Protective Effect of MFG-E8 after Cutaneous Ischemia–Reperfusion Injury

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We recently demonstrated that the secreted glycoprotein and integrin-ligand MFG-E8 promotes cutaneous wound healing by enhancing angiogenesis. Several studies have identified potential roles for MFG-E8 in regulation of ischemia–reperfusion (I/R) injury in the brain, kidney, and liver. Our objective was to assess the role of MFG-E8 in the formation of skin ulcers using a murine model of cutaneous I/R injury–cutaneous pressure ulcers. Cutaneous I/R was performed by trapping the dorsal skin between two magnetic plates for 12 hours, followed by plate removal. Expression of MFG-E8 increased in the dermis during ischemia, and then decreased after reperfusion. Administration of recombinant (r)MFG-E8 in I/R areas at the beginning of reperfusion significantly inhibited the formation of cutaneous pressure ulcers, and the number of CD31⁺ vessel and NG2⁺ pericytes in wounds were increased in I/R mice treated with rMFG-E8. The number of M1 macrophages and the amount of proinflammatory mediators monocyte chemotactic protein-1, induced nitric oxide synthase, IL-6, tumor necrosis factor-α, and IL-1β in the wound area were reduced by the administration of rMFG-E8. We conclude that MFG-E8 may inhibit the formation of pressure ulcers induced by cutaneous I/R injury by regulating angiogenesis and inflammation. Exogenous application of MFG-E8 might have therapeutic potential for cutaneous I/R injuries, including decubitus ulcers and Raynaud’s phenomenon–induced digital ulcers.

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INTRODUCTION

Pressure ulcers are increasing over the world owing to aging of the population. Pressure ulcers are significant sources of pain and distress, leading to the impairment of the quality of life of patients (Gorecki et al., 2009). Although it has long been considered that chronic tissue ischemia was a primary factor in the pathogenesis of pressure ulcers, there has been increasing evidence that ischemia–reperfusion (I/R) is associated with the pathogenesis of pressure ulcers (Salcido et al., 1994; Peirce et al., 2000; Stadler et al., 2004). Many studies of I/R injury in various organs, including the brain, kidney, and liver, suggested that the pathogenesis of I/R injury was quite complex and different from that of the injury by chronic ischemia (Carden and Granger, 2000). I/R injury is defined as a cellular injury caused by the reperfusion of blood to previously ischemic tissue, and the cascade of harmful events, including dysfunction of endothelial cells, edema, capillary narrowing, leukocyte and macrophage infiltration, production of proinflammatory cytokines, and, thereafter, the apoptosis and necrosis of tissues (Pretto 1991; Wolfson et al., 1994). Therefore, I/R injury causes more severe tissue damage compared with ischemia alone (Carden and Granger, 2000; Carmo-Araújo et al., 2007). Reactive oxygen species also have essential roles in tissue damage by reperfusion. Nitric oxide (NO) is one of the reactive oxygen species, and excessive NO or its synthase, inducible NO synthase (iNOS), is associated with the pathogenesis of I/R-induced apoptosis and tissue injury (Nathan and Xie, 1994; Reid et al., 2004; Kasuya et al. 2014). Saito et al., (2008) reported that cutaneous I/R induced the recruitment of neutrophils and macrophages and the subsequent release of proinflammatory cytokines, including IL-1β, IL-6, and tumor necrosis factor-α (TNF-α), and toxic oxygen-derived free radicals induced the apoptosis of skin fibroblasts and skin injury. In addition, they reported that monocyte chemotactant protein-1 (MCP-1) was an important factor for macrophage recruitment and it had a role in apoptosis and injury via inducing iNOS during reperfusion rather than the ischemic period (Saito et al., 2008).

The secreted glycoprotein MFG-E8, also called lactadherin and SED1, is composed of two N-terminal EGF-like domains and two C-terminal discoidin-like domains (C1 and C2) that share homology with blood coagulation factors V and VIII (Stubbs et al., 1990; Ogura et al., 1993). One EGF-like domain (E2) contains RGD integrin-binding motif, and MFG-E8 binds...
to integrin αvβ3/5 (Andersen et al., 1997; Taylor et al., 1997; Hanayama et al., 2002). MFG-E8 acts as a bridging protein between phosphatidylserine on the surface of apoptotic cells and integrin αvβ3/5 on the surface of phagocytes, and it enhances the phagocytosis and clearance of apoptotic cells (Hanayama et al., 2002; Asano et al., 2004).

With respect to the regulation of angiogenesis, there are several reports stating that the interactions of MFG-E8 with integrin αv have been implicated in the enhancement of angiogenesis in mice (Silvestre et al., 2005; Neutzen et al., 2007). Recently, we demonstrated that pericytes and/or pericyte precursors were important sources of MFG-E8 in melanoma tumors in mice, and that MFG-E8 enhanced angiogenesis in melanoma tumors and in oxygen-induced retinopathy in mice (Motegi et al., 2011a). We have also determined that MFG-E8 associated with integrin αv and platelet-derived growth factor receptor β (PDGFRβ) on the surface of pericytes after platelet-derived growth factor (PDGF) treatment, and inhibited PDGFB-stimulated degradation of PDGFRβ, resulting in the enhancement of PDGFRβ signaling mediated by integrin–growth factor receptor cross talk (Motegi et al., 2011b). Moreover, we recently demonstrated that MFG-E8 is important for cutaneous wound healing using a mouse model with full-thickness cutaneous wounds (Uchiyama et al., 2014), in which MFG-E8 was increased in granulation tissue in cutaneous wound area. Wound healing was significantly delayed in MFG-E8 knockout (KO) mice compared with wild-type (WT) mice, and recombinant mouse MFG-E8 (rMFG-E8) treatment enhanced wound healing in MFG-E8 KO mice. These results suggest that MFG-E8 could be effectively targeted with therapeutic benefit for the wound caused by ischemic disorders.

Recent studies revealed that MFG-E8 significantly reduces inflammation and protects tissue injury after I/R in several organs including the brain, liver, kidney, and gut (Matsuda et al., 2011; Wu et al., 2012; Deroide et al., 2013; Matsuda et al., 2013). These studies demonstrated that MFG-E8 mRNA and protein expression in organs, including kidney, liver, and gut, were significantly decreased by I/R, and that treatment with rMFG-E8 recovered organ dysfunction and suppressed inflammatory responses. From these previous findings and our results, we hypothesized that treatment with rMFG-E8 might prevent tissue damage and promote angiogenesis in cutaneous I/R injury. However, the possible role of MFG-E8 in cutaneous I/R injury has not been studied previously. Herein, we analyzed changes of the expression of MFG-E8 in cutaneous I/R injury, and the effect of the treatment with rMFG-E8 in cutaneous I/R injury of mouse skin.

RESULTS

Expression of MFG-E8 during cutaneous I/R in vivo

To investigate the effect of cutaneous I/R injury on MFG-E8 expression in vivo, mRNA levels of MFG-E8 expression in the skin of I/R sites during I/R injury were analyzed. Levels of MFG-E8 mRNA increased in the skin during ischemia by 2.5-fold compared with that before I/R, and then immediately decreased to the basal levels at 4 hours after reperfusion, followed by a gradual decrease by 0.5-fold at 72 hours after reperfusion compared with that before I/R (Figure 1a). These results suggest that MFG-E8 expression was enhanced by hypoxic condition owing to the ischemia.

To assess which kinds of cells in skin can contribute to the increased MFG-E8 expression during ischemia, we performed immunofluorescence staining of MFG-E8 in skin before ischemia (−12 hours), just after reperfusion (0 hour), and 72 hours after reperfusion (72 hours). We determined that MFG-E8 expression around CD31⁺ ECs and αSMA⁺ (α-smooth muscle actin) pericytes/vascular smooth muscle cells (SMCs) just after reperfusion (0 hour) was enhanced compared with that before ischemia (−12 hours) and 72 hours after reperfusion (72 hours; Figure 1b). These results suggest that ECs and pericytes/vascular SMCs might be primary sources of MFG-E8 during ischemia.

Expression of MFG-E8 in pericyte-like cells, endothelial cells, and fibroblasts treated with hypoxic condition in vitro

We previously determined that pericytes were the major sources of MFG-E8 in B16 melanoma tumors, and that MFG-E8 localized in close proximity to pericytes/vascular SMCs in the dermis of murine and human skin (Motegi et al., 2011a; Uchiyama et al., 2014). In addition, immunofluorescence staining of skin in the I/R site showed that the MFG-E8 expression levels around blood vessels were enhanced during ischemia. Therefore, we next examined whether hypoxic condition enhanced the expression of MFG-E8 in pericytes, ECs, and fibroblasts in vitro. In 10T1/2 cells, which are surrogates for pericytes and pericyte precursors, MFG-E8 mRNA levels were significantly enhanced by hypoxia in a time-dependent manner (Figure 1c). MFG-E8 mRNA levels in ECs (human umbilical vein endothelial cells) were also significantly enhanced by hypoxia in a time-dependent manner (Figure 1c). MFG-E8 mRNA levels in fibroblasts (NIH3T3) were significantly enhanced by hypoxia for 1 hour, but were not changed by hypoxia for 12 hours. In addition, in immunoblots of whole-cell lysates, protein levels of MFG-E8 expression in 10T1/2 cells were also increased by hypoxic conditions (Figure 1d). These results suggest that ischemia-induced hypoxia in the skin may enhance the expression of MFG-E8 in pericyte/vascular SMCs and ECs in I/R sites.

rMFG-E8 protected ulcer formation after cutaneous I/R

To assess the effect of rMFG-E8 on cutaneous pressure ulcers after I/R in vivo, we compared the wound area after I/R injury in normal C57BL/6 mice treated with subcutaneous injection of rMFG-E8 or phosphate-buffered saline as a control. We used a simple, reproducible, and noninvasive experimental mouse model to evaluate the pathogenesis of cutaneous pressure ulcers by I/R in vivo (Stadler et al., 2004; Saito et al., 2008). Administration of rMFG-E8 significantly inhibited the formation of cutaneous pressure ulcers after I/R (Figure 2a and b). At 3 days after reperfusion, wound areas in rMFG-E8-treated mice were 60% of the wound areas in control mice. Wound areas in rMFG-E8-treated mice were significantly smaller than those in control mice from 1 to 8 days after reperfusion. The wound closure time in control mice was...
significantly longer than that in rMFG-E8-treated mice. These results demonstrate that rMFG-E8 partially protected the formation of cutaneous pressure ulcers after I/R.

To further examine the protective effect of MFG-E8 on I/R injury, we compared the wound area after I/R in MFG-E8 WT and KO mice. Wound areas in the MFG-E8 KO mice tended to be larger than those in WT mice. At 2 days after reperfusion, wound areas in MFG-E8 KO mice were significantly larger than those in WT mice (Figure 2c). This result may partially support the results that rMFG-E8 injection protected against cutaneous I/R injury.

**MFG-E8 suppressed infiltrating macrophages, especially M1 macrophages, into cutaneous I/R area**

Infiltrating neutrophils and macrophages participate in I/R injury by regulating inflammation and angiogenesis. Therefore, we next analyzed the effect of rMFG-E8 on infiltrating neutrophils and macrophages after cutaneous I/R injury. At 1 day after reperfusion, the prominent edema in the dermis and the infiltration of inflammatory cells in the hypodermis were histologically observed (data not shown). The numbers of infiltrating MPO⁺ neutrophils in control mice were comparable to those in rMFG-E8-treated mice at 1 day after reperfusion.

**Figure 1. MFG-E8 mRNA expression during ischemia–reperfusion (I/R) injury and hypoxia treatment.** (a) Quantification of MFG-E8 mRNA levels in the I/R site from the beginning of ischemia to 72 hours after reperfusion by quantitative reverse transcriptase–PCR (RT–PCR). The end of ischemia was assigned 0. Data are relative to mRNA level in 0 h. Values were determined in n = 3 mice. (b) Expression and distribution of MFG-E8 in the skin during I/R injury (–12 hours: before ischemia, 0 hour: just after reperfusion, 72 hours: 72 hours after reperfusion). Scale bar = 20 μm. Data are representative of n = 3 independent experiments. (c) Quantification of MFG-E8 mRNA levels in pericytes, endothelial cells, and fibroblasts by quantitative RT–PCR. Cells were treated with hypoxia for the indicated times. The amount of MFG-E8 expression in nontreated cells was assigned a value of 1. Values were determined in three independent experiments. **P<0.01, *P<0.05 relative to nontreated cells. (d) MFG-E8 protein levels in 10T1/2 cells by immunoblotting. 10T1/2 cells were treated with hypoxia or normoxia conditions for 24 hours. Data are representative of n = 3 independent experiments.
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Local microenvironment influences the phagocytic and
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either classically activated macrophages (M1 macrophages) or alternatively activated macrophages (M2 macrophages; Lawrence and Natoli, 2011; Ferrante and Leibovich, 2011). M1 macrophages are observed in initial tissue damage responses, and they induce inflammation by the secretion of proinflammatory mediators, including MCP-1, NO, IL-1, IL-6, IL-12, and TNF-α (Mosser, 2003; Lawrence and Natoli, 2011). M2 macrophages have an essential role in early and middle stages of wound healing, and they induce the resolution of inflammation and promote tissue repair. Therefore, we next examined the numbers of M1/M2 macrophages in I/R areas. The number of total CD68+ macrophages and CD68+ iNOS+ M1 macrophages in I/R areas in rMFG-E8-treated mice were significantly reduced compared with those in control mice (Figure 3c). In addition, the numbers of CD68+, arginase-1+ M2 macrophages in the I/R area in rMFG-E8-treated mice were significantly reduced (Figure 3d). mRNA levels of iNOS and arginase-1 in I/R areas in rMFG-E8-treated mice were also significantly reduced (Figure 3e). Although M1 macrophage/total macrophage ratios in the wound area in rMFG-E8-treated mice were reduced, M2 macrophage/total macrophage ratios in the wound area in rMFG-E8-treated mice were not different from those in control mice, suggesting that rMFG-E8 might suppress the number of total macrophages, especially M1 macrophages, which infiltrated into the wounded area.

rMFG-E8 suppressed apoptotic cells after cutaneous I/R

I/R-induced reactive oxygen species causes apoptosis and subsequent exaggerated inflammatory responses induced by secondary necrosis (Miksa et al., 2009; Aziz et al., 2011). To examine the influence of MFG-E8 on the number of apoptotic cells in I/R areas, TUNEL staining of skin sections was performed. At 1 day after reperfusion, the number of apoptotic cells in I/R areas in rMFG-E8-treated mice were decreased compared with those in control mice (Figure 4). These results suggest that rMFG-E8 might suppress the formation and/or accumulation of apoptotic cells induced by cutaneous I/R injury.

rMFG-E8 suppressed the production of proinflammatory cytokines and chemokines after cutaneous I/R

Next we investigated the effect of MFG-E8 on the mRNA levels of proinflammatory cytokines and chemokines, including MCP-1, IL-1β, TNF-α, and IL-6, in the I/R area by real-time PCR. Treatment with rMFG-E8 significantly suppressed mRNA levels of proinflammatory cytokines and chemokines (Figure 5a–d). These results suggest that rMFG-E8 might suppress the inflammation of skin after I/R.

Next we examined the production of intracellular inflammatory cytokines and chemokines in infiltrating macrophages in the I/R sites using fluorescence-activated cell sorting analysis, because macrophages, rather than neutrophils, are major sources of proinflammatory cytokines and chemokines, including IL-6, TNF-α, and MCP-1. Similar to the results of histological analyses depicted in Figure 3, the total number of infiltrating CD68+ macrophages in I/R sites was inhibited by rMFG-E8 treatment (Figure 5e). The ratios of MCP-1+ macrophages/total macrophages, TNF-α+ macrophages/total macrophages, and IL-6+ macrophages/total macrophages in I/R sites were also inhibited by rMFG-E8 treatment (Figure 5f–h). These results suggest that rMFG-E8 might inhibit the recruitment of macrophages, as well as the production of proinflammatory cytokines and chemokines, in macrophages.
rMFG-E8 promoted angiogenesis in the I/R area after cutaneous I/R
We previously determined that blood vessel formation was inhibited in cutaneous wound area in MFG-E8 KO mice, suggesting that MFG-E8 might regulate angiogenesis in cutaneous wound healing. Therefore, we investigated the effect of rMFG-E8 on vascularity in the I/R area. At 6 days after reperfusion, the numbers of CD31⁺ ECs and NG2⁺ pericytes in the I/R areas were significantly increased compared with those in control mice (Figure 6a). The numbers of αSMA⁺ (α-smooth muscle actin) pericytes/vascular SMCs in I/R areas in rMFG-E8-treated mice tended to be more than those in control mice (Figure 6b). These results suggest that rMFG-E8 might enhance angiogenesis in I/R injury.

The effects of rMFG-E8 on IFA-induced skin inflammation
To examine whether the effects of rMFG-E8 are specific to I/R injury or globally relate to skin inflammation, we next analyzed the effect of MFG-E8 on the skin inflammation induced by incomplete Freund’s adjuvant (IFA). It has been known that IFA injection into the skin induced skin inflammatory response, such as inflammatory cells infiltration and

Figure 3. rMFG-E8 suppressed infiltrating macrophages, especially M1 macrophages, into the cutaneous ischemia–reperfusion (I/R) area. (a) The number of infiltrating neutrophils in the I/R site at 1 day after reperfusion was determined by counting myeloperoxidase-positive cells. Values were determined in six random microscopic fields in n = 3 mice per group. Scale bar = 20 μm. (b) The number of infiltrating macrophages in the I/R site at 1 day after reperfusion was determined by counting CD68-positive cells. Values were determined in six random microscopic fields in n = 3 mice per group. **P < 0.01. Scale bar = 20 μm. (c, d) Infiltration of CD68⁺ and induced nitric oxide synthase (iNOS)⁺ M1 macrophages (c) or CD68⁺ and arginase-1⁺ M2 macrophage (d) in the I/R area 1 day after reperfusion. Quantification of the CD68⁺, iNOS⁺, and arginase-1⁺ areas in six random microscopic fields in n = 3 mice per group was performed using the Image J software. The ratio of M1 or M2 macrophages (M1 or M2 macrophages/Total macrophages) in control mice was assigned a value of 1. **P < 0.01, *P < 0.05. Scale bar = 20 μm. (e) Quantification of iNOS and arginase-1 mRNA levels in I/R area at 1 day after reperfusion. n = 3 mice per group. *P < 0.05.
proinflammatory cytokines production (Vitoriano-Souza et al., 2012). IFA-injected sites were treated with subcutaneous injection of rMFG-E8 or phosphate-buffered saline as a control. The appearance of skin at 1 day after IFA injection did not differ between the two groups (Supplementary Figure S1a online). rMFG-E8 treatment did not affect the number of neutrophils, total macrophages, and M1 macrophages in the IFA-injected site at 1 day after IFA injection (Supplementary Figure S1b,c online). No significant difference in the number of apoptotic cells was observed between the two groups (Supplementary Figure S1e online). mRNA levels of iNOS, arginase-1, MCP-1, IL-1β, TNF-α, and IL-6 in IFA-injected skin in rMFG-E8-treated mice were also comparable to those in control mice at 1 day after IFA injection (Supplementary Figure S1d and S2 online). These results indicate that rMFG-E8 did not modify IFA-induced skin inflammation.

DISCUSSION

This is a study to investigate the role of MFG-E8 in cutaneous I/R injury. Using murine model (Stadler et al., 2004), we determined that MFG-E8 expression was significantly increased in the skin during ischemia, suggesting that MFG-E8 expression might be enhanced by hypoxic conditions. Immunofluorescence staining of MFG-E8 revealed that MFG-E8 expression levels around ECs and pericytes/vascular SMCs just after reperfusion (0 hour) were enhanced compared with those before ischemia (−12 hours) and at 72 hours after reperfusion (72 hours), suggesting that ECs and pericytes/vascular SMCs might be primary sources of MFG-E8 during ischemia. In addition, we confirmed that mRNA and/or protein levels of MFG-E8 in pericytes and ECs were significantly enhanced by hypoxia in a time-dependent manner. These findings suggest that hypoxia in ischemic areas might be associated with the enhancement of MFG-E8 expression in pericytes/vascular SMCs and ECs. Previous studies have reported that MFG-E8 mRNA and protein expression in organs, including the kidney, liver, and gut, were significantly decreased by I/R (Matsuda et al., 2011; Wu et al., 2012; Deroide et al., 2013; Matsuda et al., 2013). Consistent with these previous results, MFG-E8 expression in cutaneous I/R areas was decreased by 0.5-fold at 72 hours after reperfusion. The pathogenesis of the suppression of MFG-E8 expression after I/R is currently unknown; however, we suggest that I/R might cause severe damage of tissue, including pericytes/vascular SMCs and ECs, and this I/R-induced damage to the source of MFG-E8 may account for the suppression of MFG-E8 expression after I/R. Kasuya et al. (2014) reported that blood vessels in the I/R areas were reduced compared with those in marginal zones after...
We identified rMFG-E8-mediated protective mechanisms of cutaneous I/R injury, including (i) suppression of macrophages, especially M1 macrophages, infiltrating into the I/R area; (ii) suppression of apoptotic cell accumulation; (iii) suppression of proinflammatory cytokine synthesis; and (iv) enhancement of angiogenesis. We recently demonstrated that MFG-E8 regulates angiogenesis and wound healing in a cutaneous wound healing mice model (Uchiyama et al., 2014). In the present study using a murine I/R model, we additionally determined that MFG-E8 regulates the functions of M1 macrophages, including the secretion of proinflammatory cytokines.

M1 macrophages infiltrate in the early phase of response to tissue damage, and they are involved in cutaneous injury and inflammation by secreting proinflammatory mediators, including MCP-1, NO, IL-1, IL-6, IL-12, and TNF-α (Moss, 2003; Saito et al., 2008; Lawrence and Natoli, 2011). Therefore, our results indicate that MFG-E8 suppresses the M1 macrophage infiltration, through which cutaneous inflammation induced by proinflammatory mediators from M1 macrophages is restrained. With respect to M1 macrophages and MFG-E8, it has been reported that coculture of macrophages with apoptotic prostate cancer cells increased efferocytosis, elevated MFG-E8 expression levels, and induced macrophage polarization into the M2 phenotype (Soki et al., 2014). They also demonstrated that MFG-E8 enhanced the phosphorylation of STAT3, and inhibited SOCS3, a negative regulator of STAT3, therefore keeping STAT3 signaling activated and promoting M2 polarization. This study suggests that rMFG-E8 might induce M2 macrophage polarization in the I/R area, resulting in the suppression of M1 macrophage ratios. However, M2 macrophage ratios were not increased by rMFG-E8 treatment in our experiments. Therefore, further studies are required to clarify the effect of MFG-E8 on M1/M2 macrophages.

It has been well recognized that MFG-E8 acts as a bridging protein between phosphatidylserine on apoptotic cells and integrin αvβ3/5 on phagocytes, thereby enhancing phagocytosis and clearance of apoptotic cells (Hanayama et al., 2002; Asano et al., 2004). In an experimental sepsis model using cecal ligation and puncture, MFG-E8-containing exosome administration attenuated the acute systemic inflammatory response in sepsis by enhancing apoptotic cell clearance (Miksa et al., 2009). We showed that treatment with rMFG-E8 decreased I/R-induced apoptotic cell accumulation. These findings suggest that the decreased levels of MFG-E8 after cutaneous I/R may be associated with impaired phagocytosis, leading to the accumulation of apoptotic cells in the I/R area, and that the administration of rMFG-E8 may enhance phagocytosis of apoptotic cells, leading to the suppression of apoptosis and necrosis in the I/R area and protection from pressure ulcers.

We assessed vascularity in I/R areas, and found that the numbers of ECs and pericytes in the I/R areas were significantly more than those in control mice. We assume that the protective effect of rMFG-E8 on cutaneous I/R injury might be associated with both suppression of inflammation and promotion of angiogenesis.

Finally, we demonstrated that rMFG-E8 did not affect IFA-induced skin inflammation, suggesting that the inhibition of skin inflammation by rMFG-E8 treatment may be relatively restricted in cutaneous I/R injury. However, further investigation may be warranted in additional skin inflammation models.

Taken together, we conclude that MFG-E8 suppresses the formation of pressure ulcers induced by cutaneous I/R injury by regulating inflammation and angiogenesis. Exogenous MFG-E8 administration has possible therapeutic potential for
cutaneous I/R injuries, including decubitus ulcers and Raynaud’s phenomenon–induced digital ulcers.

**MATERIALS AND METHODS**
The detailed protocols and statistical analysis are described in Supplementary Materials and Methods online.

**Mice**
C57BL/6 mice were purchased from the SLC (Shizuoka, Japan). 8- to 12-week-old mice were used for all experiments. MFG-E8 KO C57BL/6 mice were generated as previously described (Neutzner et al., 2007; Motegi et al., 2011a). All experiments were approved by the Ethical Committee for Animal Experiments of the Gunma University Graduate School of Medicine and carried out in accordance with the approved guidelines.

**I/R cycles and analysis**
The I/R model that has been previously reported was used (Peirce et al., 2000; Studler et al., 2004; Saito et al., 2008). The dorsal skin was gently pulled up and trapped between two round ferrite magnetic plates that had a 12-mm diameter (113 mm²) and that were 5-mm thick (NeoMag Co, Ichikawa, Japan) for 12 hours, and then plates were removed. All of the mice developed two round ulcers separated by a bridge of normal skin. For analysis, each wound site was digitally photographed after wounding, and the wound areas were measured on photographs using Image J (version 1.48, NIH, Bethesda, MD). To assess the effects of rMFG-E8 on wound healing after cutaneous I/R injury, 400 ng of rMFG-E8 (R&D systems, Minneapolis, MN) per 50 μl of phosphate-buffered saline or 50 μl of phosphate-buffered saline as a control were injected into the dermis in the I/R site at the beginning of reperfusion.

**Real-time RT–PCR**
To analyze the mRNA levels of expression in the I/R site by real-time RT–PCR, the whole-skin samples in the I/R site were used. Real-time RT–PCR was performed as described in Supplementary Materials and Methods online.

**Statistics**
P-values were calculated using the Student’s t-test (two-sided) or by analysis of one-way analysis of variance followed by Bonferroni’s post test, as appropriate. Error bars represent s.e.m., and numbers of experiments (n) are as indicated.

**CONFLICT OF INTEREST**
The authors state no conflict of interest.

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**SUPPLEMENTARY MATERIAL**
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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