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Protective Effect of MFG-E8 After Cutaneous Ischemia-reperfusion Injury

Running Title: MFG-E8 and cutaneous I/R injury

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Abbreviations: I/R, ischemia-reperfusion; rMFG-E8, recombinant MFG-E8; ROS, reactive oxygen species; NO, nitric oxide; iNOS, induced nitric oxide synthase; TNF-α, tumor-necrosis factor-α; OIR, oxygen-induced retinopathy; PDGF, platelet-derived growth factor; PDGFRβ, PDGF receptor β; KO, knockout; Abs, antibodies; siRNA, small interfering RNA; WT, wild-type; αSMA, α-smooth muscle actine; RT-PCR, reverse transcriptase-PCR

ABSTRACT

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 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 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. Numbers of M1 macrophages and the amount of proinflammatory mediators, MCP-1, iNOS, IL-6, TNF-α and IL-1β in wound area were reduced by 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.

INTRODUCTION

Pressure ulcers are increasing over the world due to population aging. Pressure ulcers are significant sources of pain and distress, leading to the impairment of 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 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 *et al.*, 2000). I/R injury is defined as cellular injury caused by the reperfusion of blood to previously ischemic tissue, and the cascade of harmful events, including dysfunction of endothelial cells (EC), edema, capillary narrowing, leukocytes and macrophages infiltration, production of proinflammatory cytokines and thereafter, the apoptosis and necrosis of tissues (Pretto 1991; Wolfson *et al.*, 1994; Carroll *et al*., 2000). Therefore, I/R injury causes more severe tissue damage compared with ischemia alone (Parks and Granger, 1986; Carden *et al.*, 2000; Carmo-Araujo *et al.*, 2007). Reactive oxygen species (ROS) also play essential roles in the tissues damage by reperfusion. Nitric oxide (NO) is one of ROS and excessive NO or its synthase, inducible NO synthase (iNOS), is associated with the pathogenesis of I/R-induced apoptosis and tissue injury (Nathan *et al*., 1994; Reid *et al*., 2004; Kasuya *et al.* 2014). Saito *et al.* reported that cutaneous I/R induced the recruitment of neutrophils and macrophages and 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 (Saito *et al.* 2008). In addition, they reported that monocyte chemoattractant protein-1 (MCP-1) was an important factor for macrophages recruitment, and played 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 (Taylor *et al*., 1997; Andersen *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 enhances phagocytosis and clearance of apoptotic cells (Hanayama *et al*., 2002; Asano *et al*., 2004).

With respect to the regulation of angiogensis, there are several reports that interactions of MFG-E8 with integrin αv have been implicated in the enhancement of angiogenesis in mice (Silvestre *et al*., 2005; Neutzner 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 PDGF-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 our 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 brain, liver, kidney and gut (Deroide *et al.*, 2013; Matsuda *et al*., 2013; Matsuda *et al*., 2011; Wu *et al.* 2012). 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, 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 due 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 (-12h), just after reperfusion (0h) and 72 hours after reperfusion (72h). We determined that MFG-E8 expression around $CD31^+$ EC and αSMA^+ pericytes/vascular smooth muscle cells (SMC) just after reperfusion (0h) was enhanced compared to that before ischemia (-12h) and 72 hours after reperfusion (72h) (Figure 1b). These results suggest that EC and pericytes/vascular SMC 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 major sources of MFG-E8 in B16 melanoma tumors, and that MFG-E8 localized in close proximity to pericytes/vascular SMC in the dermis of murine and human skin (Motegi *et al.*, 2011a; Uchiyama *et al.*, 2014). In addition, immunofluorescence staining of skin in I/R site showed that MFG-E8 expressions around blood vessels were enhanced during ischemia. Therefore, we next examined whether hypoxic condition enhanced the expression of MFG-E8 in pericytes, EC 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 EC (HUVEC) 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 pericytes/vascular SMC and EC 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 wound area after I/R injury in normal C57BL/6 mice treated with subcutaneous injection of rMFG-E8 or PBS 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, b). At 3 days after reperfusion, wound areas in rMFG-E8-treated mice was 60% of 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 wound area after I/R in MFG-E8 WT and KO mice. Wound areas in 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.

rMFG-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 3a). However, the numbers of $CD68^+$ macrophages in rMFG-E8-treated mice were significantly decreased (Figure 3b), suggesting that rMFG-E8 might regulate accumulation or functions of macrophages.

Local microenvironment influences the phagocytic and secretory behavior of macrophages to promote development of either classically activated macrophages (M1 macrophages) or alternatively activated macrophages (M2 macrophages) (Lawrence *et al.*, 2011; Ferrante CJ and Leibovich, 2011). M1 macrophages are observed in initial tissue damage responses, and induce inflammation by the secretion of proinflammatory mediators, including MCP-1, NO, IL-1, IL-6, IL-12 and TNF-α (Mosser *et al*., 2003; Lawrence *et al*., 2011). M2 macrophages play an essential role in early and middle stages of wound healing, and 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 to those in control mice (Figure 3c). Additionally, the numbers of $CD68^+$, $arginase\text{-}1^+$ M2 macrophages in 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 macrophages/total macrophages ratios in wound area in rMFG-E8-treated mice were reduced, M2 macrophages/total macrophages ratios in 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 that infiltrated into the wounded area.

rMFG-E8 suppressed apoptotic cells after cutaneous I/R

I/R-induced ROS causes apoptosis and subsequent exaggerated inflammatory responses induced by secondary necrosis (Aziz *et al.*, 2011; Miksa *et al.*, 2009). 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 to those in control mice (Figure 4). These results suggest that rMFG-E8 might suppress 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 mRNA levels of proinflammatory cytokines and chemokines, including MCP-1, IL-1β, TNF-α and IL-6, in 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 I/R sites using FACS 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 $MCP\text{-}1^+$ macrophages/total macrophages, $TNF\text{-}\alpha^+$ macrophages/total macrophages, and $IL\text{-}6^+$ 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 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 I/R area. At 6 days after reperfusion, the numbers of $CD31^+$ EC and $NG2^+$ pericytes in I/R areas were significantly increased compared to those in control mice (Figure 6a). The numbers of αSMA^+ pericytes/vascular SMC 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 skin

induced skin inflammatory response, such as inflammatory cells infiltration and proinflammatory cytokines production (Vitoriano-Souza *et al.,* 2012). IFA-injected sites were treated with subcutaneous injection of rMFG-E8 or PBS 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 and M1 macrophages in IFA-injected site at 1 day after IFA injection (Supplemental Figure S1b,c online). No significant difference in the number of apoptotic cells was observed between the two groups (Supplemental 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 (Supplemental Figure S1d and S2 online). These results indicate that rMFG-E8 did not modify IFA-induced skin inflammation.

DISCUSSION

This is the 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 expressions around EC and pericytes/vascular SMC at just after reperfusion (0h) were enhanced compared to those at before ischemia (-12h) and at 72 hours after reperfusion (72h), suggesting that EC and pericytes/vascular SMC might be primarily sources of MFG-E8 during ischemia. In addition, we confirmed that mRNA and/or protein levels of MFG-E8 in pericytes and EC 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 SMC and EC. Previous studies have reported that MFG-E8 mRNA and protein expression in organs, including kidney, liver and gut, were significantly decreased by I/R (Deroide *et al.*, 2013; Matsuda *et al*., 2013; Matsuda *et al*., 2011; Wu *et al.* 2012). 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 SMC and EC, 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.* reported that blood vessels in I/R areas were reduced compared to those in marginal zones after reperfusion, suggesting that I/R-induced ROS might damage the blood vessels (Kasuya *et al.*, 2014).

We determined that injection of rMFG-E8 significantly inhibited the formation of ulcers after I/R, however, the differences of the formation of ulcers after I/R between

MFG-E8 WT and KO mice were not strikingly different. The reason why the KO mice have not had a more dramatic response is unknown. However, we suggest that the immediately suppression of MFG-E8 expression in I/R site after I/R in WT mice may lead to no remarkable difference of MFG-E8 expression in I/R site after I/R between WT and KO mice.

We identified rMFG-E8-mediated protective mechanisms of cutaneous I/R injury, including (i) suppression of macrophages, especially M1 macrophages, infiltrating into I/R area; (ii) suppression of apoptotic cells accumulation; (iii) suppression of proinflammatory cytokine synthesis; and (iv) enhancement of angiogenesis. We recently demonstrated that MFG-E8 regulates angiogenesis and wound healing in 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 secretion of proinflammatory cytokines.

M1 macrophages infiltrate in the early phase of response to tissue damage, and are involved in cutaneous injury and inflammation by secreting proinflammatory mediators, including MCP-1, NO, IL-1, IL-6, IL-12 and TNF-α (Mosser *et al.*, 2003; Lawrence *et al.*, 2011; Saito *et al.*, 2008). Therefore, our results indicate that MFG-E8 suppress 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 co-culture of macrophages with apoptotic prostate cancer cells increased efferocytosis, elevated MFG-E8 expression levels, and induced macrophage polarization into M2 phenotype (Soki *et al.*, 2014). They also demonstrated that MFG-E8 enhanced 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 macrophages polarization in 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 cells 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 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 I/R area and protection from pressure ulcers.

We assessed vascularity in I/R areas, and found that the numbers of EC and pericytes in 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 demonstraed 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). Eight- 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; Stadler *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 mm^2) and 5mm 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 sites were digitally photographed after wounding, and wound areas were measured on photographs using Image J (version1.48, NIH, Bethesda, MD). To assess the effects of rMFG-E8 on wound healing after cutaneous I/R injury, 400ng rMFG-E8 (R&D systems, Minneapolis, MN) per 50μl PBS or 50μl PBS 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 I/R site by real-time RT-PCR, the whole skin

samples in 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 ANOVA followed by Bonferroni's post test as appropriate. Error bars represent standard errors of the mean, and numbers of experiments (n) are as indicated.

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

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FIGURE LEGENDS

Figure 1. MFG-E8 mRNA expression during I/R injury and hypoxia treatment

(**a**) Quantification of MFG-E8 mRNA levels in I/R site from the beginning of ishemia to 72 hours after reperfusion by quantitative RT-PCR. The end of ischemia was assigned 0. Data are relative to mRNA level in 0h. Values were determined in n=3 mice. (b) Expression and distribution of MFG-E8 in the skin during I/R injury (-12h: before ischemia, 0h: just after reperfusion, 72h: 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 non-treated cells was assigned a value of 1. Values were determined in 3 independent experiments. $^{**}P<0.01$, $^{*}P<0.05$ relative to non-treated cells. (**d**) MFG-E8 protein levels in 10T1/2 cells by immunoblotting. 10T1/2 cells were treated with hypoxia or normoxia condition for 24 hours. Data are representative of n=3 independent experiments.

Figure 2. rMFG-E8 protected pressure ulcer formation after cutaneous I/R

(**a**) The size of wound area after I/R injury in normal C57BL/6 mice treated with subcutaneous injection of rMFG-E8 or PBS as a control. The size of ulcer in control mice at 3 days after reperfusion was assigned value of 100%. (Control: n=10, rMFG-E8: n=10, for each time point and group). $^{**}P<0.01$, $^{*}P<0.05$. (**b**) Photographs of wound after cutaneous I/R in control or rMFG-E8 mice at 0, 2, 4, 6, 8, and 12 days after reperfusion. (**c**) The size of wound area after I/R injury in MFG-E8 WT and KO mice. The size of ulcer in WT mice at 3 days after reperfusion was assigned value of 100%. n=5 mice per genotype. $^{*}P<0.05$.

Figure 3. rMFG-E8 suppressed infiltrating macrophages, especially M1 macrophages,

into cutaneous I/R area

(**a**) The number of infiltrating neutrophils in I/R site at 1 day after reperfusion was determined by counting myeloperoxidase positive cells. Values were determined in 6 random microscopic fields in n=3 mice per groups. Scale bar=20 μm. (**b**) The number of infiltrating macrophages in I/R site at 1 day after reperfusion was determined by counting CD68 positive cells. Values were determined in 6 random microscopic fields in n=3 mice per groups. $^{**}P<0.01$. Scale bar=20 μm. (**c, d**) Infiltration of $CD68^+$ and $iNOS^+$ M1 macrophages (**c**) or $CD68^+$ and arginase-1^+ M2 macrophage (**d**) in I/R aera at 1 day after reperfusion. Quantification of the $CD68^+$, $iNOS^+$ and arginase-1^+ areas in 6 random microscopic fields in n=3 mice per groups was performed using Image J software. The ratio of M1 or M2 macrophages (M1 or M2 macrophages/Total macrophages) in control mice was assigned values 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. $^{*}P<0.05$.

Figure 4. rMFG-E8 suppressed apoptotic cells after cutaneous I/R

The number of apoptotic cells in I/R site at 1 day after reperfusion was determined by counting both TUNEL and DAPI positive cells (Arrowhead). Values were determined in 6 random microscopic fields in n=3 mice per groups. $^{**}P<0.01$. Scale bar=20 μm.

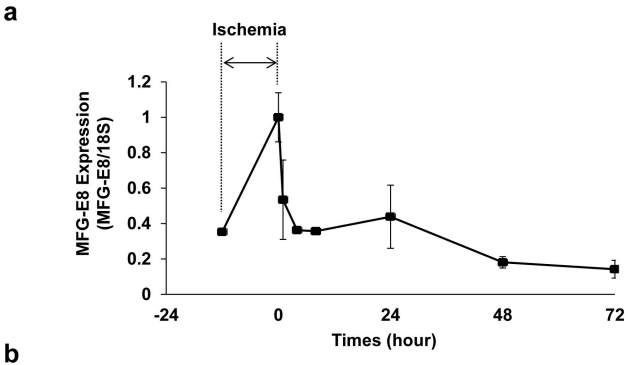
Figure 5. rMFG-E8 suppressed the production of proinflammatory cytokines after cutaneous I/R

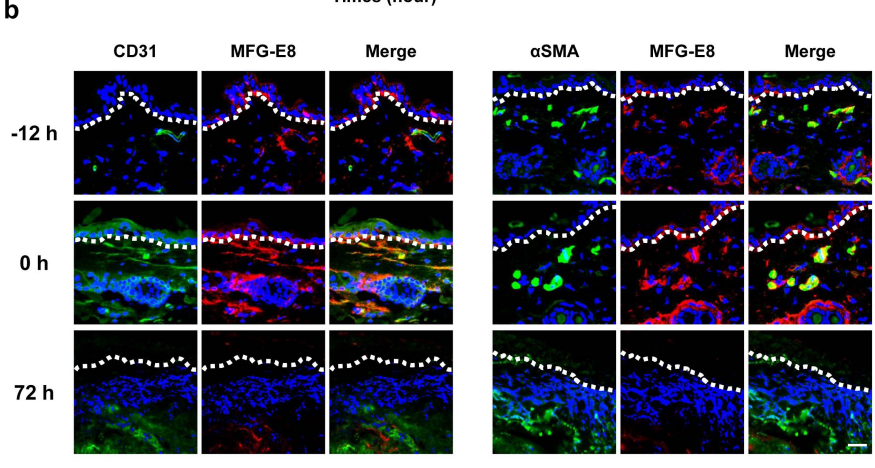
Quantification of mRNA levels of MCP-1 (**a**), IL-1β (**b**), TNF-α (**c**) and IL-6 (**d**) in I/R area at 1 day after reperfusion by quantitative RT-PCR. mRNA levels in control mice was assigned values of 1. (**e-h**) Flow cytometry analyses of the production of intracellular inflammatory cytokines and chemokines in infiltrated macrophages in I/R site. (**e**) The relative number of

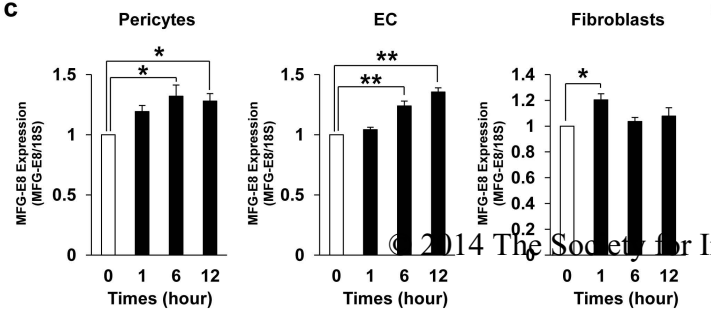
infiltrated $CD68^+$ macrophages in I/R site. Total number of macrophages in control mice was assigned values of 1. (**f**) The relative ratio of MCP-1^+ macrophages/total macrophages, (**g**) TNF-α^+ macrophages/total macrophages, and (**h**) IL-6^+ macrophages/total macrophages in I/R site. The ratio of MCP-1^+, TNF-α^+ or IL-6^+ macrophages/total macrophages in control mice was assigned values of 1. n= 3 mice per group. $^{**}P<0.01$, $^{*}P<0.05$.

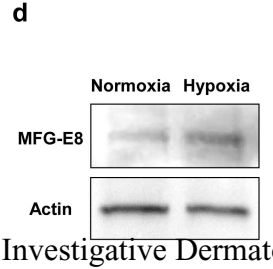
Figure 6. rMFG-E8 promoted angiogenesis in I/R area after cutaneous I/R

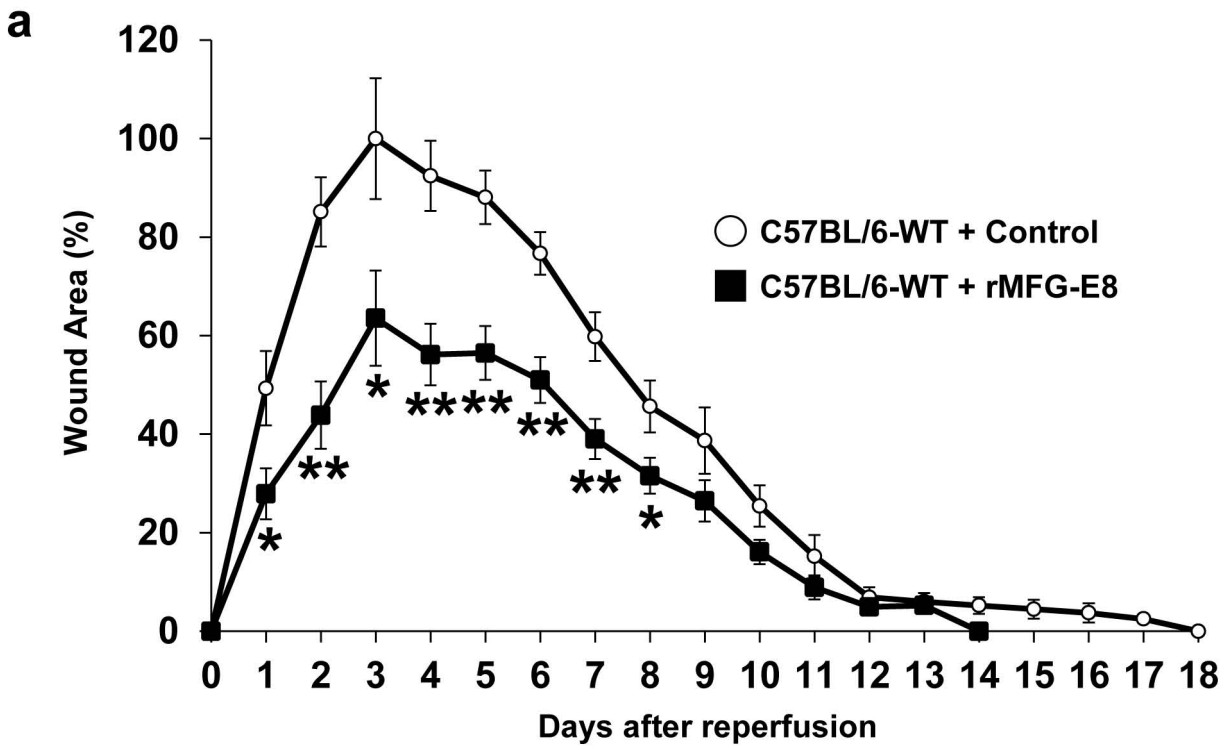
(**a**) The amount of $CD31^+$ EC and $NG2^+$ pericytes in cutaneous I/R area at 6 days after reperfusion. (**b**) The amount of αSMA^+ myofibroblast or pericytes in cutaneous I/R area at 6 days after reperfusion. Quantification of the $CD31^+$, $NG2^+$ and αSMA^+ areas in 6 random microscopic fields in n=3 mice per groups was performed using Image J software. Positive area in control mice was assigned a value of 1. $^{**}P<0.01$, $^{*}P<0.05$.

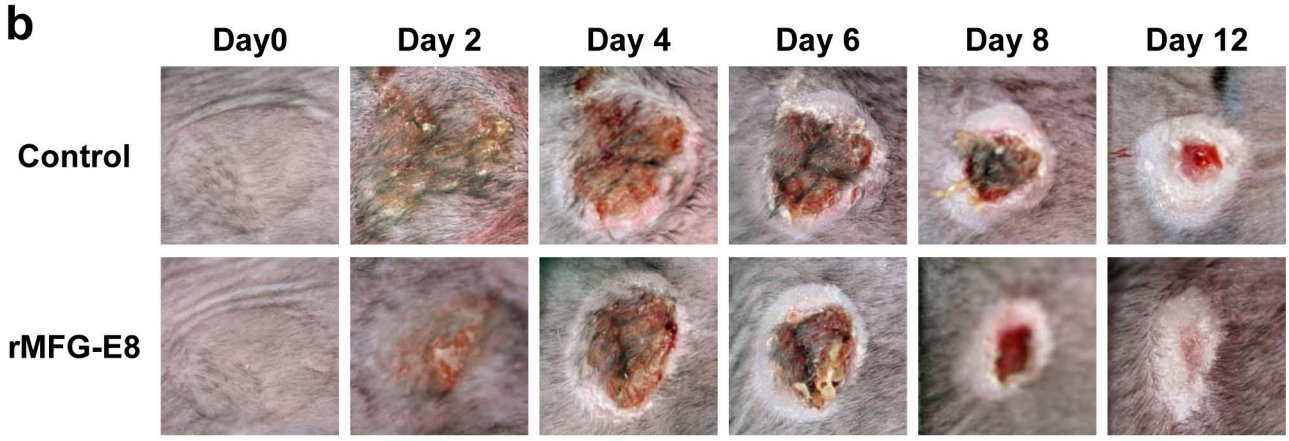


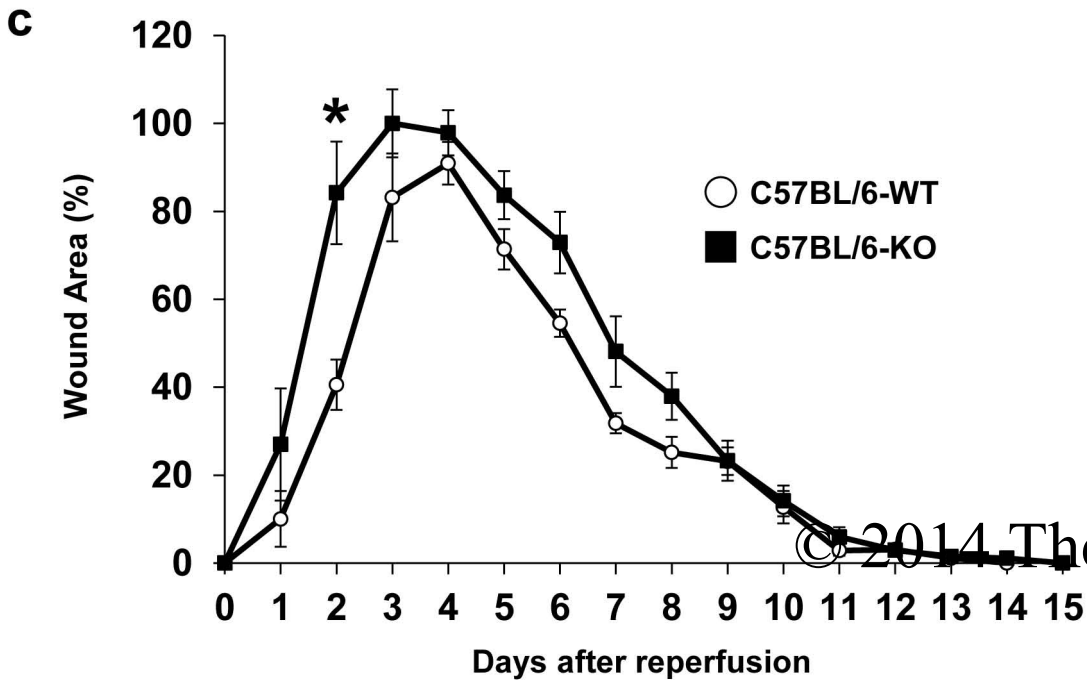


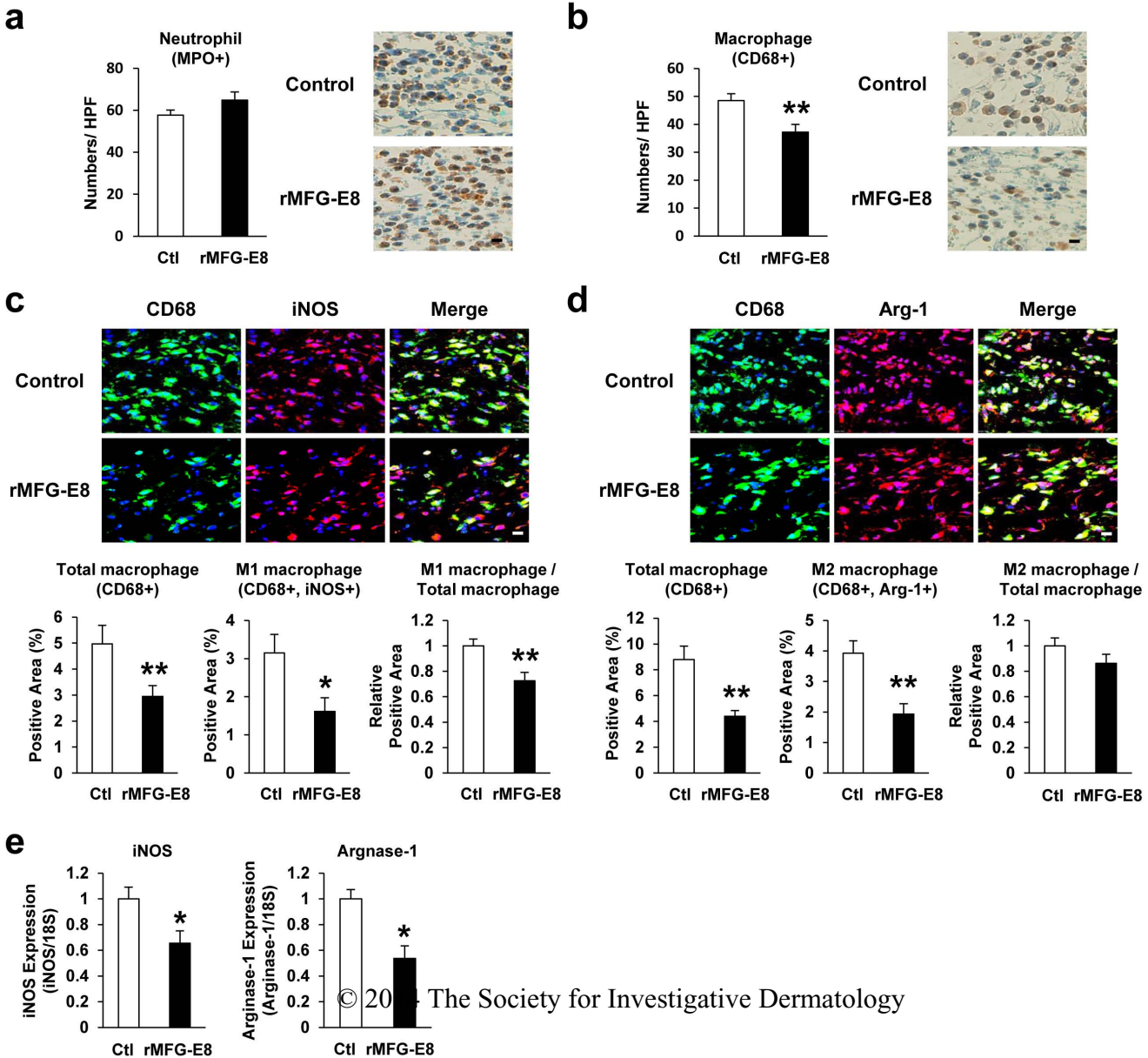


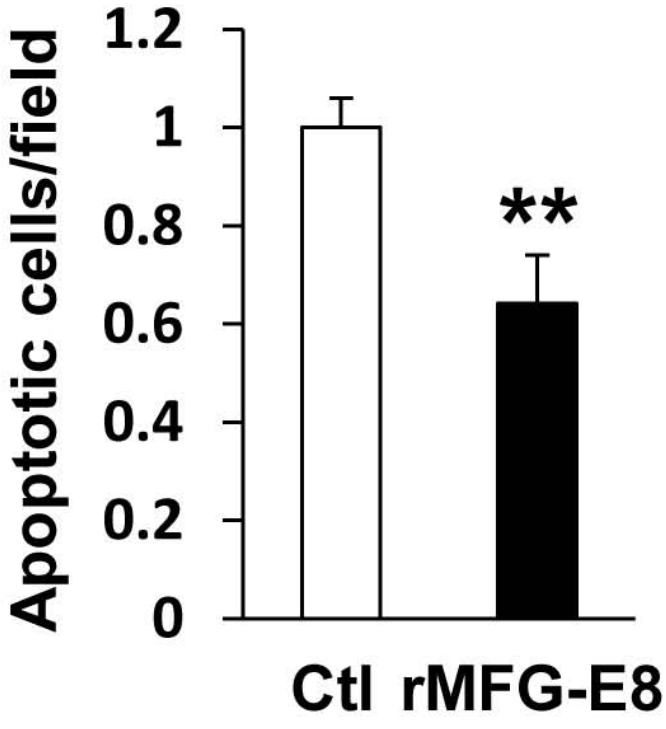








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0.6
0.4
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0
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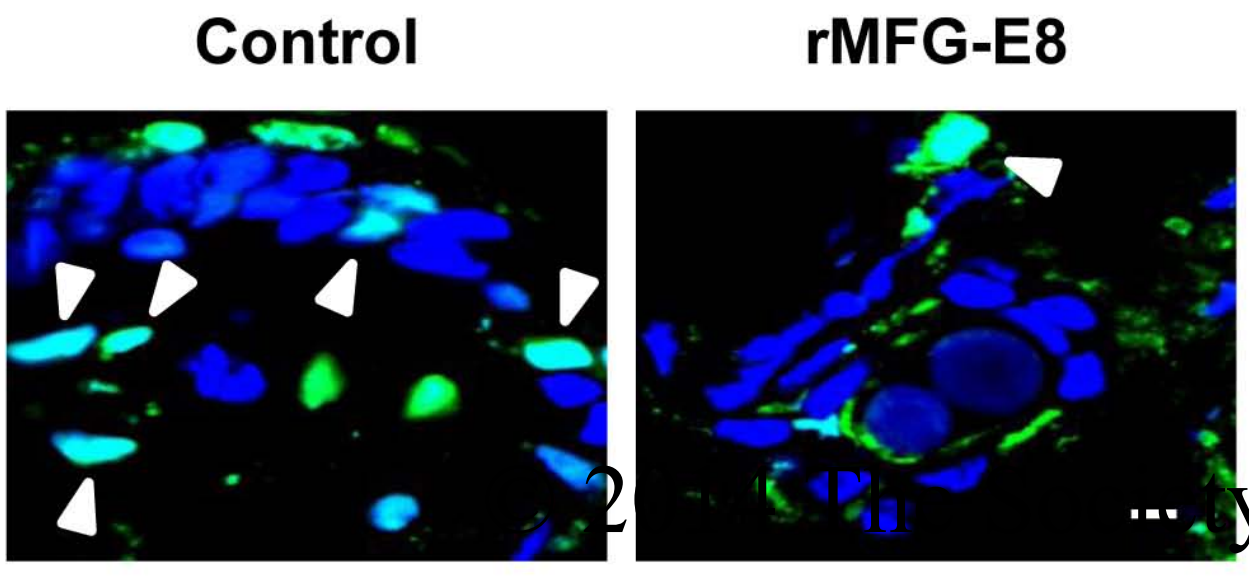
Ctl
rMFG-E8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