

# Effect of a Stable Angiotensin-(1–7) Analogue on Progenitor Cell Recruitment and Cardiovascular Function Post Myocardial Infarction

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**Background**—Angiotensin-(1–7) improves cardiac function and remodeling after myocardial infarction (MI). This may involve recruitment of hematopoietic progenitor cells that support angiogenesis. However, angiotensin-(1–7) is rapidly metabolized in plasma and tissue. The authors investigated in mice the effect of a metabolically stable angiotensin-(1–7) analogue, cyclic angiotensin-(1–7), on progenitor cell recruitment and on the heart post MI, when given in the angiogenesis phase of remodeling.

**Methods and Results**—Angiogenic progenitor cell recruitment was measured by using flow cytometry 24 and 72 hours after a daily bolus injection of cyclic angiotensin-(1–7) in healthy C57BL/6 mice. Further, mice underwent MI or sham surgery and subsequently received saline or 2 different doses of cyclic angiotensin-(1–7) for 3 or 9 weeks. Cyclic angiotensin-(1–7) increased circulating hematopoietic progenitor cells at 24 hours but not 72 hours. Post MI, cyclic angiotensin-(1–7) diminished cardiomyocyte hypertrophy and reduced myogenic tone, without altering cardiovascular function or cardiac histology at 9 weeks. Importantly, cyclic angiotensin-(1–7)-treated mice had reduced cardiac capillary density at 3 weeks after MI but not after 9 weeks. Finally, cyclic angiotensin-(1–7) decreased tube formation by cultured human umbilical vein endothelial cells.

**Conclusions**—Our results suggest that cyclic angiotensin-(1–7), when given early after MI, recruits progenitor cells but does not lead to improved angiogenesis, most likely because it simultaneously exerts antiangiogenic effect in adult endothelial cells. Apparently, optimal treatment with cyclic angiotensin-(1–7) depends on the time point of onset of application after MI. (*J Am Heart Assoc.* 2015;4:e001510 doi: 10.1161/JAHA.114.001510)

**Key Words:** angiotensin-(1–7) • cardiac function • endothelial progenitor cells • hemodynamics • myocardial infarction • renin-angiotensin system • vascular function

The renin-angiotensin system (RAS) is a major physiological regulator of blood pressure and volume homeostasis, and consequently it is involved in the pathogenesis of cardiovascular diseases.<sup>1</sup> The classic treatment of heart

failure and hypertension is directed at inhibition of the angiotensin II (Ang II)-producing enzymes renin and angiotensin-converting enzyme (ACE) and of the Ang II type 1 (AT1) receptor. In an attempt to further optimize pharmacological RAS modulation, alternative intervention strategies have emerged; among them, stimulation of angiotensin-(1–7) [Ang-(1–7)] function is one of the most attractive.<sup>2</sup> Ang-(1–7) is generated by ACE2. By stimulating its own receptor, the AT1–7/Mas receptor,<sup>3</sup> Ang-(1–7) exerts antifibrotic, antiproliferative, antithrombotic, and antihypertensive effects—that is, effects that generally oppose those of Ang II.

The beneficial effects of Ang-(1–7) or ACE2 upregulation in cardiovascular tissue are well documented. In rat models, Ang-(1–7) infusion or ACE2 overexpression preserves cardiac and endothelial function in heart ischemia and failure, prevents renal and cardiovascular anomalies in hypertension and diabetes, and exerts protective effects in cardiopulmonary disease.<sup>3–8</sup> A779, a Mas receptor antagonist, blocks Ang-(1–7)-stimulated collagen production in cardiac cells and attenuates the antihypertensive and antiproteinuric effects of captopril and hydralazine.<sup>9–11</sup> After myocardial infarction (MI),

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Ang-(1–7) prevented endothelial dysfunction<sup>12</sup> and alterations in the structure and hemodynamic function of the heart.<sup>13</sup> The antihypertrophic effect of Ang-(1–7) was also observed in rats displaying cardiac hypertrophy after isoproterenol exposure.<sup>13</sup> In vitro, the hypertrophic effect of isoproterenol was blocked by AVE-0991, an Ang-(1–7) receptor agonist.<sup>14</sup> Moreover, Ang-(1–7) increases the formation of endothelial progenitor cells from bone marrow (BM)-derived hematopoietic cells through AT1–7/Mas receptors in vitro as well as cardiac endothelial growth factor–positive cells in vivo in a mouse MI model<sup>15</sup> and promotes tube formation in cultured pig BM-derived angiogenic cells at low concentrations.<sup>16</sup> Studying the Ang-(1–7)–AT1–7/Mas receptor axis is therefore a crucial point for improving cardiac function and remodeling, in particular in relation to neovascularization.

Although beneficial effects of Ang-(1–7) have been observed in animal models of cardiovascular disease, its native form also stimulates AT1 receptors at high concentrations and is rapidly metabolized in tissue and plasma.<sup>3</sup> Consequently, Ang-(1–7) may not be optimum for use in patients. Hypothetically, cyclic Ang-(1–7) [cAng-(1–7)], an Ang-(1–7) analogue that is resistant to metabolism and acts as a specific agonist for AT1–7/Mas receptors, is a more promising compound for clinical cardiovascular therapy.<sup>17</sup> We recently showed beneficial effects of 8-week treatment of cAng-(1–7) on endothelial function and cardiac remodeling on MI rat model,<sup>18</sup> suggesting that cAng-(1–7) is a promising new agent in treating MI. In this study and our seminal study with native Ang-(1–7),<sup>12</sup> therapy was started 2 weeks after the induction of MI. This might not be the optimal time point for stimulation of angiogenesis by recruitment of progenitor cells, which, in patients, takes place in the first week after MI.<sup>19</sup> Moreover, in the previous study with cAng-(1–7), the animals did not develop heart failure, possibly due to too small infarct size. Therefore, it is still not known if cAng-(1–7) can prevent heart failure, which remains a therapeutic challenge. On the basis of all previous data, we hypothesize that cAng-(1–7) will recruit angiogenic progenitor cells and will improve cardiac neovascularization, thereby suppressing early remodeling and preventing heart failure.

## Methods

### Animal Studies

Male wild-type C57Bl/6 mice were fed standard mouse chow and water, available ad libitum. Housing was at room temperature with a 12-hour light/12-hour dark cycle. After at least 1 week of acclimatization in the caretaking facility, the mice underwent either a sham procedure or coronary ligation to induce left ventricular MI. Osmotic minipumps (model 1004; Alzet) were implanted subcutaneously

immediately after surgery for MI and the mice received saline, 5  $\mu\text{g kg}^{-1} \text{day}^{-1}$  [cAng-(1–7)-5], or 50  $\mu\text{g kg}^{-1} \text{day}^{-1}$  of cAng-(1–7) [cAng-(1–7)-50] continuously. The cAng-(1–7) doses were chosen on the basis of pharmacokinetic studies in the rat; a 10-fold range was applied because currently no data are available in mice.<sup>20,21</sup> A 9-week treatment study investigated the effect of cAng-(1–7) on chronic heart failure, while a 3-week study focused on its effects on early cardiac remodeling. The cAng-(1–7) was provided by Lanthiopharm BV and Tarix Pharmaceuticals. After 3 or 9 weeks, hemodynamic function was measured with use of a 1F pressure–volume catheter (PVR-1035; Millar) under isoflurane anesthesia.<sup>22</sup> To measure the effect of cAng-(1–7) on progenitor cell recruitment, 2 additional studies were performed in noninfarcted male wild-type C57Bl/6 mice, the first enabling us to get an impression of the time frame of effects and to make a comparison versus native Ang-(1–7), and the second to confirm possible effects at the most relevant time point. In the first study, mice ( $n=4/\text{group}$ ) received daily subcutaneous injections of saline, cAng-(1–7) (50  $\mu\text{g/kg}$ ), or native Ang-(1–7) (500  $\mu\text{g/kg}$ ). After 24 or 72 hours, the mice were killed, and blood and bone marrow samples were collected for fluorescence-activated cell sorting analysis. Because in the first study cAng-(1–7) tended to increase c-kit–, Sca-1–, and Flk-1–positive cells after 24 hours in blood, in the second study we focused on cAng-(1–7) (50  $\mu\text{g/kg}$ ) effects versus saline after 24 hours and increased the number to 13 per group. For both the first and second study, the percentage of progenitors in the total pool of mononuclear cells was first assessed. Data were normalized by expressing them as percent of the mean percentage of the saline-treated animals.<sup>23</sup> All animal studies were performed according to Dutch guidelines and approved by the institutional animal care committee.

### Cardiac Histology

The hearts were collected and fixed in 4% paraformaldehyde solution. After fixation, the slices were dehydrated and paraffin-embedded. Gomori's staining was used to visualize individual cardiomyocytes. Only transversally cut cells in the surviving myocardium of the left ventricle showing a nucleus were used to determine the cardiomyocyte area. Sirius red staining was used to determine MI size and fibrosis. Fibrosis was scored outside the infarction and infarction border zone. Cardiac vascular density was assessed by the use of lectin staining (Isolectin B<sub>4</sub> [BSI-B<sub>4</sub>], L5391; Sigma). Briefly, endogenous peroxidase was blocked with 0.3% hydrogen peroxide, the lectin antibody was diluted in 1% bovine serum albumin in PBS, and the sections were incubated overnight at 4 °C with this solution. Positive staining was visualized by using diaminobenzidine, and nuclei were counterstained with

hematoxylin. Negative controls were obtained by omitting the antibody from the incubation procedure.

## Vascular Function

Thoracic aortic endothelial function was measured with a Mulvany myograph as described previously.<sup>18</sup> Briefly, mice thoracic aortas (diameter  $\approx 800$   $\mu\text{m}$ ) were cut in ring segments of  $\approx 2$ -mm length. The aortic rings were mounted in Mulvany myograph organ baths and preincubated for 30 minutes, after which tension was normalized by adjusting the diameter length to 90% of the length at which the equivalent of 100 mm Hg blood pressure is reached. Subsequently, acetylcholine concentration–response curves were constructed after precontraction with U46619 (100  $\mu\text{mol/L}$ ).

## Myogenic Tone

Mesenteric arteries were used to determine the myogenic tone as described previously.<sup>24</sup> Briefly, each pressure level from 20 to 160 mm Hg was maintained for 5 minutes, and the vessel diameter was then measured. Myogenic tone was expressed as  $[(D_1 - D_2)/D_1] \cdot 100$ , where  $D_1$  is the difference in diameter between consecutive pressure points in  $\text{Ca}^{2+}$ -free buffer and  $D_2$  is the difference in diameter in  $\text{Ca}^{2+}$ -containing buffer.

## Fluorescence-Activated Cell Sorting Analysis

Mononuclear cell suspensions ( $\approx 2 \times 10^7$  cells/mL) of blood and BM were obtained through density centrifugation using Lympholyte-Mouse (CL5030; Cedarlane). Isolated cells were incubated with antibodies against Flk-1 (VEGF-R2, Ly-73, 560070; BD Pharmingen), c-kit (CD117, 553869; BD Pharmingen), and Sca-1 (Ly6A/E, 553335; BD Pharmingen), following a previously used protocol that avoided overlap with  $\text{Lin}^-$  cells in the dot plots.<sup>23</sup> Flow cytometric analysis with a mononuclear gate was performed on a FACSCanto or BD LSR II (BD Biosciences). Data analysis occurred with the help of Flowjo and Infinicyte software.

## Endothelial Tube Formation

Primary cultures of human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 medium (Lonza). Cells from passages 4 to 7 were used throughout the study. The 15-well  $\mu$ -slides (Ibidi GmbH) were coated with 10  $\mu\text{L}$  of Matrigel (Cell Biolabs). To analyze the effect of cAng-(1–7) on endothelial tube formation in vitro,  $3 \times 10^3$  and  $6 \times 10^3$  cells per well were incubated with  $10^{-10}$  to  $10^{-7}$  mol/L cAng-(1–7) for 6 hours. The total number of tubes was measured using CL Vision32 software (Clemex Analyse).

## Statistical Analysis

The results are expressed as mean  $\pm$  SEM. Shapiro–Wilkinson normality test was used to distinguish normal from nonparametric distribution. Normally distributed data were tested by using Student *t* test or 1-way ANOVA, followed by post-hoc evaluation according to Dunnett (for comparisons between  $\geq 2$  groups). Nonparametric data were tested by using a Mann–Whitney *U* test. Differences in concentration–response curves to acetylcholine and myogenic tone curves were tested by general linear model for repeated measures, including all concentrations and pressures.  $P < .05$  was considered significant.

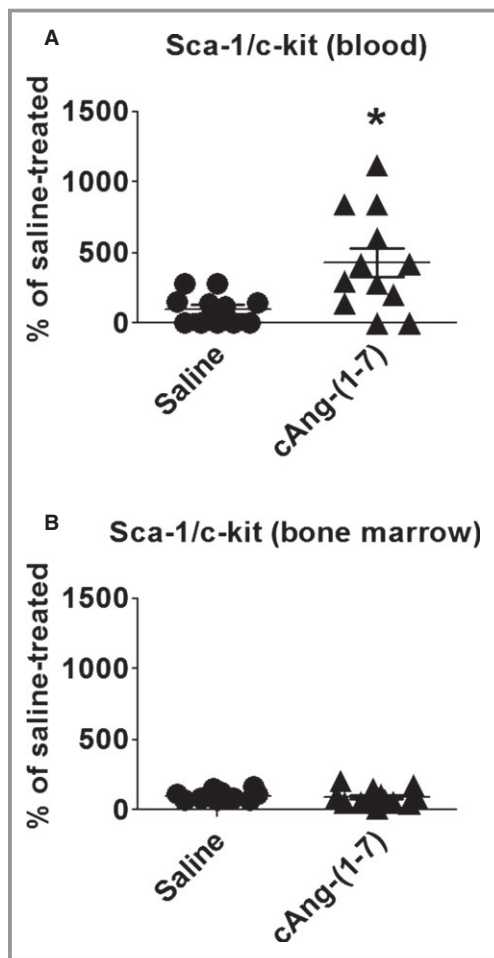
## Results

### Fluorescence-Activated Cell Sorting Analysis of Progenitor Cells

After a 24-hour treatment with cAng-(1–7), levels of cells double positive for Sca-1 and c-kit were significantly increased in blood samples from noninfarcted animals (Figure 1A). Double-positive cells in BM samples remained similar at this time point (Figure 1B). At 24 hours, cAng-(1–7)-treated animals presented with no effects on levels of cells positive for the endothelial marker Flk-1 in blood ( $72.69 \pm 15.38$  versus  $97.67 \pm 32.86$ ,  $P = \text{NS}$ ) or in BM ( $100 \pm 10.69$  versus  $120.3 \pm 15.67$ ,  $P = \text{NS}$ ). No triple-labeled cells were observed, most likely due to the already very low amounts of Sca-1/c-kit double-positive and Flk-1 single-positive cells. Further, after cAng-(1–7) treatment for 72 hour, the Flk-1, and c-kit/Sca-1 double-positive cell levels were no longer different from those in saline-treated mice (data not shown). Native Ang-(1–7) did not change progenitor cell levels ( $n = 4$ ; data not shown).

### Weight Relations and Histological Characteristics

All animal groups showed similar body weights (BWs) during the 3- and 9-week treatments. However, at 3 weeks, the heart weight (HW)-to-BW ratio tended to be higher in the MI+saline group than in the sham group ( $P = 0.069$ ; Table 1). Remarkably, a 3-week treatment with cAng-(1–7)-50 increased this ratio even further (Table 1), and results after 9 weeks were similar. However, significance was not reached due the small number per group. We therefore combined all MI animals into 1 group and subdivided them according to treatment [saline versus cAng(1–7)]. Under those conditions, HW:BW was significantly increased after MI, and cAng(1–7) did not alter this. Repeating the analysis with tibia length instead of BW yielded the same result (data not shown). Left ventricular cardiomyocyte area was higher in the MI+saline group than in the sham group, and this difference disappeared in the



**Figure 1.** Fluorescence-activated cell sorting analysis results of double-positive cells for Sca-1 and c-kit markers from blood samples (A) and bone marrow (B) from noninfarcted animals treated with saline or cAng-(1-7) for 24 hours ( $n=11$  to  $12$ ). cAng-(1-7), noninfarcted animals were treated with  $50 \mu\text{g kg}^{-1} \text{day}^{-1}$  of cAng-(1-7). Data have been expressed as a percentage of the mean percentage in saline-treated animals (see Methods section). Percentages of measured cells of total mononuclear cells in saline-treated mice were 0.24%, 2.00%, 0.20%, and 0.005% in blood samples vs 1.03%, 1.75%, 0.46%, and 0.08% in bone marrow samples for the c-kit, Sca-1, Flk-1, and Sca-1/c-kit double-positive cells, respectively.  $*P<0.05$  vs saline (Mann-Whitney  $U$  test). FACS indicates fluorescence-activated cell sorting.

presence of cAng-(1-7) (Figure 2A and 2B), although the difference between saline and cAng-(1-7)-treated MI animals could not be statistically confirmed. Coronary ligation led to large infarctions and increased intercellular fibrosis outside the infarct area, and both remained unaffected by cAng-(1-7) (Figure 3). Vascular density near the infarcted area did not change after MI (Figure 4) but tended to decrease in the

**Table 1.** Animal Data

	Animals	BW, g	HW:BW, mg/g
3-Week treatment			
Sham	7	27.84±0.93	4.14±0.11
MI+saline	5	27.44±1.22	4.98±0.30
MI+cAng-(1-7)-50	8	26.50±0.57	5.69±0.24*
9-Week treatment			
Sham	12	30.38±1.01	4.45±0.19
MI+saline	12	30.59±0.48	4.88±0.17
MI+cAng-(1-7)-5	9	30.22±0.63	5.16±0.24
MI+cAng-(1-7)-50	8	30.26±0.61	5.05±0.22

Data are expressed as mean±SEM. BW indicates body weight; HW, heart weight; MI, myocardial infarction; MI+cAng-(1-7)-5, infarcted animals treated with  $5 \mu\text{g kg}^{-1} \text{day}^{-1}$  of cAng-(1-7); MI+cAng-(1-7)-50, infarcted animals treated with  $50 \mu\text{g kg}^{-1} \text{day}^{-1}$  of cAng-(1-7).

\* $P<0.05$  vs sham group (1-way ANOVA followed by Dunnett post-hoc test).

surviving myocardium remote from the infarction area after saline treatment (Figure 4C, not significant) 3 weeks after MI. Treatment with cAng-(1-7) led to a further decrease, becoming statistically significant compared with sham-operated controls (Figure 4C). At 9 weeks after MI, the differences in vascular density had disappeared (Figure 4B and 4D).

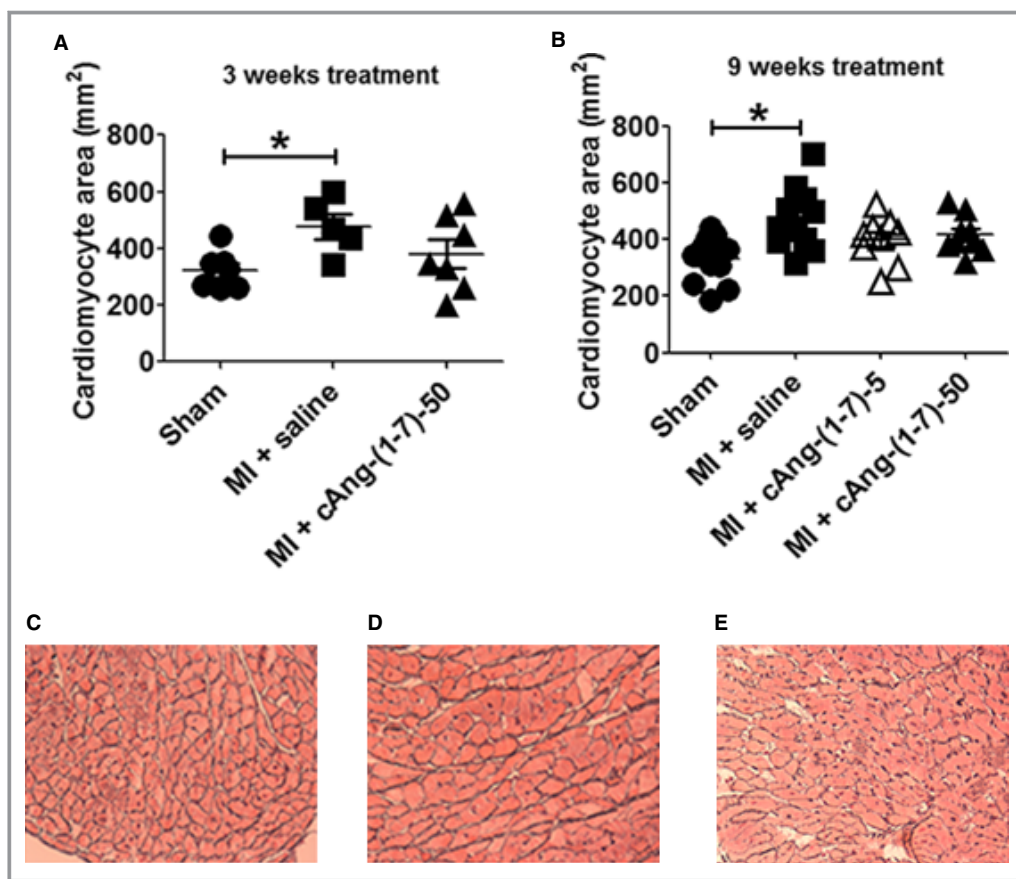
## Hemodynamics

Both infarcted saline- and cAng-(1-7)-treated groups showed no differences with regard to blood pressure versus sham animals (Tables 2 and 3). Cardiac output and stroke volume were significantly decreased 3 weeks after MI in the saline- and cAng-(1-7)-treated animals and restored to normal at 9 weeks after MI (Table 2). Ejection fraction also tended to be decreased in the 3-week group, but this was not significant, most likely due to a low number of observations. Furthermore, heart rate, end-systolic and end-diastolic pressures, and cardiac contractility were not altered by MI or cAng-(1-7) treatment after 3 weeks (not shown) or 9 weeks (Table 3).

## Vascular Function and Myogenic Tone

There is a longstanding general consensus that increased peripheral vascular resistance after MI importantly contributes to the development of heart failure.<sup>25</sup> Therefore, endothelial function and myogenic tone, 2 main contributors to vascular resistance,<sup>26,27</sup> were measured. The aortic endothelial function was not significantly altered by the infarction after 9 weeks, and cAng-(1-7) treatment showed no additional effects (Figure 5A). However, pressure-induced contraction of mesenteric arteries tended to be enhanced at 9 weeks after MI versus noninfarcted animals (not statistically





**Figure 2.** Histological analysis of ventricular cardiomyocyte area on 3-week (A) and 9-week (B) treated groups (n=5 to 12). Representative pictures of sham (C), saline (D), and cAng-(1-7)-treated animals (E). MI+cAng-(1-7)-5, infarcted animals treated with  $5 \mu\text{g kg}^{-1} \text{day}^{-1}$  of cAng-(1-7). MI+cAng-(1-7)-50, infarcted animals were treated with  $50 \mu\text{g kg}^{-1} \text{day}^{-1}$  of cAng-(1-7). \* $P < 0.05$  vs sham (1-way ANOVA followed by Dunnett post-hoc test). MI indicates myocardial infarction.

detectable at these low numbers of observations), while cAng-(1-7) treatment prevented this effect (Figure 5B).

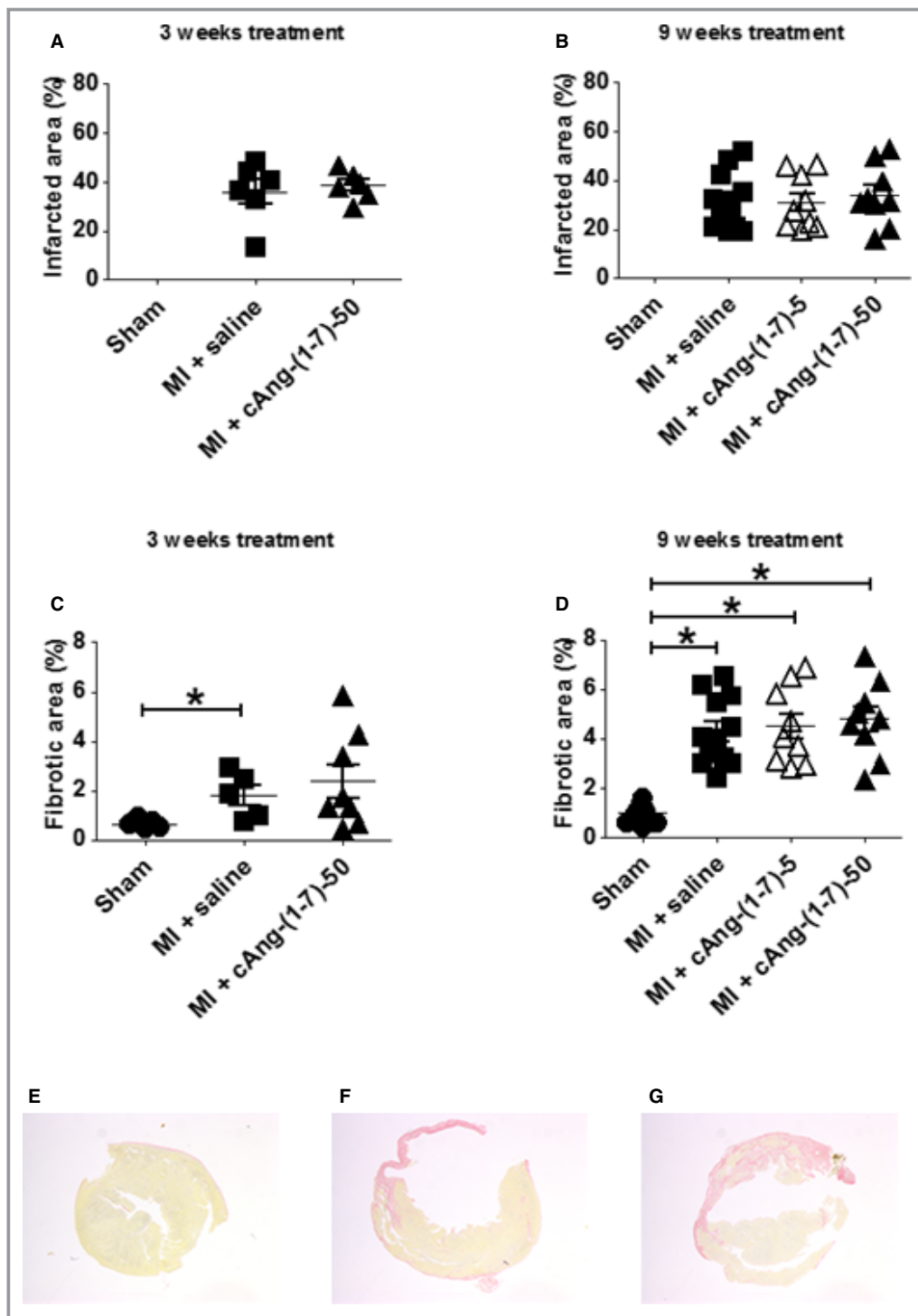
### Endothelial Tube Formation

HUVECs cultured for 6 hours formed fewer tubes in the presence of cAng-(1-7) treatment in a dose-dependent manner (Figure 6:  $P < 0.001$  GLM-repeated measures for dose).

### Discussion

In the present study, we explored if cAng-(1-7) would be able to improve recruitment of angiogenesis-supporting progenitor cells and thus improve neovascularization of the surviving myocardium after MI to prevent heart failure. The results show that although cAng-(1-7) can increase circulating hematopoietic progenitor cells after 24 hours, the effects lasted only shortly and had disappeared after 72 hours. Moreover, neither circulating Flk-1-positive cells nor vascular

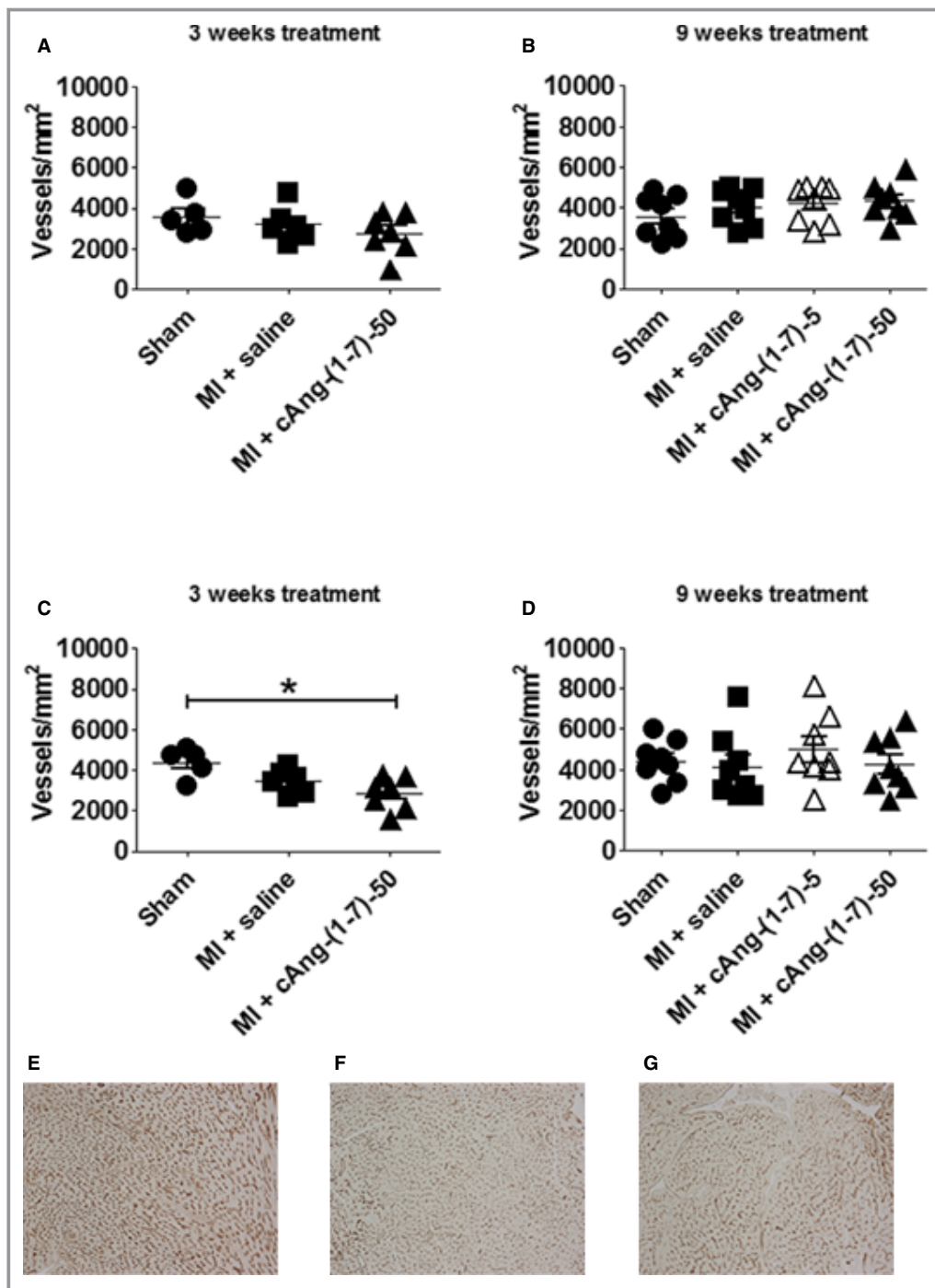
density in the myocardium of infarcted mice increased after long-term cAng-(1-7) administration. Rather, vascular density was reduced by cAng-(1-7) 3 weeks after MI in the surviving hypertrophied myocardium remotely from the scar tissue and normalized again at 9 weeks after MI. cAng-(1-7) reduced myocyte hypertrophy and prevented the increase of peripheral myogenic tone without showing an effect on aortic endothelial function. Despite the large infarctions, hypertrophy, and increased fibrosis, cardiac function did not appear to be worsened 9 weeks after MI, so an effect on heart failure could not be effectively measured. Because myocyte dimensions were increased 9 weeks after MI but vascular density was similar to that of sham-operated animals, the surviving mice had apparently compensated for cardiac hypertrophy by increasing neovascularization, thus maintaining proper hemodynamic function. Implicitly, this means that cAng-(1-7) delayed restoration of vascular density, as observed at 3 weeks post MI. This is most likely due to its antiangiogenic effect on adult endothelial cells (reduced tube formation by HUVECs), its short-lasting (1-day) effect on Sca-1- and



**Figure 3.** Histological analysis of myocardial infarction (MI) (n=5 to 12). MI area on 3-week (A) and 9-week (B) treated groups. Fibrosis on 3-week (C) and 9-week (D) treated groups. Representative photomicrographs of sham- (E), saline- (F), and cAng-(1-7)-treated animals (G). MI+cAng-(1-7)-5, infarcted animals treated with  $5 \mu\text{g kg}^{-1} \text{day}^{-1}$  of cAng-(1-7). MI+cAng-(1-7)-50, infarcted animals treated with  $50 \mu\text{g kg}^{-1} \text{day}^{-1}$  of cAng-(1-7). \* $P < 0.05$  vs sham (1-way ANOVA followed by Dunnett post-hoc test).

c-kit-positive progenitor cells, and the absence of an effect on Flk-1-positive cells. Because cAng-(1-7) demonstrates potentially beneficial effects such as reduction of hypertrophy and a

decrease in myogenic tone, the compound is still eligible for exploration as a drug to prevent heart failure after MI. However, our results suggest that it is optimum to administer



**Figure 4.** Cardiac vascular density determined by lectin staining ( $n=5$  to  $8$ ). Vessel density at infarcted border area on 3-week (A) and 9-week treated groups (B). Vessel density at remote myocardium area on 3-week (C) and 9-week (D) treated groups. Representative photomicrographs of sham- (E), saline- (F), and cAng-(1-7)-treated animals (G). MI+cAng-(1-7)-5, infarcted animals treated with  $5 \mu\text{g kg}^{-1} \text{day}^{-1}$  of cAng-(1-7). MI+cAng-(1-7)-50, infarcted animals treated with  $50 \mu\text{g kg}^{-1} \text{day}^{-1}$  of cAng-(1-7). \* $P<0.05$  vs sham (1-way ANOVA followed by Dunnett post-hoc test). MI indicates myocardial infarction.

cAng-(1-7) after the cardiac angiogenesis phase, as we did in our previous studies.<sup>12,18</sup>

The increase in hematopoietic progenitor cells due to cAng-(1-7) is in accordance with previous studies showing

that Ang-(1-7) analogues improve restoration of circulating blood cells in models of hematopoietic stress.<sup>28-31</sup> Our current results, which do not represent conditions of hematopoietic stress, indicate that cAng-(1-7) swiftly recruits

**Table 2.** Hemodynamic Data After 3-Week Treatment

	3-Week Treatment		
	Sham	MI	
		Saline	cAng-(1-7)-50
Animals, n	6	4	4
HR, min <sup>-1</sup>	515.60±15.12	534.30±21.58	454.30±42.04
SV, µL	10.41±1.85	4.65±0.95*	3.26±0.85*
CO, mL/min	5201±896.9	2487±489.5*	1883±244.1*
EF, %	49.63±8.27	25.47±6.24	19.35±10.84

Data are expressed as mean±SEM. MI indicates myocardial infarction; MI+cAng-(1-7)-50, infarcted animals treated with 50 µg kg<sup>-1</sup> day<sup>-1</sup> of cAng-(1-7); HR, heart rate; SV, stroke volume; CO, cardiac output; EF, ejection fraction.

\*P<0.05 vs sham group (1-way ANOVA, Dunnett post-hoc test).

progenitor cells, causing a tendency to lower such cells in the BM. Apparently, this recruitment does not result in de novo progenitor cell formation, so the effect is short lasting. We were unable to detect an increase in Flk-1-positive cells. It is therefore unlikely that cAng-(1-7) increases hematopoietic cells that support angiogenesis. Although our previous study predicted such an effect of native Ang-(1-7), showing an increase in c-kit- and vascular endothelial growth factor-positive cardiac cells,<sup>15</sup> native Ang-(1-7) also has antiangiogenic properties in HUVECs, and on this basis it is now exploited as anticancer therapy.<sup>32,33</sup> Conversely, Ang-(1-7) analogues have shown beneficial effects on wound repair, which is largely dependent on angiogenesis.<sup>34</sup> Apparently, HUVECs as a model for tube formation might be predictive for tumor and cardiac angiogenesis but not for all other vascular beds. An example is stroke, in which native Ang-(1-7) has

shown benefit, although this was largely contributable to an anti-inflammatory effect.<sup>35-37</sup>

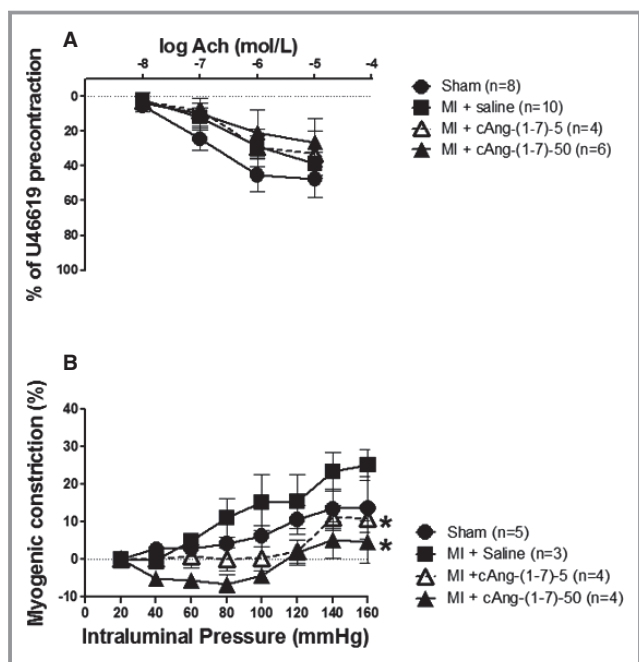
In the present study, we observed a decrease in left ventricular myocyte size. This observation is in agreement with our recent study in a rat model in which treatment with cAng-(1-7) was started 2 weeks after MI.<sup>18</sup> The previous study showed a decrease in HW:BW after cAng-(1-7) treatment. This indicates that indeed the effect of the drug is optimal when started later after MI. Still, fibrosis could not be prevented, which is in disagreement with effects of native Ang-(1-7) in the DOCA salt, Ang II infusion, and isoproterenol infusion models of cardiac hypertrophy.<sup>38-41</sup> However, these models are either blood pressure dependent or are not based on ischemia, or they are models of lifetime overexpression of Ang-(1-7), which is not translatable into clinical use in MI. One study in which cyclodextrin-enveloped Ang-(1-7) was applied shows reduced collagen I mRNA levels, but the investigators did not evaluate fibrosis or mention the ventricular region that was sampled for PCR analysis, which precludes comparison.<sup>42</sup> A study with AVE0991, a claimed Ang-(1-7) analogue, shows that this drug can reduce fibrosis in infarcted hearts, which is partly reversible with A779; however, the microphotographs in this study confusingly represent the infarcted zone.<sup>43</sup> Studies dedicated to the effects of ischemia-induced fibrosis show that ACE2 overexpression can reduce fibrosis, without demonstrating that this is due to enhanced Ang-(1-7) formation.<sup>44,45</sup> Therefore, although ACE2/Ang-(1-7)/Mas stimulation has shown promise as an antifibrotic treatment in several conditions,<sup>46-48</sup> the role of Ang-(1-7) in ischemia-induced fibrosis is still unclear. At this point, we cannot determine if native Ang-(1-7) would be more effective against fibrosis than cAng-(1-7). Moreover,

**Table 3.** Hemodynamic Data After 9-Week Treatment

	9-Week Treatment			
	Sham	MI		
		Saline	cAng-(1-7)-5	cAng-(1-7)-50
Animals, n	10	5	7	7
HR, min <sup>-1</sup>	503.10±12.09	472.30±27.86	488.20±10.52	506.70±7.74
ESP, mm Hg	79.72±5.93	79.14±9.35	75.63±5.21	90.71±9.85
EDP, mm Hg	6.60±2.59	8.27±3.03	7.33±3.05	6.18±1.20
SV, µL	8.83±0.90	7.06±1.45	10.14±1.49	6.27±0.89
CO, mL/min	4584±530.8	3441±836.7	5012±782.0	3190±466.0
EF, %	42.93±6.14	40.99±4.38	42.65±8.32	43.27±12.28
dP/dT max, mm Hg/s	6514±470.2	6692±903.8	6050±282.4	6090±565.2
dP/dT min, mm Hg/s	-6391±691.0	-7170±915.2	-5405±392.3	-6231±1111

Data are expressed as mean±SEM. MI indicate myocardial infarction; MI+cAng-(1-7)-5, infarcted animals treated with 5 µg kg<sup>-1</sup> day<sup>-1</sup> of cAng-(1-7); MI+cAng-(1-7)-50, infarcted animals treated with 50 µg kg<sup>-1</sup> day<sup>-1</sup> of cAng-(1-7); HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; SV, stroke volume; CO, cardiac output; EF, ejection fraction; dP/dT min, peak rate of pressure decline; dP/dT max, peak rate of pressure rise.

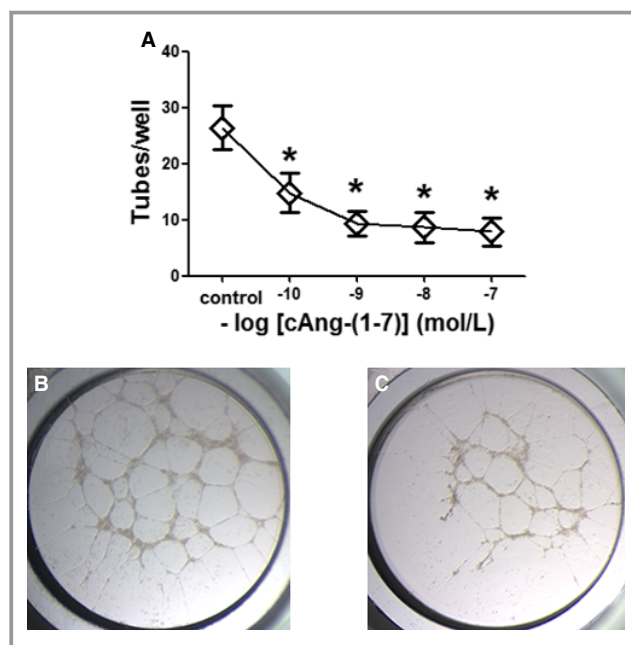




**Figure 5.** Aortic endothelial function and mesenteric myogenic tone on 9-week treated groups (n=3 to 10). A, Concentration–response curve of U46619-precontracted thoracic aorta to acetylcholine (Ach) on 9-week treatment groups. B, Myogenic tone performed on mesenteric arteries from animals treated for 9 weeks. MI+cAng-(1-7)-5, infarcted animals treated with 5  $\mu\text{g kg}^{-1} \text{ day}^{-1}$  of cAng-(1-7). MI+cAng-(1-7)-50, infarcted animals treated with 50  $\mu\text{g kg}^{-1} \text{ day}^{-1}$  of cAng-(1-7). \* $P < 0.05$  vs MI+saline (general linear model for repeated measures). MI indicates myocardial infarction.

MI increases vascular permeability, leading to edema.<sup>49</sup> Because fibrosis and HW:BW did not differ after cAng-(1-7) treatment, despite the reduced left ventricular myocyte size, it seems reasonable to conclude that the increased HW:BW was due to edema.

In our previous studies in rats, we observed improvement in endothelial function.<sup>12,18</sup> We were not able to observe this in the present study in mice, possibly because of the lack of clear endothelial dysfunction in the aortic segments used in the present study. However, we have observed a normalization of myogenic tone in mesenteric arteries. It is well known that after MI, animals can develop an increased myogenic tone and that blockade of AT1 receptors can abolish this increase instantaneously.<sup>27</sup> The effect of long-term Ang-(1-7) on myogenic tone was not previously studied. We show that after MI, also in the absence of heart failure, myogenic tone has a tendency to increase and that after MI, the myogenic tone is effectively reduced by long-term cAng-(1-7) treatment. Both AT1 receptor signaling and myogenic tone are well known to be modulated by NO released from the endothelium.<sup>27</sup> Furthermore, Ang-(1-7) antagonizes Ang II–induced vasoconstrictions in organ bath experiments independently



**Figure 6.** Tube formation assay performed on human umbilical vein endothelial cells cultured for 6 hours with cAng-(1-7) at densities of  $3 \times 10^3$  cells/well and  $6 \times 10^3$  cells/well (n=6 to 8). Representative pictures of control (B) and cAng-(1-7) human umbilical vein endothelial cells treated cells (C). \* $P < 0.05$  vs control (1-way ANOVA followed by Dunnett post-hoc test).

from NO when given at concentrations  $> 1 \mu\text{mol/L}$ .<sup>50,51</sup> This can involve inhibition of transactivation of epithelial growth factor receptors, which is importantly involved in myogenic tone.<sup>52,53</sup> To our knowledge, the effect of long-term Ang-(1-7) administration in vivo on ex vivo vascular responses to Ang II, and its relation to plasma levels, has not been evaluated yet. Nor is there any direct evidence for the involvement of Mas receptors in myogenic tone. It is, therefore, not clear how Ang-(1-7)/Mas signaling decreases myogenic tone, but this might be a relevant question in relation to treatment of cardiovascular disease.

## Conclusions

This study suggests that cAng-(1-7) given early after MI might not lead to improved angiogenesis, mainly due to an antiangiogenic effect on adult endothelial cells. It is recommended that after MI, Ang-(1-7) analogues are administered after the neovascularization phase. However, the full potential of cAng-(1-7) as an experimental drug still needs to be explored in diverse cardiac disease models and to be compared with the effects of other Ang-(1-7)/Mas–stimulating drugs such as ACE2 activators, Nor-Leu3 Ang-(1-7), CGEN856S, cyclodextrin-encapsulated Ang-(1-7), and AVE0091.<sup>2</sup> Abolishment of myogenic tone, as a mechanism to reduce workload, is an important new topic in such studies.

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## Disclosure

None.

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