Blockade of EMAP II Protects Cardiac Function after Chronic Myocardial Infarction by Inducing Angiogenesis

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Abstract

Promoting angiogenesis is a key therapeutic target for protection from chronic ischemic cardiac injury. Endothelial-Monocyte-Activating-Polypeptide-II (EMAP II) protein, a tumor-derived cytokine having anti-angiogenic properties in cancer, is markedly elevated following myocardial ischemia. We examined whether neutralization of EMAP II induces angiogenesis and has beneficial effects on myocardial function and structure after chronic myocardial infarction (MI). EMAP II antibody (EMAP II AB), vehicle, or non-specific IgG (IgG) was injected ip at 30 min and 3, 6, and 9 days after permanent coronary artery occlusion in mice. EMAP II AB, compared with vehicle or non-specific antibody, significantly, $p<0.05$, improved the survival rate after MI, reduced scar size and attenuated the development of heart failure, i.e., left ventricular ejection fraction was significantly higher in EMAP II AB group, fibrosis was reduced by 24%, and importantly, more myocytes were alive in EMAP II AB group in the infarct area. In support of an angiogenic mechanism, capillary density (193/HPF vs. 172/HPF), doubling of the number of proliferating endothelial cells, and angiogenesis related biomarkers were upregulated in mice receiving EMAP II AB treatment as compared to IgG. Furthermore, EMAP II AB prevented EMAP II protein inhibition of *in vitro* tube formation in HUVECs. We conclude that blockade of EMAP II induces angiogenesis and improves cardiac function following chronic MI, resulting in reduced myocardial fibrosis and scar formation and increased capillary density and preserved viable myocytes in the infarct area.

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Keywords

EMAP II inhibition; myocardial infarction; cardioprotection; angiogenesis

1. Introduction

Myocardial infarction (MI), caused by coronary artery disease, is the leading cause of morbidity and mortality in the United States. After coronary artery occlusion, the recovery of myocardial function is largely dependent on the heart's ability to re-vascularize the peri-infarcted region. Growth factors such as vascular endothelial growth factor (VEGF) were found to be important in promoting angiogenesis within the peri-infarcted region[1–6]. Consequently, angiogenesis has been studied as a promising therapeutic target in ischemic cardiovascular disease for some time, but despite the huge amount of data supporting this mechanism in experimental settings, little has moved forward to clinical therapy, making the discovery of novel pro-angiogenic mechanisms critical.

Accordingly, we studied a novel candidate, an EMAP II antibody, which blocks the Endothelial-Monocyte-Activating-Polypeptide-II (EMAP II) mediated pathway. EMAP II is a tumor-derived pro-inflammatory cytokine with anti-angiogenic properties[7–13] and inhibits VEGF-induced pro-angiogenic signaling in human umbilical vein endothelial cells (HUVEC)[14], making it an important target for retarding tumor progression [15–18]. In the heart, EMAP II protein expression was increased 24hrs after MI and remained elevated for 6 weeks after MI [19]. If this intrinsic mechanism could be neutralized, it might provide a novel angiogenic mechanism, and protect ischemic myocardium. Indeed, after permanent CAO in hearts without preformed collaterals, angiogenesis is essentially the only mechanism to protect the ischemic myocardium. Therefore the goal of the present investigation was to determine in a mouse MI model, induced by permanent coronary artery occlusion (CAO), whether blocking the EMAPII pathway with an EMAP II neutralizing antibody induces angiogenesis and protects cardiac function after permanent CAO.

2. Materials and Methods

2.1 Surgical animal model of myocardial infarction

We performed left anterior descending (LAD) coronary artery occlusion (CAO) in 3–6 month-old male C57BL/6 mice for myocardial infarction (MI). Briefly, mice were anesthetized with pentobarbital sodium (60 mg/kg, ip). The left side of the chest was opened, and the LAD artery was permanently ligated with 8-0 prolene suture through the third intercostal space incision under proper ventilation. Regional ischemia was confirmed by visual inspection (color change) and ECG monitoring (ST elevation). Sham-operated control mice underwent the same procedure without CAO. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Eighth Edition 2011). These studies were approved by the Institutional Animal Care and Use Committee in the New Jersey Medical School.

2.2 EMAP II Antibody (AB) treatment

We used a rabbit anti-EMAP II (rEMAP II) antibody (polyclonal monospecific antibody based on immunoblotting of plasma and cell extracts; anti-EMAP II IgG blocked the activity of rEMAP II in cell culture assays) [19]. EMAP II antibody was produced as previously described [20]. In brief, a peptide sequence of 13 amino acid residues located within a homologous region of the human and murine forms of mature EMAP II were used to generate an antibody. This peptide was synthesized and the antibody produced by Zymed Laboratories, Inc. The antibody is specific to EMAP II identified by producing a single band on a western blot that is blocked after being incubated with excess EMAP II. In addition, the antibody used in these publications and in these studies is from a single rabbit and was protein A purified and sterile filtered. EMAP II dosing and injection interval of EMAP II antibody treatment is based on ^{125}I EMAP II antibody labeled clearance studies as previously described [20]. Functionality of the antibody from a single rabbit was validated in vitro in a lung co-culture system markedly increased endothelial cell growth and tube formation [21] and in vivo [20]. Mice were selected randomly for treatment with either vehicle (rabbit IgG) or EMAP II AB (2mg/kg) via intraperitoneal injection by an investigator who was not involved in the surgery. EMAP II AB was injected at 30 min, day 3, day 6, and day 9 after CAO surgery.

2.3 Measurement of cardiac function by echocardiography

To measure LV function, two-dimensional transthoracic echocardiography was performed in mice using ultrasonography (Accuson 256, Siemens Medical Solutions) with a 13-MHz linear ultrasound transducer under anesthesia with 1.25 % Tribromoethanol (Avertin, 290 mg/kg, ip) Measurement of LV end-diastolic and end systolic diameters and wall thickness and LV ejection fraction and fractional shortening were taken from the myocardium proximal to the infarct. Following echocardiography, the heart tissue was harvested after the mice were euthanized using CO_2 inhalation.

2.4 Measurement of capillary density and proliferating endothelial cells (ECs)

Four weeks after CAO, the heart was immediately excised, fixed with 10% neutralized buffered formalin and embedded in paraffin for histological studies. 5μm thickness of serial heart tissue sections were used for histological assessment and immunohistochemical staining. In order to discriminate proliferation in non-myocytes and myocytes, tissue sections were counterstained with wheat germ agglutinin (WGA) Alexa Fluor-488 conjugate (Invitrogen) or troponin I, a myocyte marker, as described in our previous study [22].

For measurement of capillary density, ECs were stained with isolectin-IB$_4$ from *Griffonia simplicifolia* (InVitrogen), and quantitative analysis was accomplished using Image-Pro Plus software after taking pictures under 40×magnification with an Olympus microscope. Relative capillary density was calculated as capillary numbers/HPF (high power field).

Tissue sections were double-immunostained with rabbit-anti Ki67 antibody (Neomaker) or anti-phosphohistone H3 (PHH3) (Cell Signaling) as a proliferation marker with isolectin-IB$_4$ as an EC marker. Antigen retrieval was performed in 10mM citrate buffer (pH 6.0) with boiling under pressure for 10 min. After the blocking process with Protein Block Serum-

Free solution (DAKO), the tissue sections were incubated with Ki67 or PHH3 for 1hr at 37°C followed by detection with goat anti-rabbit Alexa 594 conjugated (Invitrogen). The myocardium was further incubated with isolectin-IB$_4$ Alexa Fluor-488 conjugated antibody (Invitrogen) overnight at 4°C. Sections were mounted using ProLong Gold antifade reagent with DAPI (Invitrogen). The proliferation of ECs were expressed as the number of Ki67+Isolectin+ cells per mm^2 or PHH3+isolectin+ cells per mm^2. Vessels in the infarct area were immunostained with α-smooth muscle actin (α-SMA) (Abcam). Only vessels with ring structure were counted to distinguish arterial vessels from myofibroblasts, which also stain with α-SMA.

2.5 In vitro EC tube formation assay

Human microvascular endothelial cells from the heart (HMVEC-Cs) were purchased from Lonza (Basel, Switzerland). Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC (Manassas, VA, USA). Matrigel (reduced growth factor, BD Bioscience) was placed in 12-well tissue culture plates allowed to gel at 37°C for 30 min. Cells were cultured on the Matrigel with either vehicle (rabbit nonspecific IgG 1μM), EMAP II protein (1μM), or EMAP II AB (1μM) treatment for 20hrs. For an hypoxic condition, cells were incubated in a hypoxia chamber for 5hrs.

2.6 Measurement of interstitial fibrosis

To examine the collagen deposition after MI, tissue sections were stained with Picric acid Sirius Red (PSR) after 1 and 4 weeks with vehicle or EMAP II AB treatment. Quantitative analysis of interstitial fibrosis was accomplished using Image-Pro Plus software after taking pictures under an Olympus microscope at 20× magnification.

2.7 Measurement of scar size after chronic MI

For measuring scar size after 4weeks MI, LV was cross-sectioned at two levels below the coronary artery ligation position, and paraffin-embedded. Each level of tissue was divided by five sub-levels, and a 5μm serial cut was performed. Tissue slides were stained with PSR and scar size was measured with ImageJ software. For measuring viable myocytes in the infarcted area after 4wks MI, LV was stained with troponin I, with isolectin-IB$_4$ and DAPI after longitudinal-sectioning of the heart with a four chamber-view. The number of viable myocytes in the infarct area was determined by counting troponin I + myocytes in the infarct area.

2.8 Myocyte size and number

To measure myocyte cross-sectional area, tissue sections were co-stained with WGA and DAPI, and quantitated using ImagePro-Plus software. The total number of myocytes of each group was measured by the method of Kajstura et al.[23].

2.9 Quantitative RT-PCR

Specific primers and probes (derived with FAM and TAMRA, ordered from IDT DNA Company) were designed for the transcripts of interest. The optimal combination of primers and probes for a qPCR assay was determined with the Primer Express software (Applied

Biosystems). Following reverse transcription of the mRNA of interest from 50ng of total RNA, the cDNA was used for quantitative PCR (qPCR) (40 cycles of a 10-s step at 95°C and a 1-min step at 60°C) using the SybrGreen method on a 7700 ABI-Prizm Sequence Detector (Applied Biosystems, Foster City, CA). Values are reported per 18s rRNA transcript to correct for sample-to-sample RNA loading variations.

Genes and primer sequences used in this study:

Gene	Forward (5′ 3′) →	Reverse (5′ 3′) →
Rn18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGC
Ephb2	GCCGTGGAAGAAACCCTGAT	GTTCATGTTCTCGTCGTAGCC
Ephb4	GGAAACGGCGGATCTGAAATG	TGGACGCTTCATGTCGCAC
Cdh1	CAGTTCCGAGGTCTACACCTT	CCAGGACATGGCCTCTCTC
Icam1	CGCTGTGCTTTGAGAACTGTG	ATACACGGTGATGGTAGCGGA
Vcam1	TTGGGAGCCTCAACGGTACT	GCAATCGTTTTGTATTCAGGGGA
Itga5	TGCAGTGGTTCGGAGCAAC	TTTTCTGTGCGCCAGCTATAC
Vegfa	CTGCCGTCCGATTGAGACC	CCCCTCCTTGTACCACTGTC
Pecam1	ACGCTGGTGCTCTATGCAAG	GTGAAGGAGTTTTCCTCAGCC
Kdr	TTTGGCAAATACAACCCTTCAGA	GCTCCAGTATCATTTCCAACCA

2.10 Western blot

Western blot was performed to determine the time course of EMAP II expression after MI as previously described [1]. Briefly, tissue was obtained from the same region in the heart in every animal, rinsed in 50mM Tris pH 7.4, 0.9 N NaCl, and 0.01% NaN₃, homogenized in the presence of proteinase inhibitors. The homogenates were cleared by centrifugation at 14,000 *g* for 20 min; the protein concentration was determined by Bradford analysis (Bio-Rad, CA), and the samples were normalized by protein content. Equal amounts of protein were electrophoresed on a 12% SDS–PAGE gel, transferred to a nitrocellulose membrane. After blocking with 5% milk for 1 hour, the membrane was probed with a rabbit anti-EMAP II antibody (1:200) at 4°C for 1 hour, rinsed with TBST X3, then probed with HRP conjugated anti-rabbit secondary antibody (1:10000, Bio-Rad, CA). Specific binding was detected using a chemiluminescence substrate (Pierce, Rockford, IL) and blue basic autorad film (BioExpress, Belgium). Quantitative analysis was accomplished using a GS-800 calibrated densitometer (Bio-Rad, CA).

2.11 Statistical analysis

All data are expressed as mean ±SE. Comparisons between vehicle and the EMAP II AB treated groups were performed by the Student's *t*-test. For statistical analysis of data from multiple groups, we used ANOVA followed by the Student-Newman-Keuls or Bonferroni post hoc analysis. For data that did not meet the ANOVA assumptions, the Kruskal-Wallis test was applied using GraphPad-Prism 5.0 (GraphPad-Software, San Diego, CA). The data for uncensored survival time was analyzed using the Wilcoxon and the log-rank test. $p < 0.05$ was taken as a minimal level of significance.

3. Results

3.1 EMAP II protein was increased in the heart after MI

Previous studies in the rat heart have shown that EMAP II protein expression was increased 24hrs after MI and remained elevated for 6 weeks post MI[10]. To confirm that EMAP II protein expression was also induced by MI in the mouse heart, we did a time course study of EMAP II expression after MI. As shown in Figure 1C, EMAP II protein expression was increased, $p < 0.05$, by 1.9 fold 24 hours after MI in the mouse heart, and remained elevated, $p < 0.05$, at 1 week (1.3 fold), 2 weeks (1.5 fold) and 4 weeks (1.2 fold).

3.2 EMAP II AB significantly improved the survival rate after MI

To test whether EMAP II AB increased survival of mice after chronic myocardial infarction (MI), permanent CAO was induced in 3–6 month-old male C57BL/6 mice, either treated with vehicle (rabbit IgG) or EMAP II AB (2 mg/kg) via intraperitoneal injection. EMAP II AB was injected at 30 min, day 3, day 6 and day 9 after CAO. As shown in Figure 1A, the Kaplan-Meyer survival study indicated that the survival rate in mice treated with EMAP II AB was significantly improved after MI.

3.3 EMAP II AB protected cardiac function after MI

Blockade of EMAP II improved LV systolic function after chronic MI induced by permanent CAO: LV ejection fraction and fractional shortening were not different in sham animals treated with vehicle or EMAPII AB. LV ejection fraction was reduced significantly more ($p < 0.05$) in the vehicle group at 1 week ($53 \pm 2.2\%$) and 4 weeks ($35 \pm 2.4\%$) after MI than in the EMAP II AB group ($61 \pm 1.2\%$ at 1 week and $53 \pm 4.4\%$ at 4 weeks, respectively) from baseline values in sham operated mice ($72 \pm 0.5\%$, n=4) (Figure 1B and Table 1). Fractional shortening was also significantly improved in the EMAP II AB group compared to the vehicle group, by levels similar to those for LV ejection fraction after 4 weeks MI (Table 1). Heart weight/tibial length (HW/TL) was not different in sham animals treated with vehicle or EMAPII AB. At 4 wks after MI, HW/TL was slightly, but not significantly greater in the EMAPII treated mice (8.3 ± 0.4) compared with those receiving vehicle (8.0 ± 0.2), potentially due to the increased numbers of myocytes in the MI zone. Lung weight/TL was not different in either sham or after 4 wks MI in the two groups. It is important to recognize that the beneficial effects of EMAP II AB on LV function after chronic MI would have been even more dramatic, if all animals had survived, since those that died (more in the vehicle group) most likely had worse LV function.

3.4 EMAP II AB reduced fibrosis and scar size and preserved myocytes after MI

We then explored the underlying histological changes after EMAP II AB treatment. The scar size was decreased, $p < 0.05$, after 4wks MI with the EMAP II AB ($20.8 \pm 6.2\%$, n=6) compared with the vehicle group ($32 \pm 7.9\%$, n=6) as shown by PSR staining (Figure 2A). Fibrosis adjacent to the infarct was also reduced in the EMAP II AB group, compared with the vehicle group at both 1 and 4 weeks after MI (Figure 2B). The number of viable myocytes in the infarct area was determined by counting troponin I+ myocytes in the infarct area. More preserved myocytes were seen in the EMAP II AB group in the infarct area,

shown with H&E staining, after 4wk MI (Figure 3A). We used double immunostaining of Ki67 as a proliferation marker, and WGA as a myocyte marker, and DAPI to identify the nucleus, to study whether blockade of EMAP II affects the number of proliferating myocytes after MI. Proliferating myocytes were increased with EMAP II AB as shown in Figure 3B.

3.5 EMAP II AB increased angiogenesis after chronic MI

We then used double immunostaining of Ki67 or phosphohistone H3 (PHH3) as proliferation markers, and isolectin-IB$_4$ as an endothelial cell (EC) marker to study whether blockade of EMAP II affects angiogenesis after MI. Using immunostaining of CD 34 as an endothelial progenitor cell marker, vWF as a vessel endothelial cell marker, we found that the progenitor endothelial cells were increased after EMAP II AB treatment (Figure 4A). The number of Ki67-positive EC was also increased in the EMAP II AB group compared to vehicle after 4 week MI (Figure 4B). The EMAP II AB group showed increased capillary density in the non-ischemic zone of the heart compared to vehicle (193 ± 5/HPF *vs.* 172 ± 4/ HPF) (n=5), $p<0.05$ (Figure 4C). EMAP II AB treatment also increased the number of blood vessel smooth muscle cells after chronic MI (Figure 4D).

3.6 EMAP II AB promotes angiogenesis *in vitro*

Finally, we used an *in vitro* tube formation model to confirm that EMAP II AB could promote angiogenesis. HMVEC-Cs were cultured on the Matrigel with vehicle or EMAP II AB treatment for 20hrs. Hypoxia, where cells were incubated in a hypoxia chamber for 5 hours, was used to mimic *in vivo* MI. As shown in Figure 5A, EMAP II AB significantly increased tube formation as compared with vehicle. Next, to confirm this and also to more closely mimic the *in vivo* condition, we used EMAP II protein to see whether it could inhibit *in vitro* tube formation, and whether EMAP II AB could reverse the anti-angiogenic effect of EMAP II protein. As shown in Figure 5B, EMAP II protein did inhibit the *in vitro* tube formation, and this phenomenon was reversed by EMAP II AB, indicating that the protective role of EMAP II AB was through its neutralization of the anti-angiogenic effect by EMAP II protein. Most of angiogenesis related biomarkers were also upregulated after EMAP II AB treatment (Figure 6). These results strongly suggest that the beneficial effects of EMAP II AB after chronic CAO are due to enhanced angiogenesis.

4. Discussion

In hearts without preformed collateral vessels, it is almost impossible to protect the ischemic myocardium in the absence of angiogenesis. In this study, we found that EMAP II AB reduced MI scar size and significantly improved the survival rate and cardiac function after chronic MI through an EMAP II angiogenic mechanism. EMAP II is an anti-angiogenic protein that has been identified in the myocardium during fetal development [24], and has been shown to inhibit metastatic tumor growth with its anti-angiogenic properties [15, 17, 18, 20]. Furthermore, EMAP II was shown to be expressed with transcription products localizing predominantly to the perivascular stroma region in the normal rat myocardium and was upregulated 2-fold in the heart 6 hours following MI and remained elevated following MI in the peri-infarct region [19].

The underlying hypothesis of this study was that blocking the anti-angiogenic properties of EMAP II after MI would result in increased angiogenesis, which would in turn protect the heart against the development of heart failure after MI. Indeed, the mice treated with EMAP II antibody exhibited reduced mortality from MI (Figure 1A) and protected the ischemic heart, as reflected by reduced infarct scar and adjacent fibrosis resulting in a significant preservation of cardiac function.

The reduced scar and preservation of cardiac function were most likely due to the angiogenesis that was induced by the EMAP II antibody, as demonstrated by increased capillary density, upregulated angiogenesis related biomarkers and increased vessel tube formation *in vitro, which has also been demonstrated* in developmental models [20, 21]. Furthermore, we have previously reported that inhibition of VEGF signaling is one possible anti-angiogenic mechanism of EMAP II [14]. In that report, we showed that EMAP II protein efficiently and dose dependently binds to VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2). In addition, EMAP II protein inhibited binding of VEGF to VEGFR1 and VEGFR2. In the current study, we did a comprehensive qPCR study of angiogenesis markers and determined that most of these markers, such as VEGFA and VEGFR2 (KDR), were significantly up-regulated after treatment with a neutralizing EMAP II AB treatment, but not nonspecific IgG, as shown in Figure 6. An *in vitro* vessel tube formation study with HMVEC-Cs cultured under hypoxia, showed that EMAP II AB significantly increased tube formation as compared with vehicle (Figure 5A). In addition, we examined *in vitro* vessel formation with HUVEC cells treated with either EMAP II protein, EMAP II AB, EMAP II protein + EMAP II AB, or EMAP II protein + nonspecific IgG. We found that EMAP II protein significantly inhibited vessel formation, and this inhibition could be neutralized and rescued by EMAP II AB, but not by nonspecific IgG, as shown in Figure 5B. This supports the concept that VEGF mediated angiogenesis is a key mechanism of action of EMAP II AB neutralization of EMAP II protein.

As a further result of the angiogenesis, the infarct area after 4 week MI demonstrated significantly more viable myocytes in the EMAP II AB group than in the vehicle group, as assessed by dual staining with troponin I and isolectin-IB$_4$. The numbers of proliferating myocytes were also increased at both 1 and 4 weeks after MI, as assessed by Ki67 [25, 26]. Myocyte regeneration and proliferation are controversial topics [27–32]. On one hand several investigators report that this mechanism is important to repairing the infarcted heart [33–38]. On the other hand others have reported that this mechanism does not occur [39, 40]. One other point of view is that it occurs but just at lower levels [41, 42]. Although it could be argued whether or not myocyte regeneration or proliferation occurred in the present study, it is clear that the numbers of Ki67 positive myocytes did increase in the ischemic zone of the mice treated with EMAP II antibody, and most importantly, the numbers of myoctes within the ischemic zone were significantly greater in those animals with antibody treatment.

In summary, neutralization of the anti-angiogenic cytokine, EMAP II, preserved LV function by promoting angiogenesis with reduction of scar size and fibrosis, and preservation of myocytes in the infarct area after chronic MI. Since EMAP II increases naturally during chronic MI, it could play a role in limiting angiogenesis with chronic

myocardial ischemia and conversely, blocking this adverse effect could be a potential novel therapeutic target for chronic myocardial ischemic disease.

5. Conclusion

Neutralization of EMAP II induces angiogenesis and improves cardiac function following chronic MI, resulting in reduced myocardial fibrosis and scar formation and increased capillary density and most importantly, preserved viable myocytes in the infarct area.

Acknowledgments

Abbreviations

EMAP II	Endothelial Monocyte Activating Polypeptide II
CAO	Coronary Artery Occlusion
MI	Myocardial Infarction
EF (%)	Ejection Fraction (%)
HMVEC-Cs	Human Microvascular Endothelial Cells from the Heart
HUVECs	Human Umbilical Vein Endothelial Cells

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Highlights

1. First *in vivo* study showing that blockade of EMAP II by a neutralizing antibody significantly improved survival rate after chronic myocardial infarction

2. Blockade of EMAP II improves cardiac function following chronic myocardial infarction, resulting in reduced myocardial fibrosis and scar formation.

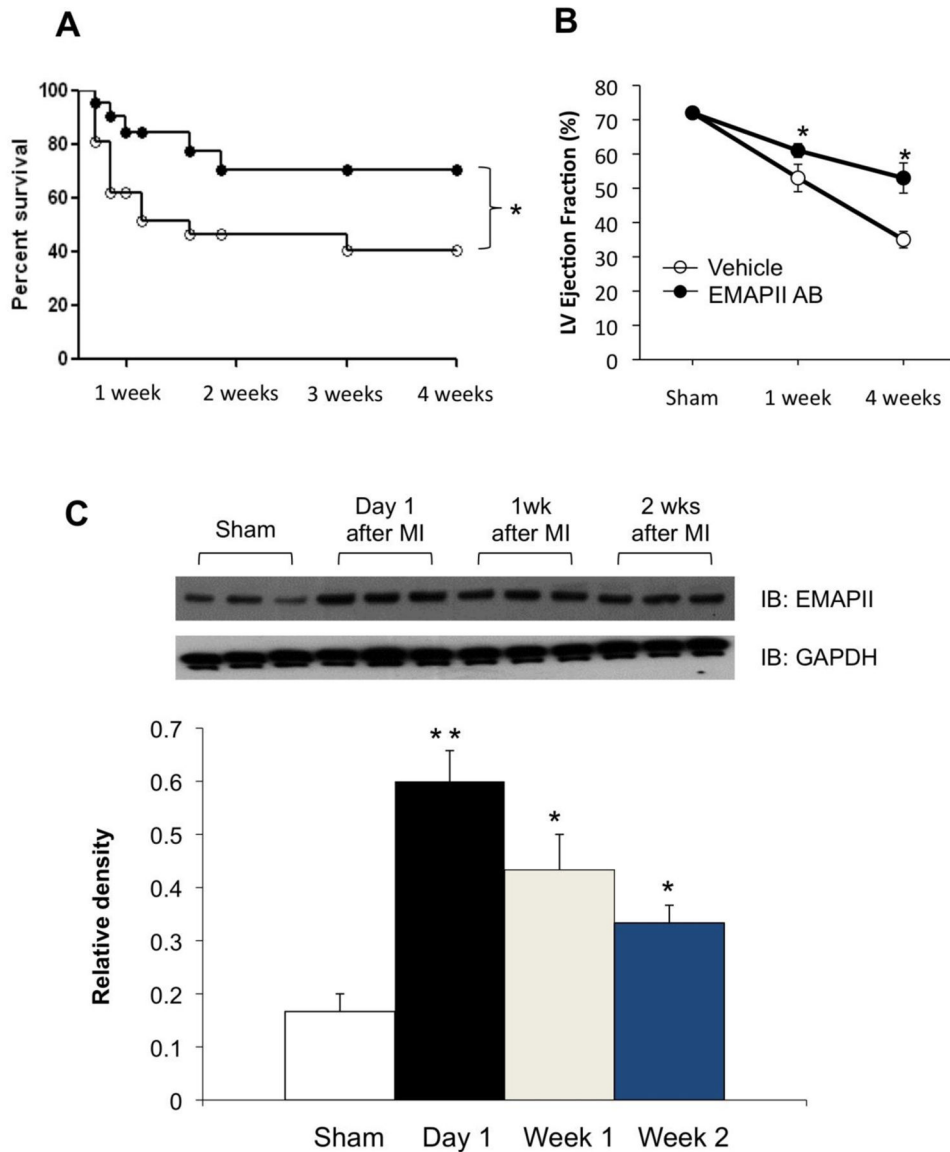3. The underlying EMAP II mechanism involves angiogenesis as demonstrated both *in vitro* and *in vivo*.

Figure 1.
EMAP II AB promotes survival and preserves heart function after chronic myocardial infarction (MI). (*A*) Kaplan-Meyer survival curve of mice treated with either EMAP II AB or vehicle after 4 week chronic MI. *: p<0.05 compared with vehicle, n=30 for EMAP II AB group, n=28 for vehicle group. (*B*) EMAP II AB treated mice have preserved left ventricle ejection fraction after both 1 and 4 weeks chronic MI. *:p<0.05 compared with vehicle, n=10 for EMAP II AB group, n=7 for vehicle group. (*C*) Dynamics of EMAPII expression after chronic MI. EMAP II expression was upregulated one day after MI surgery and continues to be highly expressed at least two weeks after MI as shown with representative western blot (top panel) and quantification (bottom panel). **: p<0.01, *: p<0.05, all compared with Sham.
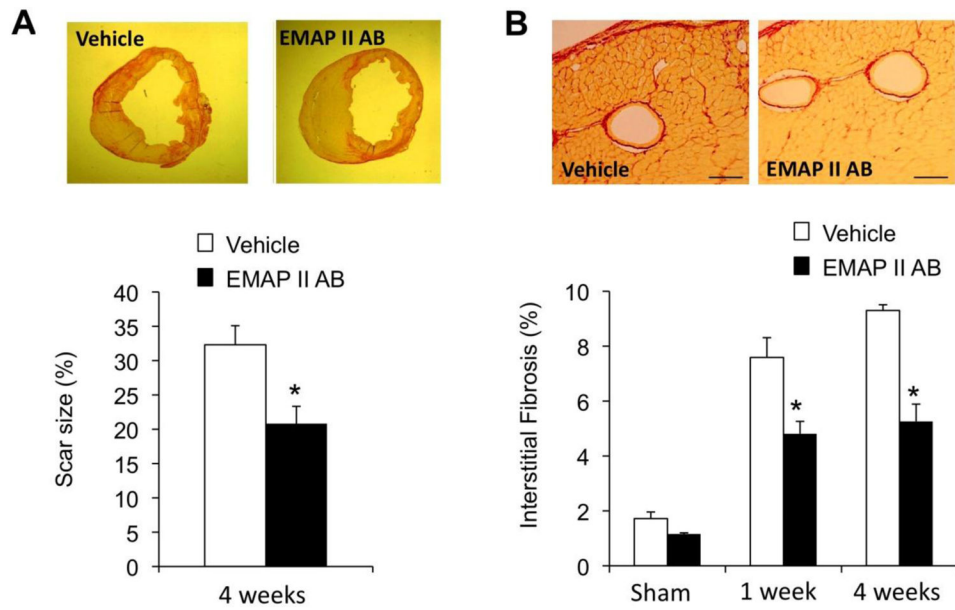
Figure 2.
EMAP II AB improved LV structure with reduced scar size and collagen deposition. (*A*) The scar size was decreased after EMAP II AB treatment for 4 weeks. Upper panels: Representative images of infarct rings after 4wk MI as shown by PSR staining; lower panel: quantitative data. (*B*) The interstitial fibrosis (%) in the adjacent area was less in the EMAP II AB group after both 1 and 4 week MI as shown by PSR staining. Upper panels: representative images after 1 wk MI; lower panel: quantitative data.
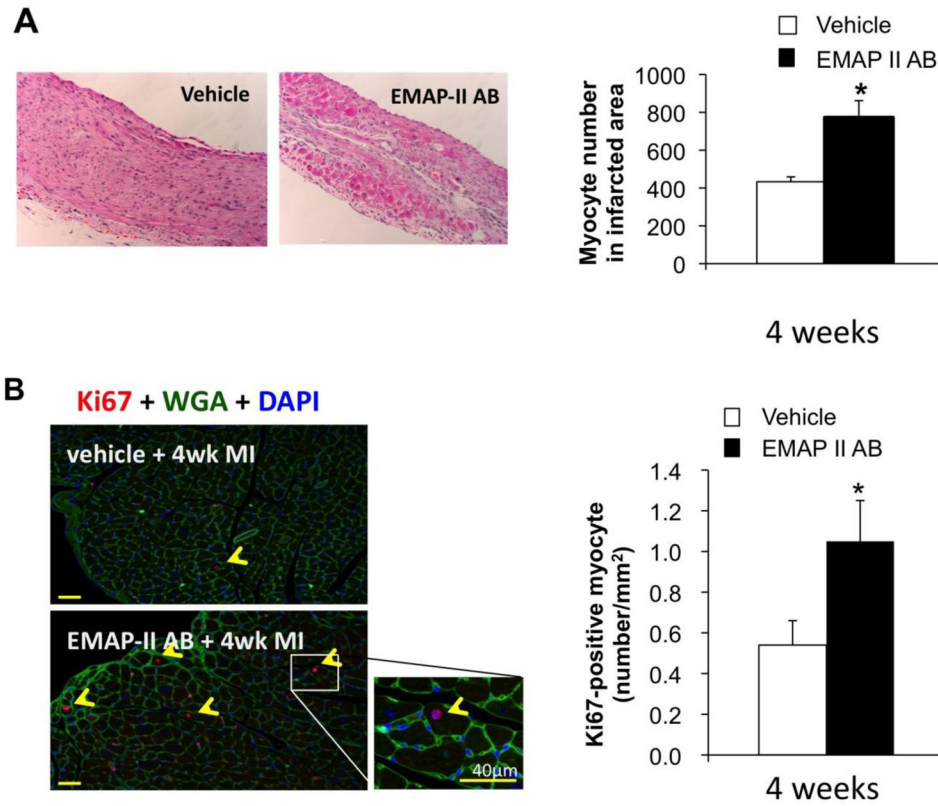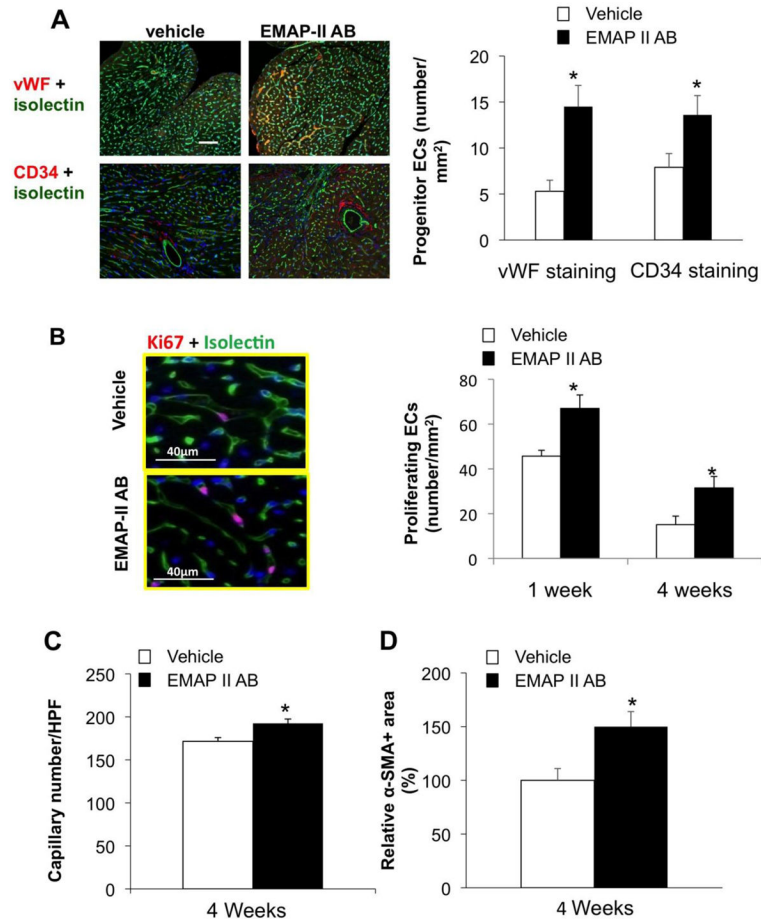
Figure 3.
EMAP II AB preserved myocytes and increased proliferating myocytes after chronic MI. (*A*) More preserved myocytes were seen in EMAP II AB as shown with H&E staining of infarct area after 4 week MI. Left panels: H&E staining images (original magnification, ×10); right panel: quantitative data. (*B*) Proliferating myocytes were increased with EMAP II AB as shown by Ki67-WGA-DAPI triple staining (left panel) and quantification (right panel). *: $p<0.05$ compared with vehicle. n=6 for each group.

Figure 4.
EMAP II AB increased angiogenesis after chronic MI. (*A*) EMAP II AB increased progenitor ECs as shown by increased numbers of vWF+ and CD34+ endothelial cells. Left panels: vWF and CD34+ staining images; right panel: quantitative data. (*B*) EMAP II AB increased proliferating ECs as shown by representative photographs of Ki67+ endothelial cells (left panel) and quantification (right panel). (*C*) EMAP II AB increased capillary density. (*D*) EMAP II AB also increased blood vessel smooth muscle cells after chronic MI. *: p<0.05 compared with vehicle. n=5 for each group.
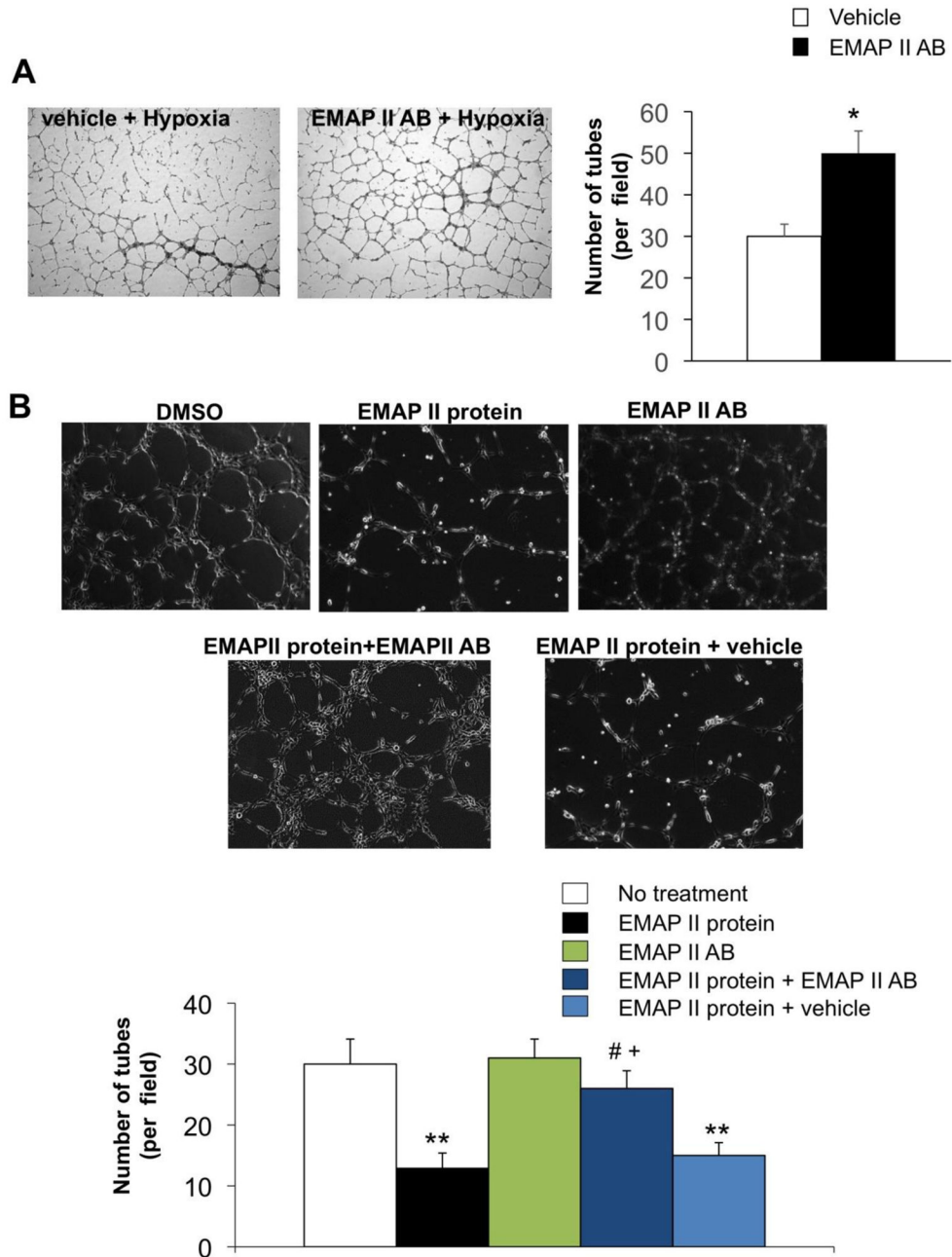
Figure 5.

EMAP II AB promotes angiogenesis demonstrated with *in vitro* tube formation assay. (*A*) EMAP II AB stimulates more tube formation in cultured HMVEC-Cs under hypoxia as shown by representative photographs (left panel; original magnification, ×10) and quantification (right panel). *:p<0.05 compared with vehicle (*B*) EMAP II protein inhibited tube formation in culture HUVECs which could be rescued by EMAP II AB. as shown by representative photographs (upper panel) and quantification (lower panel). **:p<0.01 compared with no treatment; #: p<0.05 compared with EMAP II protein; +:p<0.05

compared with EMAP II protein + vehicle. Data presented are representative of experiments repeated independently three times.
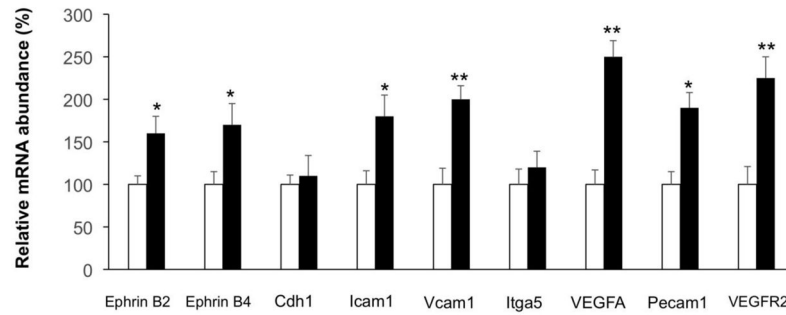
Figure 6.
EMAP II AB upregulated angiogenesis related biomarkers after chronic MI as determined by quantitative PCR. Results are expressed as the mean ± SEM. *: p<0.05 compared with vehicle, **: p<0.01 compared with vehicle. n=5 per group.

Table 1

Echocardiographic Data

	Sham	4 wk MI	
	Vehicle + EMAPII	Vehicle	EMAP-II AB
n	8	7	10
LV EF (%)	72 ± 1.0	35 ± 2.4[*]	53 ± 4.4[*†]
LV FS (%)	35 ± 0.8	13 ± 1.1[*]	23 ± 2.4[*†]
LVEDD(mm)	3.84 ± 0.05	5.51 ± 0.31[*]	5.25 ± 0.22[*]
LVESD(mm)	2.51 ± 0.05	4.78 ± 0.29[*]	4.08 ± 0.25[*]
Heart Rate (bpm)	440 ± 5	400 ± 18	375 ± 13[*]

[*] p<0.05 *vs.* Sham

[†] p<0.05 *vs.* Vehicle after 4wk MI

(One way ANOVA with Bonferroni post hoc analysis)