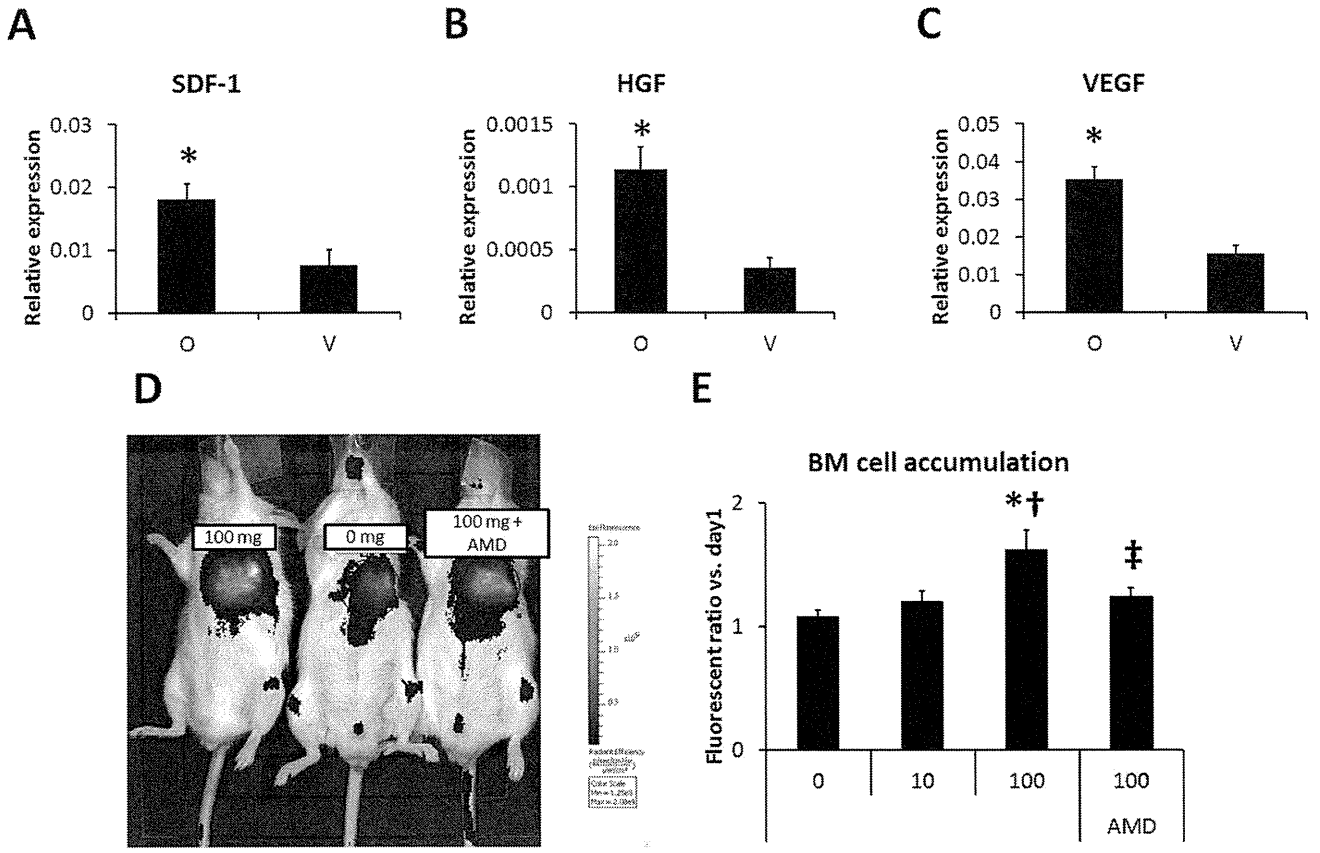

Figure 2. ONO-1301 enhanced SDF-1 secretion and BMC migration via SDF-1/CXCR4 signaling after MI. A–C) The SDF-1, HGF, and VEGF expression at the border zone of the infarcted area was measured by quantitative RT-PCR. The expression levels of these cytokines were higher in the ONO-1301-treated (O) group compared to the vehicle (V) group. (O group, n = 7; V group, n = 7–8; *$P<0.05$ vs. V group). The expression relative to GAPDH is shown. D) BMC migration to ONO-1301-treated infarcted myocardium was evaluated using IVIS. Representative picture of IVIS at day 3. Left: 100 mg/Kg, Center: 0 mg/Kg, Right: 100 mg/Kg+AMD3100 (AMD). E) The number of accumulated BMCs was greater in the 100 mg/kg ONO-1301-treated infarcted heart compared to the 0 and 10 mg/kg ONO-1301-treated infarcted heart. When BMCs treated with AMD were injected, the BMC accumulation decreased in the 100 mg/Kg ONO-1301-treated infarcted heart compared with the untreated-BMC-injected heart (0 mg/Kg, n = 4; 10 mg/Kg, n = 8; 100 mg/Kg, n = 5; 100 mg/Kg+AMD3100, n = 4; *$P<0.05$ vs. 0 mg/Kg, †$P<0.05$ vs. 10 mg/Kg, ‡$P<0.05$ vs. 100 mg/Kg).
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of GFP with either of these markers was observed (figure S3 in File S1).

Therapeutic Effects of ONO-1301 Administration on Cardiac Performance, Survival, and LV-remodeling at 4 Weeks Post-MI

ONO-1301 was detected in the plasma of blood samples from the ONO-1301-treated group 3 weeks after treatment (figure S4 in File S1). The cardiac functions in the MI mice with and without following ONO-1301 treatment were evaluated. Mortality was substantial until 14 days post-LAD ligation in the vehicle group, and similar mortality levels were observed with non-treated MI mice [11]. In contrast, in the ONO-1301-treated group, there was little mortality 7 days after MI, and thus a difference in survival (Fig. 4A). Cardiac performance was evaluated by 2D echocardiography 4 weeks after implantation. The LVEDA was smaller in the ONO-1301-treated group than in the vehicle group, but the difference was not significant. In contrast, the LVESA was significantly smaller, and the LVFAC was significantly greater, in the ONO-1301-treated group than in the vehicle group (Fig. 4B). In the histological analysis, the vehicle group showed a typical MI with a large anterior LV scar and dilatation of the LV cavity. By comparison, the LV of the ONO-1301-treated group was less dilated, and the anterior wall was thicker (Fig. 4C, D). The infarcted area and percent fibrosis were significantly smaller in the ONO-1301-treated than in the vehicle-treated group (Fig. 4C, E–G).

Discussion

Here, we showed that ONO-1301 promotes BMC accumulation in the injured myocardium. *In vitro*, ONO-1301 enhanced SDF-1 expression, and BMC migration was greater to conditioned medium obtained from ONO-1301-stimulated cells. The enhanced migration was diminished by blocking SDF-1/CXCR4 signaling. Consistent with the *in vitro* experiments, ONO-1301 enhanced the SDF-1 expression of myocardial tissue. High ONO-1301 accelerated the BMC accumulation after MI in a SDF-1/CXCR4-dependent manner. Some BMCs in the infarcted myocardium differentiated into capillary structures within 7 days. Furthermore, the sustained-release delivery of ONO-1301 in the infarcted myocardium also led to functional improvements following MI. Our data suggest that ONO-1301 is a novel inducer of BMC recruitment, and that ONO-1301 treatment may be a promising therapeutic strategy for the clinical treatment of MI.

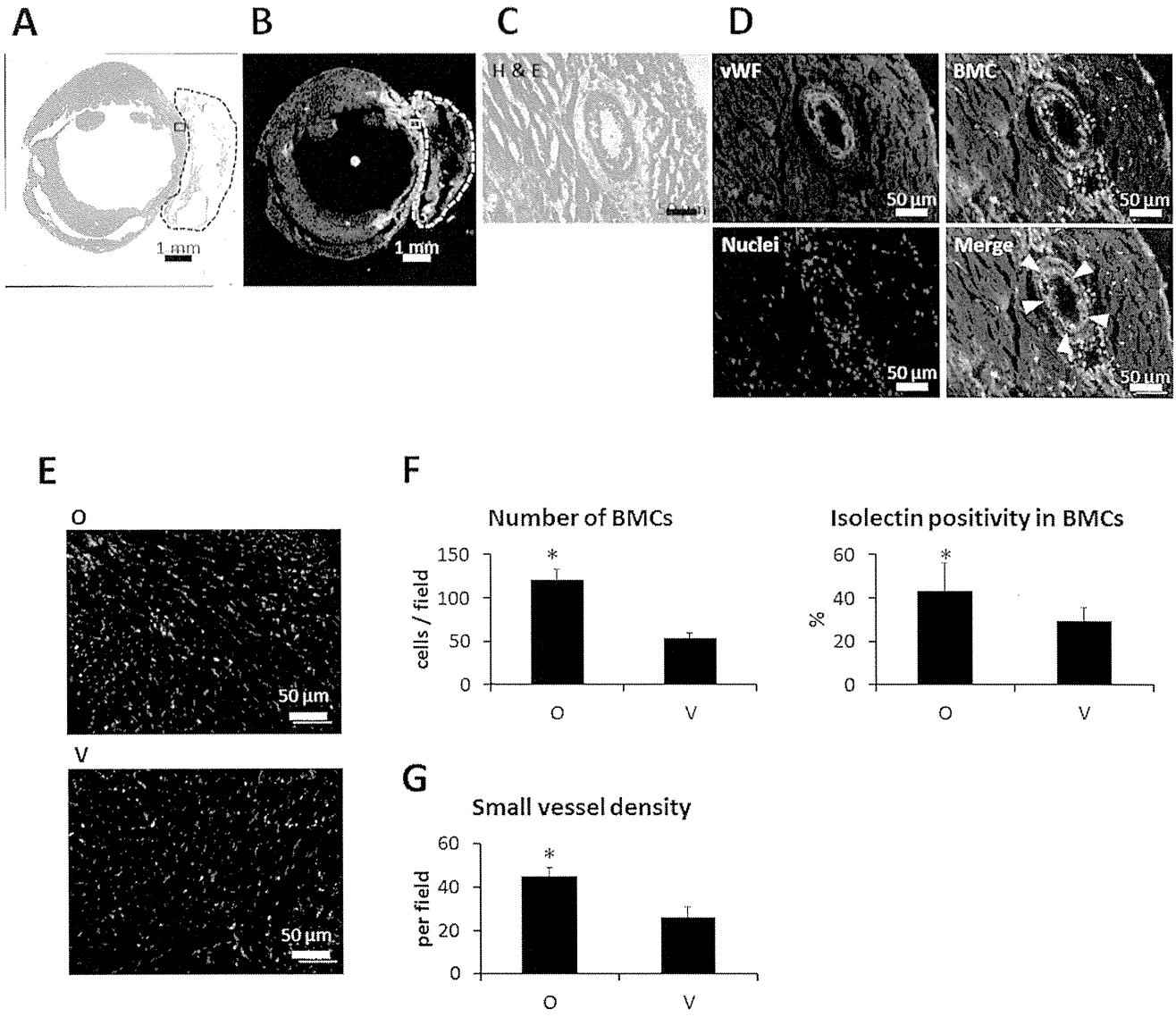

Figure 3. BMCs differentiated into capillary structures in the infarcted area after MI and ONO-1301 treatment. Representative macro image of H and E staining seven days after MI and ONO-1301 treatment. The transplanted sheet is enclosed by a dashed line. A) Serial section of A. The BMCs displayed GFP. B) High-magnification image of the boxed region in A. C) Serial section of C. Arrowheads indicate vWF-expressing BMCs. Red indicates vWF; green, BMCs; and blue, nuclei. D) Representative images of isolectin-stained BMCs seven days after MI and ONO-1301 treatment. E) BMC accumulation and percentages of isolectin-positive BMCs. The number of BMCs that accumulated in the infarcted myocardium was greater in the ONO-1301-treated (O) group than in the vehicle (V) group. The percentage of isolectin-positive BMCs was also greater in the O group than in the V group. *$P<0.05$ vs. V group. F) Small vessel density. Small vessels were detected by CD31 immunostaining. The density of small vessels in the O group was greater than in the V group. *$P<0.05$ vs. V group.
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It is difficult to understand the whole mechanism underlying the functional improvements induced by ONO-1301. It was already reported that ONO-1301 enhances the expression of angiogenic factors HGF and VEGF, leading to angiogenesis and the suppression of fibrosis progression [7,8,9]. In this study, we discovered an alternative mechanism for ONO-1301's therapeutic efficacy in the acute MI mouse, in which the upregulation of SDF-1 promotes BMC accumulation. Stem-cell recruitment and homing are regulated by the interplay of cytokines, chemokines, and proteases. In particular, the SDF-1/CXCR4 axis is central for the mobilization of stem cells from the bone marrow and their homing to ischemic tissues [12]. In the case of ischemic insult, SDF-1 is released by the injured tissue and stimulates the mobilization of progenitor cells from the bone marrow [1,13]. Furthermore, prostaglandins have been reported to facilitate BMC mobilization via upregulation of CXCR4 expression [14,15]. In our experimental setting, ONO-1301 was detected from peripheral blood samples 3 weeks after treatment (Fig. S4 in File S1), suggesting that ONO-1301 may similarly act on the bone marrow to promote the BMC mobilization. Thus, BMC recruitment in the injured myocardium may be enhanced by the upregulation of SDF-1 in cardiac fibroblasts and by the direct upregulation of CXCR4 in BMCs located in the bone marrow. In addition, recent reports show the possibility of endogenous regeneration in the injured heart, including proliferation of postnatal cardiomyocytes and cardiac stem cells [16,17,18,19]. While we were unable to

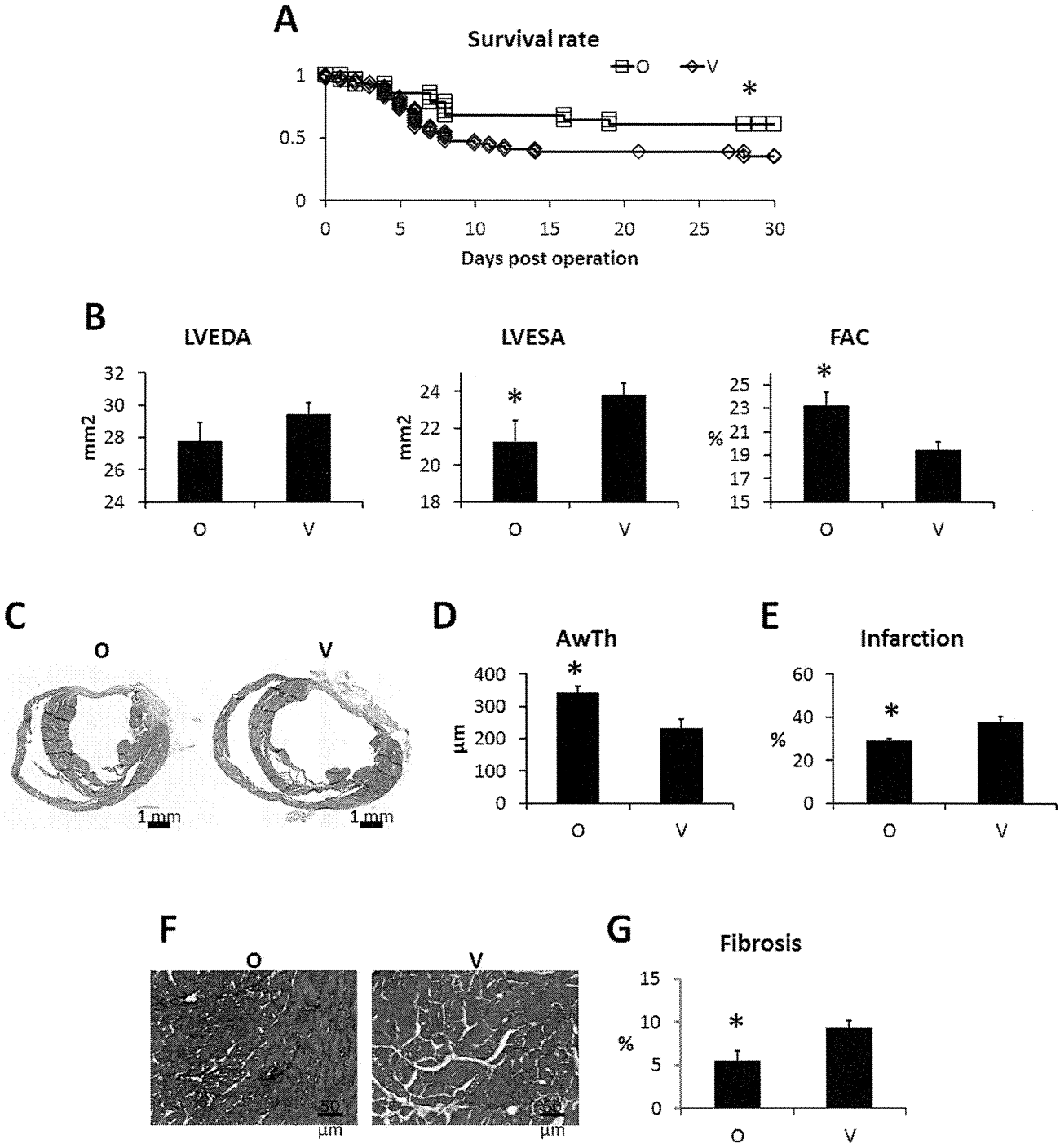

Figure 4. ONO-1301 treatment improved the cardiac performance and survival rate after MI. Survival rates after treatment. The ONO-1301-treated (O) group (n = 33) showed significantly better survival than the vehicle (V) group (n = 48). *$P<0.05$ vs. V group. A) Evaluation of cardiac performance 4 weeks after treatment. In the O group, the LVESA was smaller, and the FAC was significantly higher compared to the V group (O group, n = 22; V group, n = 20; *$P<0.05$ vs. V group). B) Representative macro images from each group. C) Quantification of anterior wall thickness. Anterior wall thickness was significantly thicker in the O group (n = 6) compared to the V group (n = 4). *$P<0.05$ vs. V group. D) Quantification of percent infarction. Infarction was significantly smaller in the O group (n = 6) compared to the V group (n = 4). *$P<0.05$ vs. V group. E) Representative Masson trichrome staining images at the border zone. F) Quantification of fibrosis. Fibrosis at the border zone was significantly smaller in the O group (n = 6) compared to the V group (n = 4). *$P<0.05$ vs. V group.
doi:10.1371/journal.pone.0069302.g004

detect newly-generated cardiomyocytes derived from BMCs in this study, it would be interesting to evaluate the possibility of cardiomyogenesis involving other cell types.

We observed massive BMC accumulation 7 days after MI, including in the infarcted ventricular wall, where they provided structural support in place of the necrotic cardiomyocytes. The

BMCs recruited into the infarcted myocardium may contain various kinds of somatic stem cells, such as endothelial progenitor cells [20], bone marrow-derived stem cells [21], and bone marrow mononuclear cells [2], which have potent therapeutic effects in heart failure [22]. Furthermore, bone marrow-derived mesenchymal stem cells secrete prostaglandin [23], which may act like ONO-1301 and amplify the effects of the ONO-1301-mediated therapy. Kawabe et al. clearly showed that prostaglandin facilitates the recruitment of endothelial progenitor cells [24]. Although further analysis is needed, the enhanced accumulation of BMCs may predispose the damaged heart tissue to better restoration following MI.

Many reports have shown that granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) also induce BMC mobilization, with therapeutic effects in animal models [25]. However, G-CSF therapy in unselected patients with acute MI did not lead to functional improvements beyond those achieved with conventional therapy. In addition, the administration of GM-CSF in cancer patients has been shown to transiently increase the LV end-systolic dimensions and decrease cardiac contractility [25,26]. The lack of efficacy of G-CSF therapy in clinical trials may be due, at least in part, to its poor initiation and duration; such therapies are likely to be most beneficial during the early phase after acute MI. Although conventional prostacyclin and its analogs are chemically and biologically unstable, ONO-1301 is a long-acting prostacyclin agonist that exerts stable effects *in vivo*, because it lacks a prostanoid structure. Furthermore, we used a slow-release form of ONO-1301, made by polymerizing it with poly-lactic and glycolic acid; this ONO-1301 could still be detected in the blood 3 weeks after its administration (figure S4 in File S1).

Furthermore, in our *in vitro* analysis, although we used normal human dermal fibroblasts to examine the SDF-1/CXCR-4-dependent BMC migration, the reactivity to ONO-1301 stimulation will differ depending on the cell type. For example, the G-CSF expression was upregulated in some kinds of cells (unpublished data). Thus, together with the upregulation of multiple beneficial cytokines such as HGF and VEGF, because of the longer duration of its activity, ONO-1301 may be more potent than conventional protein-based therapies.

Our data showed that ONO-1301 treatment was a potent inducer of BMC homing. Of the BMCs that accumulated in the infarcted myocardium, 43 percent expressed isolectin, an endothelial cell marker, but the other BMCs had a fibroblastic morphology, and did not express cardiac-lineage or cardiofibroblast markers (Figure S3 in File S1). ONO-1301 administration resulted in the attenuation of cardiac dysfunction, with enhanced BMC accumulation. Further study is required to elucidate the mechanism, but we speculate that paracrine effects of factors released by the BMCs play pivotal roles in the therapeutic efficacy, rather than the transdifferentiation of the BMCs into the cardiac or vascular lineage. The effect of cardioprotective and angiogenic factors secreted by the accumulated BMCs and the direct stimulation of ONO-1301 itself may synergistically increase the angiogenesis and cardioprotection, leading to improved therapeutic results.

In summary, ONO-1301 may be a powerful, long-acting activator of multiple cytokines. In particular, SDF-1 may enhance the BMC accumulation in a SDF-1/CXCR4-signaling-dependent manner, leading to an attenuation of the cardiac dysfunction following MI. Our findings suggest that the method involving a sustained release of ONO-1301 may be adapted as a novel drug delivery system for treating heart failure.

Supporting Information

File S1.
(DOCX)

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Author Contributions

Conceived and designed the experiments: YI SM Y. Sawa. Performed the experiments: YI KI NS. Analyzed the data: YI AS. Contributed reagents/materials/analysis tools: Y. Sakai. Wrote the paper: YI SM SF Y. Sawa.

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