus, we speculate that a diabetic wound environment associated with an inadequate number of macrophages, as well as their aberrant activation, may have contributed to the diminished capacity of macrophages to express GPNMB at the site of injury.

The presence of GPNMB-positive macrophages at the site of injury has been shown to be closely associated with the repair process in mouse models of liver and kidney injury (Katayama et al., 2015; Kumagai et al., 2015, Li et al., 2010), suggesting that GPNMB may act as a tissue repair factor at the site of injury. Our results from skin injury models in C57BL/6 wild-type and *Gpnmb*-mutant mice are in line with these findings and further provide important insight into the functional role of macrophage-derived GPNMB in promoting the recruitment of MSCs to the site of injury. We further show that wound-infiltrated MSCs are capable of directing the polarization of macrophages toward the M2-like phenotype. Our finding is consistent with published studies showing that the transfer of exogenous MSCs could modulate inflammatory response by promoting a M2 phenotype of macrophages (Xie et al., 2016; Zhang et al., 2010). Collectively, our study indicates that a dynamic crosstalk between macrophages and MSCs is mediated by GPNMB and that this contributes to the repair process in acute wounds (Figure 4d). We further hypothesize that this crosstalk is dysregulated in a diabetic wound environment and that this may contribute to the

diminished recruitment of MSCs to the wound. However, it remains to be further elucidated whether the attenuated recruitment of MSCs to the wounds is responsible for delayed healing in diabetic wounds.

What is the origin of endogenous MSCs that are recruited to the wound in response to GPNMB signaling? We have observed that increased recruitment of MSCs to the wound with the topical treatment of rGPNMB is associated with a concomitant decrease in the number of MSCs in the bone marrow compartment and an increase in numbers in the wound (see Supplementary Figure S9 online). This suggests that GPNMB signaling may elicit the mobilization of MSCs from the bone marrow to the local site of skin injury. However, it should be noted that the extent of increase in MSCs in the wound were not directly correlated with the change in numbers in the bone marrow (~40% decrease in bone marrow at day 3 v. ~200% increase in wound at day 5) (see Supplementary Figure S9), which suggests that certain mechanisms other than the contributions from bone marrow MSCs might act on the response as well. One plausible explanation is the role played by pericytes, because pericyte MSCs share the same positive expressions of Sca-1, CD29, and CD146 with bone marrow MSCs (Wong et al., 2015). Accumulating evidence suggests that MSC-like cells are derived from pericytes at the perivascular niche and that they function in response to localized injury for regeneration (Birbrair et al., 2015; Caplan, 2008; da Silva Meirelles et al., 2016; Mills et al., 2013; Wong et al., 2015). Our preliminary data suggest that GPNMB can also act on human pericytes for their enhanced migration in response to rGPNMB, albeit to a lesser extent for pericytes than bone marrow MSCs (see Supplementary Figure S10 online). Thus, we do not rule out the potential role played by pericyte MSCs in our observed response, which remains to be further elucidated.

In conclusion, we report the unprecedented role of GPNMB as a critical regulator in the crosstalk between macrophages and MSCs in the process of wound healing. The strategy to manipulate the number and functional capacities of endogenous MSCs by potentiating GPNMB activity is a promising therapeutic for promoting wound repair.

MATERIALS AND METHODS

Animals

Wild-type C57BL/6, *Gpnmb*-control *D2J/Gpnmb*$^{+}$, *Gpnmb*-mutant *DBA/2J* (*D2J*), diabetic *db/db* (BKS.Cg-*Dock7*^{m} $^{+/+}$ *Lepr*^{db}/J), and nondiabetic *db/*$^{+}$ (heterozygotes *Lepr*^{db}/$^{+}$) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Male mice at 8–12 weeks old were used for all the experiments. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Kent State University.

Excisional skin wounding, topical treatment of rGPNMB, and wound size measurement

Skin wounding was performed as previously described (Kim et al., 2014). A single circular wound was made into the dorsal skin of mice using an 8-mm sterile biopsy punch. The wound was then gently dressed with sterile collagen sponge (Medline, Mundelein, IL) soaked with sterile saline for the saline control group or rGPNMB (2 μg per wound; R&D, Minneapolis, MN) for the rGPNMB treatment group. Photographs of wounds were taken with a digital camera, in which the camera was held at the same distance from the wound. The extent of wound closure at a given time point was normalized as a percentage change with respect to the initial value at day 0. The detailed procedure is described in the Supplementary Materials online.

Immunofluorescence microscopy

Immunofluorescence analysis was performed with frozen sections of tissue from wounded skin as described in the Supplementary Materials.

MACS of cells from wounds of mice

Cells were dissociated from excisional wounds of mice using an enzymatic digest with collagenase I, collagenase XI, and hyaluronidase (Sigma-Aldrich, St. Louis, MO). Then macrophages and MSCs were sorted by MACS as described in the Supplementary Materials.

Flow cytometry

The flow cytometric identification of M1- or M2-polarized macrophages and MSCs ($CD11b^{-}CD45^{-}CD31^{-}CD146^{+}Sca1^{+}CD29^{+}$ cells) were performed using cells dissociated from mouse wound, as described in the Supplementary Materials.

Colony forming unit-fibroblast and tri-lineage differentiation assays

The colony forming unit-fibroblast and tri-mesenchymal lineage differentiation assays were performed with MACS-sorted $CD11b^{-}CD45^{-}CD31^{-}CD146^{+}Sca1^{+}CD29^{+}$ cells from skin wounds, as described in the Supplementary Materials.

Administration of BMDMs to the wounds of mice

Macrophages were prepared and cultured from bone marrow of *D2J* or *D2J/Gpnmb*$^{+}$ mice. The transfer of BMDMs to the site of injury was performed as described (Bannon et al., 2013; Pannell et al., 2016; Thuraisingam et al., 2010). Briefly, a total of 40 μl of BMDMs (1×10^{6} macrophages) were transferred to the collagen sponge cut into an 8-mm–diameter circle by fully soaking the dressing into the solution with BMDMs. Immediately after skin wounding (at day 0) in the *D2J* mouse, the BMDM-soaked wound dressing was gently applied to the wound of the mouse. The detailed procedure is described in the Supplementary Materials.

Co-culture of macrophage and MSCs

MSCs were MACS-sorted from wounds of C57BL/6 mice and co-cultured with M0-polarized macrophages isolated from bone marrow of C57BL/6 mice. Then, macrophages were stained by phycoerythrin-conjugated anti-CD206 and antigen-presenting allophycocyanin CD86 antibodies (BioLegend, San Diego, CA), and mean fluorescence intensity was quantified by flow cytometry. The detailed procedure is described in the Supplementary Materials.

Quantitative PCR analysis

Total RNA was isolated from wound cells or wound-sorted macrophages. The primers for *Gpnmb*, M1 marker (*iNOS*), M2 markers (*Arg-1*, *CD206*), proinflammatory cytokines (*Tnf-α*, *Il-1β*), and pro-healing factors (*Igf-1*, *Vegf*) were synthesized by Integrated DNA Technologies (Coralville, IA) (see Supplementary Table S1). The detailed procedure is described in the Supplementary Materials.

ELISA

Wound tissues collected at indicated time points were homogenized, and the protein levels of GPNMB (R&D, Minneapolis, MN), IL-1β, TNF-α, IGF-1, and VEGF (BioLegend, San Diego, CA) were measured using ELISA kits, as described in the Supplementary Materials.

Statistics

All data were expressed as the mean ± standard error of the mean. The Student unpaired *t* test was used for comparisons between two groups. One-way analysis of variance, followed by Bonferroni post hoc test, was used for comparisons between multiple groups. *P* less than 0.05 was considered statistically significant.

CONFLICT OF INTEREST

BY, FS, and MK declare a pending patent application based on the research in the article.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2017.08.034.

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