Cardiomyocyte ADAM17 Is Essential in Post–Myocardial Infarction Repair by Regulating Angiogenesis

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Background—A disintegrin and metalloproteinase 17 (ADAM17) is a membrane-bound enzyme that mediates shedding of many membrane-bound molecules, thereby regulating multiple cellular responses. We investigated the role of cardiomyocyte ADAM17 in myocardial infarction (MI).

Methods and Results—Cardiomyocyte-specific ADAM17 knockdown mice (ADAM17floxed/α-MHC-Cre; f/f/Cre) and parallel controls (ADAM17floxed; f/f) were subjected to MI by ligation of the left anterior descending artery. Post MI, f/f/Cre mice showed compromised survival, higher rates of cardiac rupture, more severe left ventricular dilation, and suppressed ejection fraction compared with parallel f/f-MI mice. Ex vivo ischemic injury (isolated hearts) resulted in comparable recovery in both genotypes. Myocardial vascular density (fluorescent-labeled lectin perfusion and CD31 immunofluorescence staining) was significantly lower in the infarct areas of f/f/Cre-MI compared with f/f-MI mice. Activation of vascular endothelial growth factor receptor 2 (VEGFR2), its mRNA, and total protein levels were reduced in infarcted myocardium in ADAM17 knockdown mice. Transcriptional regulation of VEGFR2 by ADAM17 was confirmed in cocultured cardiomyocyte–fibroblast as ischemia-induced VEGFR2 expression was blocked by ADAM17-siRNA. Meanwhile, ADAM17-siRNA did not alter VEGF bioavailability in the conditioned media. ADAM17 knockdown mice (f/f/Cre-MI) exhibited reduced nuclear factor-κB activation (DNA binding) in the infarcted myocardium, which could underlie the suppressed VEGFR2 expression in these hearts. Post MI, inflammatory response was not altered by ADAM17 downregulation.

Conclusions—This study highlights the key role of cardiomyocyte ADAM17 in post-MI recovery by regulating VEGFR2 transcription and angiogenesis, thereby limiting left ventricular dilation and dysfunction. Therefore, ADAM17 upregulation, within the physiological range, could provide protective effects in ischemic cardiomyopathy.

Key Words: cardiac remodeling ■ inflammation ■ myocardial infarction ■ tumor necrosis factor-alpha convertase ■ vascular endothelial growth factor receptor-2

A disintegrin and metalloproteinase 17 (ADAM17), also known as tumor necrosis factor (TNF)-α-converting enzyme, is a ubiquitously expressed membrane-bound sheddase that cleaves many membrane-bound molecules, including cytokines, growth factors, and receptors to activate or inactivate various cellular signaling pathways. ADAM17 is expressed in multiple organs and cell types, including the cardiomyocytes in the heart. ADAM17 levels are elevated in the heart of patients with myocarditis, dilated cardiomyopathy, and hypertrophic obstructive cardiomyopathy, whereas its levels in serum are elevated in acute myocardial infarction (MI) and advanced congestive heart failure. Elevated ADAM17 levels correlate negatively with left ventricular systolic function and post-MI outcomes. However, it is not known whether the elevated ADAM17 contributes to disease progression or is a compensatory response to counteract disease pathogenesis. Angiogenesis, formation of new vessels in the ischemic myocardium, is a compensatory mechanism that contributes to cardiac repair and functional recovery post MI. ADAM17 has been reported to be involved in the regulation of angiogenesis through different mechanisms that vary in an organ- and disease-dependent fashion. We used cardiomyocyte-specific ADAM17 knockdown mice to examine the role of cardiac ADAM17 in post-MI myocardial repair.

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Methods

Detailed Methods are available in the Data Supplement.
Experimental Animals
ADAM17−/−floxed (f/f) mice, a kind gift from Carl Blobel (NY), and α-myosin heavy chain (α-MHC)-Cre (Cre) mice (Jackson Laboratories) were crossed to generate cardiomyocyte-specific ADAM17 knockdown (ADAM17−/−floxed/α-MHC-Cre or f/f/Cre) and the littermate controls (ADAM17−/−floxed or f/f). MI induction and echocardiography were conducted as before. All experiments were conducted in accordance with the guidelines of the University of Alberta Animal Care and Use Committee and the Canadian Council of Animal Care.

Immunohistochemistry was used to detect CD31, phospho-vascular endothelial growth factor receptor 2 (VEGFR2; Tyr1175), ADAM17, neutrophil, and macrophage as before. Lectin immunohistochemistry was used using fluorescein-conjugated Ricinus communis agglutinin I (lectin; Vectorlabs) perfusion method was used to assess microvascular density in sham and MI heart. mRNA expression analyses were performed by Taqman reverse transcription polymerase chain reaction as before. Electrophoretic mobility shift assay was used to detect DNA–nuclear factor-κB (NFκB) interaction using the LightShift chemiluminescent electrophoretic mobility shift assay kit (Thermo Scientific) according to the manufacturer’s instructions. ELISA was used to detect VEGF, and TNF-α levels.

Ex vivo ischemia reperfusion of isolated hearts was performed as before. In vitro cocultures of adult cardiomyocyte and adult cardiofibroblast (cMyo–cFB) cocultures were generated as before. In vitro gene silencing was achieved by transfection with Silencer Select Negative Control-siRNA (Life Technologies) or Silencer Select ADAM17 siRNA (Life Technologies) using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer’s instructions.

Statistical Analyses
All statistical analyses were performed using the IBM SPSS Statistics 19 software. Mortality and rupture incidents were compared using the Kaplan–Meier survival curve followed by the Log-rank test. Averaged values represent means±SEM. Normal data distribution was confirmed with Shapiro–Wilk Statistic test for all

![Figure 1](https://example.com/figure1.png)

**Figure 1.** ADAM17−/−floxed/α-MHC-Cre (f/f/Cre) mice exhibit more severe post-myocardial infarction (MI) outcomes compared with parallel ADAM17−/−floxed (f/f) mice. A, Survival (i) and rate of left ventricular (LV) rupture incidence (ii) post MI or post sham (sh) in indicated genotypes (n=23–30; Kaplan–Meier survival curves followed by the Log-rank test). B, Trichrome-stained heart cross-sections at 1 week post sham/MI. C, Parameters of heart function (ejection fraction [EF], fractional area change [FAC], and Wall motion score index) and structure (LV diastolic volume) assessed by echocardiography (n=7–12 per group per genotype). A significant difference was detected for both factors, MI and genotype, and their interaction (l) or only for one factor (MI; ii–iv). Two-way ANOVA with Bonferroni multiple comparison test. *P<0.05 vs corresponding sham and #P<0.05 vs corresponding f/f group. Inf indicates infarct; non, noninfarct; and peri, peri-infarct.
We first examined how ADAM17 levels are altered in the myocardial tissue after MI. In the nonischemic myocardium, ADAM17 was detected primarily but not limited to the membrane of cardiomyocytes as it overlapped with wheat germ agglutinin membrane staining. However, dispersed distribution was observed in the infarct and peri-infarct myocardium (Figure 1 in the Data Supplement). To investigate the role of cardiomyocyte-bound ADAM17 in post-MI remodeling, we used mice with reduced ADAM17 in the cardiomyocytes, t/t/α-MHC-Cre (t/t/Cre) mice. Compared with control mice with intact ADAM17 expression (ADAM17<sub>flox/flox</sub> or t/t), t/t/Cre mice showed 40.1% reduction in mRNA expression, 64.5% reduction in protein, and 31.4% reduction in ADAM17 activity (Figure IIAi, IIB, and IIC in the Data Supplement). Noncardiomyocyte cells from t/t/Cre hearts showed no change in ADAM17 mRNA, protein, or activity. Expression of other ADAMs remained unaltered in cMyc and nonmyocytes of both genotypes (Figure IIAii–IIV in the Data Supplement).

After MI, t/t/Cre mice showed compromised survival rate (35.5% versus 65.2% in t/t mice) and higher cardiac rupture incidence (55.8% versus 27.3% in t/t mice; Figure IAI and IAii in the Data Supplement). However, the infarct size was comparable between the 2 genotypes at 1 week post MI (Figure 1B). Heart function measured by echocardiography showed similar cardiac dysfunction and left ventricular dilation between genotypes at 3 days post MI (Figure III in the Data Supplement). However, at 1 week post MI, t/t/Cre mice exhibited exacerbated cardiac dysfunction as detected by a greater reduction in ejection fraction (Figure 1Ci), fractional area change (Figure 1Cii), higher wall motion score index (Figure 1Ciii), and greater left ventricular dilation (Figure 1Civ) compared with parallel t/t-MI mice. Heart rates were not significantly different between the genotypes after sham (410±16 beats per minute in t/t/Cre versus 398±17 beats per minute in t/t) or MI.
ADAM17 mRNA was significantly reduced post MI in both genotypes but to a greater extent in f/f/Cre hearts (Figure 1Di). Other ADAMs were altered similarly between the 2 genotypes post MI including increased ADAM9 in the infarct, reduced ADAM10 in the peri- and noninfarct, and elevated ADAM12 in the infarct and peri-infarct myocardium (Figure 1Dii–1Div).

ADAM17 Deficiency Suppresses Post-MI Angiogenesis But Does Not Alter Inflammation

To determine the mechanism underlying the exacerbated post-MI recovery in f/f/Cre mice, we first examined whether ADAM17 knockdown hearts are more susceptible to ischemic injury. Using the isolated heart Langendorff ischemia-reperfusion model, we found that both genotypes showed comparable rate and amplitude of recovery after a 30-minute ischemic period during reperfusion (Figure IV in the Data Supplement). Therefore, ADAM17 deficiency does not enhance acute susceptibility to ischemia or ischemia-reperfusion injury in the isolated heart ex vivo, as such, the adverse post-MI events in f/f/Cre mice are related to the myocardial response in vivo.

Among the systemic events, angiogenesis and inflammation are critical in influencing post-MI myocardial repair and remodeling. We assessed the vascular density in the infarct and peri-infarct myocardium using fluorescent-labeled lectin perfusion (whole animal) and CD31-staining. Although vascular density was found to be reduced after MI in both genotypes, this reduction was significantly greater in the infarct myocardium of f/f/Cre hearts compared with f/f mice at 1 week post MI (Figure 2A and 2B). Myocardial vascular density in sham-operated f/f/Cre mice was comparable with f/f mice. Inflammation is an early post-MI response that can influence the fate of cardiomyocytes and infarct expansion.26 Assessment of the infiltrating inflammatory cells at 3 days post MI showed similar densities of neutrophils (A) and macrophages (B) in the infarct and peri-infarct regions of both genotypes (Figure 3).

ADAM17 Regulates Post-MI Angiogenesis Through Induction of VEGFR2

Angiogenesis is mediated through activation of VEGFR2 by its primary ligand, VEGF, which is then phosphorylated (p-VEGFR2) and translocated to the nucleus.27
Consistent with the lower vascular density in f/f/Cre-MI hearts, p-VEGFR2 density was markedly reduced in the infarct myocardium of these mice compared with f/f-MI mice (Figure 4A). Superimposing the p-VEGFR2 and lectin staining revealed that p-VEGFR2 detection was not limited to the endothelial cells in the vascular wall within the infarct and peri-infarct areas (Figure V in the Data Supplement). Furthermore, total VEGFR2 protein (Figure 4B) and mRNA expression (Figure 4C) were also significantly reduced in f/f/Cre hearts post MI. Activation of NFkB has been reported to induce VEGFR2 gene expression. Among the substrates shed by ADAM17, TNF-α activates TNF-α receptor 1, and triggers nuclear translocation of NFkB. We performed electrophoretic mobility shift assay to detect nuclear translocation and DNA binding of NFkB and found that the NFkB–DNA binding was indeed suppressed in the f/f/Cre compared with f/f hearts within 3 hours of MI (Figure 4D). Consistently, TNF-α levels in the myocardial tissue (i) and serum (ii) were lower in the f/f/Cre-MI mice at this time point (Figure 4E). These data collectively suggest

Figure 4. Reduced mRNA, protein, and activity of vascular endothelial growth factor receptor 2 (VEGFR2) in cardiomyocyte A disintegrin and metalloproteinase 17 (ADAM17) knockdown mice. A, Representative immunofluorescence staining (i) and averaged phospho-VEGFR2 (p-VEGFR2) density (ii) in the infarct (inf), peri-infarct (peri), and noninfarct (non) regions of the myocardium at 1 week post myocardial infarction (MI; n=17–30 fields per group; 4 mice per genotype). Western blot showing total VEGFR2 protein (B) and VEGFR2 mRNA levels by Taqman reverse transcription polymerase chain reaction (C) in the infarct, peri-infarct, and noninfarct regions at 1 week post sham (sh) and post MI (n=6 per group per genotype). D, Electrophoretic mobility shift assay showing DNA-bound nuclear factor-κB (NFκB) at 3 hours post sham/MI. E, Tumor necrosis factor-α (TNF-α) levels in myocardial homogenate (i) and serum (ii) at 3 hours post sham/MI (n=5 per group per genotype). Two-way ANOVA with Bonferroni multiple comparison test. Significant differences were detected for both factors, MI and genotype, and their interaction (Aii, C, Ei, and Eii). Graphs represent mean±SEM. *P<0.05 vs corresponding sham group and #P<0.05 vs corresponding ADAM17flx/flx (f/f) group. HPRT indicates hypoxanthine phosphoribosyltransferase; and f/f/Cre, ADAM17flx/flx/α- MHC-Cre.
A disintegrin and metalloproteinase 17 (ADAM17) downregulation did not alter proteolytic activities post myocardial infarction (MI). Expression profile of matrix metalloproteinases (MMPs; A) and tissue inhibitor of metalloproteinases (TIMPs; B), and total proteolytic activities in ADAM17<sup>flx/flx</sup> (f/f) and ADAM17<sup>flx/flx</sup>/α-MHC-Cre (f/f/Cre) mice at 1 week post MI. C. Total collagenase (i) and gelatinase (ii) activities in f/f and f/f/Cre mice post MI. MT-MMP: membrane-type matrix metalloproteinase. n=6 per group per genotype. Two-way ANOVA with Bonferroni multiple comparison test. Significant differences were detected for one factor (MI; A, II, AIII, AIV, AIX, BIII-BVIII, CIII, and CIV); for 2 factors, MI and genotype, and their interaction (AIII); and for both factors (AIII, AIV, and AIX). Graphs represent mean±SEM. *P<0.05 vs corresponding sham (sh) group and #P<0.05 vs corresponding f/f group. HPRT indicates hypoxanthine phosphoribosyltransferase; non, noninfarct; and peri peri-infarct.
that ADAM17 can regulate VEGFR2 expression through an NFkB-mediated pathway.

Proteolytic Activities Are Not Altered With ADAM17 Downregulation After MI

The extracellular matrix and its remodeling are major contributors to post-MI remodeling and tissue angiogenesis. As such, we investigated whether the reduced angiogenesis in ADAM17-knockdown hearts was associated with altered levels or activity of the proteolytic enzymes that regulated extracellular matrix turnover, the matrix metalloproteinases (MMPs), or their inhibitors, tissue inhibitor of metalloproteinases (TIMPs). Expression analysis of MMPs, including the gelatinases, MMP2 and -9; stromelysins, MMP3, -10 and -11; collagenases MMP-13 and MT1-MMP (MMP14), MT2-MMP (MMP15), and MMP28 showed that MMP3 was increased to a greater degree in the infarct region of f/f/Cre hearts, whereas the increase in MMP9 and MMP10 did not reach statistical significance compared with f/f-MI hearts (Figure 5Ai–5Aiv). Meanwhile, mRNA expressions of other MMPs were altered similarly in both genotypes after MI (Figure 5Ai). Expression of TIMPs (Figure 5B) showed that the rise in TIMP2 in the infarct region of f/f hearts was not matched by a similar change in f/f/Cre hearts (ii), whereas TIMP3 levels were reduced in f/f/Cre hearts post MI (iii). Measurement of total proteolytic activities showed a similar rise in collagenase (i) and gelatinase (ii) activities post MI (Figure 5C). Therefore, reduced ADAM17 levels did not alter proteolytic activities in the myocardium post MI.

ADAM17 Silencing Reduced VEGFR2 Expression But Not VEGFα Bioavailability In Vitro

To further confirm the role of ADAM17 in VEGFR2 expression under ischemic conditions and to also examine whether the reduced VEGFR2 activation could be because of reduced VEGFα bioavailability in the absence of ADAM17 sheddase activities, we generated cocultures of adult cMyo and cFB (to simulate the i n vivo myocardial scenario where these cell types interact) and used ADAM17-small interfering RNA (ADAM17-siRNA) to inactivate ADAM17. ADAM17-siRNA reduced ADAM17 mRNA and protein levels by ≈86% compared with negative control-siRNA (Figure VIA in the Data Supplement). Specificity for the siRNA system was confirmed by unaltered levels of other ADAMs (ADAM9, -10, and -12; Figure IVB in the Data Supplement). To exclude the compensatory contribution of the ADAM17 from cFB, ADAM17-siRNA–treated cFBs were cocultured with cMyo from f/f/Cre mice and, subsequently, subjected to ischemic conditions. Ischemia triggered mRNA expression of VEGFR2 in control cocultures (normal ADAM17 levels), but this increase was suppressed in ADAM17-deficient cocultures (Figure 6Ai). Expression of VEGFR1 and VEGF, were induced similarly by ischemia and not altered after ADAM17 silencing (Figure 6Aii and 6Aiii). In addition, ADAM17 silencing did not alter the ischemia-induced VEGFα shedding into the conditioned media (Figure 6B). These data suggest that the primary effect of ADAM17 under ischemic conditions is through regulation of VEGFR2 expression rather than enhancing bioavailability of VEGFα.

Figure 6. Targeted A disintegrin and metalloproteinase 17 (ADAM17) silencing in cocultured cardiomyocyte–cardiofibroblast in vitro suppressed ischemia-induced rise in vascular endothelial growth factor receptor 2 (VEGFR2) mRNA levels. A, mRNA levels of VEGF receptors, VEGFR1 and VEGFR2, and VEGFα in cocultured adult cardiomyocyte–cardiofibroblasts transfected with ADAM17-siRNA (or negative siRNA control), and subjected to ischemia. B, VEGFα protein levels shed into the conditioned media. n=6 per group; 3 independent cultures from 3 individual hearts; 2-way ANOVA. Significant differences were detected for one factor (ischemia) and its interaction with ADAM17-siRNA (Ai), for ischemia only (Aii and B), for both factors (ischemia and ADAM17-siRNA) and their interaction (Ai). *P<0.05 vs corresponding nonischemic condition and P<0.05 vs corresponding (negative) siRNA control. HPRT indicates hypoxanthine phosphoribosyltransferase; and RFU, relative fluorescence units.
ADAM17 is a membrane-bound enzyme that can mediate catalytic shedding of many growth factors and cytokines, and it has been correlated with adverse outcomes in cardiomyopathies.\textsuperscript{4,6–10} In this study, we demonstrate the key role of cardiomyocyte ADAM17 in post-MI remodeling and recovery. ADAM17 knockdown in cardiomyocytes was sufficient to suppress VEGF expression and activation, impairing angiogenesis in the infarct and peri-infarct myocardium, leading to exacerbated left ventricular dilation and dysfunction. The role of endothelial ADAM17 in angiogenesis has been reported in the retina through enhanced VEGF\textsubscript{A} shedding and bioavailability.\textsuperscript{17} In this study, we report the significance of cardiomyocyte ADAM17 in regulating postischemic angiogenesis by regulating VEGFR2 transcription and activation independent from VEGF\textsubscript{A} bioavailability.

Angiogenesis has the potential to rescue the ischemic myocardium at early stages after MI and can prevent the transition to heart failure.\textsuperscript{11,12} ADAM17, particularly in endothelial cells, has been shown to promote angiogenesis through different mechanisms.\textsuperscript{16,17,30,31} In this study, we examined the contribution of cardiomyocyte ADAM17 in post-MI repair and found that reduced ADAM17 in cardiomyocytes suppresses ischemia-induced angiogenesis leading to exacerbation of cardiac dysfunction and dilation. In attempting to identify the cell types that exhibit VEGF\textsubscript{A} activation within the infarct region, we found that although a fraction of the p-VEGFR2–positive cells colocalized with endothelial (lectin-positive) cells, p-VEGFR2 was also detected in nonendothelial cells. It is highly unlikely that these cells are inflammatory cells because by 1 week post MI, inflammatory cells have already dissipated, and at this time point, the infarct region is devoid of cardiomyocytes. It has been reported that vascular trauma can mobilize bone marrow endothelial progenitor cells (with limited CD31 expression) into the peripheral circulation to enhance angiogenesis.\textsuperscript{32} VEGF\textsubscript{A}–positive cardiomyocyte progenitor cells have also been reported to contribute to the formation of the cardiomyocytes and endothelial cells.\textsuperscript{33} It is plausible that after tissue injury, as a part of recapitulation of fetal gene expression pattern, such progenitor cells are migrated to and proliferated within the damaged myocardium to promote tissue repair.

MMPs and the overall proteolytic activities can regulate extracellular matrix remodeling and can thereby contribute to angiogenesis. However, ADAM17 knockdown did not alter the proteolytic activities in the myocardium post MI. TIMP3 has been reported to be the physiological inhibitor of ADAM17.\textsuperscript{34} Reduced TIMP3 levels in the f/f/Cre hearts may be balanced by the reduced ADAM17 levels in these hearts.

ADAM17, also known as TNF-\(\alpha\)-converting enzyme, can shed the membrane-bound TNF-\(\alpha\) and generate soluble/activate TNF-\(\alpha\) that then binds to and activates TNF-\(\alpha\) receptor-1 and the downstream signaling pathways, mainly the NF\(\kappa\)B pathway.\textsuperscript{35} Activation of NF\(\kappa\)B can induce VEGF\textsubscript{A} expression via complement 32.\textsuperscript{36} We found that ADAM17 knockdown led to reduced TNF-\(\alpha\) levels and NF\(\kappa\)B–DNA binding post MI, which could underlie the reduced VEGF\textsubscript{A} expression in f/f/Cre-MI hearts. In addition to NF\(\kappa\)B–binding elements, there are other potential cis-acting sequences in the VEGF\textsubscript{A} promoter, such as Sp-1– and Ap-2–binding sites.\textsuperscript{36} Whether these transcription factors are also involved in the regulation of VEGF\textsubscript{A} expression post MI will require further investigation. It has been reported that p-VEGFR2 can bind to the Sp-1 responsive element of its promoter and upregulates its own expression through a positive feedback mechanism.\textsuperscript{27}

ADAM17 is responsible for ectodomain shedding of multiple transmembrane molecules. In addition to TNF-\(\alpha\) and VEGF, ADAM17 can also mediate shedding of many EGFR ligands, including HB-EGF and transforming growth factor-\(\alpha\).\textsuperscript{37} adhesion molecules, and some receptors of these ligands, such as TNF-\(\alpha\) receptor-1, VEGFR\textsubscript{2},\textsuperscript{31} and transforming growth factor-\(\beta\) receptor-1.\textsuperscript{38} Because shedding of the receptors will result in the opposite outcome of ligand shedding, the role of ADAM17 in mediating cellular events can be variable and unpredictable. Activation of EGFR leads to cell proliferation and hypertrophy, but these phenotypes were unaltered in ADAM17-knockdown mice post MI, and as such, we did not pursue this pathway further. Meanwhile, ADAM17-mediated shedding of VEGF\textsubscript{A} and the resulting activation of VEGF\textsubscript{A} could have provided an alternative explanation for the differential angiogenesis observed in f/f/Cre mice after MI. We found that the role of ADAM17 in post-MI angiogenesis is primarily through expression and activation of VEGF\textsubscript{A} rather than shedding of VEGF\textsubscript{A}.

In conclusion, this study provides the first evidence for the role of cardiomyocyte ADAM17 in post-MI angiogenesis through induction of VEGF\textsubscript{A}. Considering the critical effect of angiogenesis in ischemic myocardium, intact levels of ADAM17 are essential for optimal post-MI remodeling. In addition, although ADAM17 overexpression has been reported to promote angiogenesis in the brain,\textsuperscript{16} considering the broad spectrum of ADAM17 substrates, excess overexpression of ADAM17 could also result in adverse effects, and therefore, a physiological balance should be targeted to achieve optimal outcomes.

Limitations
This study provides evidence on the adverse outcomes of reduced cardiomyocyte ADAM17 on cardiac structure and function early post MI; however, the long-term effects of ADAM17 downregulation will require further investigation. Our in vitro coculture system of cMYO and cFB confirms that under ischemic conditions, ADAM17 regulates the expression of VEGF\textsubscript{A} but not shedding of VEGF\textsubscript{A}. However, the absence of endothelial cells in this coculture system may pose a limitation in directly extending these findings to the angiogenic events in vivo.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Myocardial infarction (MI) results in nonuniform adverse remodeling of the heart. The myocardium directly downstream from a blocked coronary artery undergoes ischemia, cell loss, and formation of a fibrotic scar that can expand leading to left ventricular dilation, dysfunction, and eventually heart failure. The cellular events in the peri-infarct myocardium can influence the extent of this injury and the overall cardiac response to MI. The adaptive increase in angiogenesis is a critical response to minimize infarct expansion and the propensity to develop heart failure. By using cardiomyocyte-specific A disintegrin and metalloproteinase 17-knockdown mice, we demonstrate that A disintegrin and metalloproteinase 17, a multifunctional membrane-bound enzyme with the ability for ectodomain shedding of many cell–surface ligands and receptors, regulates post-MI angiogenesis. The loss of cardiomyocyte A disintegrin and metalloproteinase 17 compromised the expression and activation of vascular endothelial growth factor receptor 2, the main angiogenic pathway in the heart, resulting in reduced infarct-related angiogenesis without affecting myocardial inflammation or changes in the extracellular matrix. We provide evidence that orchestration of the post-MI angiogenesis response requires a cue from the cardiomyocytes to trigger a response from the endothelial cells. Therefore, preserving A disintegrin and metalloproteinase 17 levels in the ischemic myocardium could serve as a potential therapeutic approach to reduce the adverse remodeling, which ensues MI.
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SUPPLEMENTAL MATERIAL
DETAILED METHODS

Experimental Animals and In Vivo Myocardial Infarction Protocol

ADAM17<sup>flox/flox</sup> (f/f) mice were a kind gift from Carl Blobel (The Hospital for Special Surgery, NY),<sup>1</sup> alpha-myosin heavy chain (αMHC)-Cre (Cre) mice purchased from Jackson Laboratories. These two mice (both C57BL/6) were cross-bred to generate cardiomyocyte-specific ADAM17 knockdown (ADAM17<sup>flox/flox</sup>/αMHC-Cre or f/f/Cre) and the littermate controls (ADAM17<sup>flox/flox</sup> or f/f). Myocardial infarction (MI) was induced in 10-12 weeks old male f/f and f/f/Cre mice by left anterior descending artery (LAD) ligation as before.<sup>2</sup> Parallel sham-operated mice from each genotype served as controls. Heart function was assessed by echocardiography at 3-day and 1-week post-MI/sham as before.<sup>2,3</sup> Hearts were excised, flash-frozen in OCT medium, or were fixed in 10% formalin and processed for immunohistochemical analyses, or separated into infarct, peri- and non-infract regions and flash-frozen for molecular analyses. All experiments were conducted in accordance with the guidelines of the University of Alberta Animal Care and Use Committee (ACUC) and the Canadian Council of Animal Care.

Cardiac Function Assessment

Cardiac structure and systolic function were determined by noninvasive transthoracic echocardiography in anesthetized mice (0.75% isoflurane) as before.<sup>4,5</sup> Modified parasternal long axis EKV loops were also used to measure ejection fraction (EF) by Simpson’s method. M-mode images were used to measure left ventricular (LV) chamber sizes and wall thicknesses. Fractional area change (FAC), a measure of LV global systolic function, was measured by two-dimensional echocardiography in the apical four-chamber view by measuring the fractional change in the area inscribed by the LV endocardium at peak diastole and peak systole.<sup>6</sup> The wall
motion score index (WMSI) was calculated based on the *American Society of Echocardiography* recommended assessment of wall motion function. An increase in WMSI (>1) indicates suppressed LV systolic wall motion.

**Histochemical and immunostaining (Trichrome, Lectin, CD31, Phospho-VEGFR2 (Tyr1175), ADAM17, Neutrophil and Macrophage Staining)**

Freshly excised hearts were arrested in diastole in 1M KCl, fixed in 10% formalin, paraffin-embedded and processed for trichrome staining as before. Lectin immunofluorescence assay using fluorescein conjugated Ricinus communis agglutinin I (RCA; lectin; Vectorlabs) perfusion method was used to assess microvascular density in sham and MI heart as before. Briefly, heparinized mice were anesthetized and 0.2 mg of lectin in 100 µL saline was injected into the jugular vein. After 18 minutes of lectin circulation, 0.2 mg of papaverine HCl (Vasodilator, Sigma Aldrich) in 50 µL saline was injected to promote maximal dilation of microvessels and circulated for 2 minutes. The hearts were then excised, and frozen in OCT mounting medium. Five-µm thick cryosections were cut, fixed with 4% paraformaldehyde for 20 minutes followed by rehydration (PBS, 30 minutes). Sections were mounted in Prolong Gold antifade mounting medium with DAPI (Life Technologies). Images were captured using a fluorescence microscope (Olympus IX81), analyzed using Metamorph software (Version 7.7.0.0) and vascular density reported as percentage of lectin-positive vessels per field.

Vascular endothelial cells were stained in OCT-embedded hearts by immunofluorescence staining using rat anti-CD31 primary antibody (BD Pharmingen) and Cy3 conjugated goat anti-rat secondary antibody (Life Technologies) as before. Briefly, 5-µm thick OCT-embedded
cryosections were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X100, and blocked with 4% bovine serum albumin. Sections were then incubated with primary antibody overnight (4°C in a humidified chamber), then washed and incubated with secondary antibody for 1 hour at 37°C. Sections were then visualized and images captured using a fluorescence microscope, and CD31-positive vessels were quantified (Metamorph software) and reported as ‘percent per field’.

Activation of VEGFR2 was assessed by immunofluorescence staining of phosphorylated VEGFR2 using rabbit anti-Phospho-VEGFR2 (Tyr1175) primary antibody (Cell Signaling Technology) and Alexa Fluor594 Donkey Anti-Rabbit secondary antibody (Life Technologies) as describe for CD31-staining. ADAM17 staining was performed on paraffin-embedded sections with primary anti-ADAM17 antibody (EMD Millipore), followed by an Alexa Fluor® 594-conjugated secondary antibody, and then double stained with Oregon Green 488 conjugated wheat germ agglutinin (WGA) to stain the cell membranes. Neutrophil staining was performed using anti-mouse neutrophil antibody with Cy3-labeled anti-rat secondary antibody, and nuclear DAPI staining, visualized by fluorescent microscopy as before. Macrophages were stained with rat anti-mouse F480 antibody (BD Pharmingen: cat#550292, clone M3/84) and biotinylated rabbit anti-rat IgG linking antibody (Cell Signaling) followed by HRP conjugated streptavidin labeling reagent, and visualized by light microscopy as before.

**mRNA Expression, Western blots and Activity Assays**

Total RNA was extracted using TRIzol Reagent (Life Technologies) and mRNA expression levels for ADAM9, ADAM10, ADAM12, ADAM17, Vascular Endothelial Growth Factor A
(VEGFA), VEGF receptor-1 (VEGFR1) and VEGFR2, MMPs -2, -3, -9, -10, -11, -13, -28, MT1-MMP, MT2-MMP, and TIMPs 1-4 were detected by TaqMan RT-PCR as before. Taqman primer/probe cocktails were purchased from Life Technologies Corporation (Supplemental Table I). Hypoxanthine-guanine phosphoribosyltransferase-1 (HPRT) was used as an internal control as before.

Membrane protein was extracted from the myocardium as before and used for Western blot analyses to detect ADAM17 (Santa Cruz Biotechnology), VEGFR2 (Cell Signaling Technology) using specific antibodies, and an anti-rabbit secondary antibody (Cell Signaling Technology) as previously described.

To measure ADAM17 activity, fresh aliquote of the membrane proteins (5µg), and fluorogenic quenched ADAM17 substrate peptide ((7-methoxycoumarin-4-yl)acetyl-PLAQAV-N-3-(2,4-dinitrophenyl)- L-2,3-diaminopropionyl-RSSSR-NH2) (R&D Systems) were used according to the manufacturer’s instructions. Total collagenase and gelatinase activities were measured using tissue homogenates and fluorescent-labeled collagen or gelatin substrates (Invitrogen) as before.

**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear protein fractions were extracted with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to manufacturer’s instructions. DNA–NFκB interaction was detected using the LightShift chemiluminescent EMSA kit (Thermo Scientific) according to the manufacturer’s instructions. Briefly, nuclear protein was incubated with biotin-labelled DNA probes containing the consensus NFκB sequence 5′-gatc-
CAGGGCTGGGGATTCCCCATCTCCACAGG-3′ (sense). Then, DNA–protein complexes were run on a 5% native polyacrylamide gel, transferred to a positively charged nylon membrane (Thermo Scientific), cross-linked to the membrane, and detected by use of horseradish peroxidase-conjugated streptavidin and chemiluminescence. The specific band was confirmed by reducing the binding reaction with a NFκB p65 antibody (Santa Cruz).

Enzyme-Linked Immunosorbent Assay (ELISA)

Shedding of VEGFₐ was measured in the conditioned media using mouse VEGF Immunoassay kit (R&D Systems) according to the manufacturer’s instructions. TNFα levels were measured in myocardial homogeneates, extracted by CelLytic™ M extraction reagent (Sigma), or serum following 3 hours of sham or MI surgery, using Mouse TNF alpha ELISA Ready-SET-Go! kit (eBioscience) according to the manufacturer’s instructions.

Ex vivo Ischemia-Reperfusion Protocol

Male (12-11 weeks old) mice of either genotype were heparinized and anesthetized, and hearts were excised and perfused on a Langendorff apparatus at a constant pressure of 80 mmHg with oxygenated modified Krebs-Henseleit solution as before. A water-filled balloon was inserted into the LV, and LV pressure and contraction/relaxation kinetics were monitored using the PowerLab system (ADInstruments, Australia). After 15 minutes of baseline recording, hearts were subjected to 30 minutes of global ischemia followed by 45 minutes of reperfusion.
Isolation and Co-culture of Adult Cardiomyocytes (cMyo) and cardiofibroblasts (cFB), siRNA Transfection, and In Vitro Ischemia

CMyo and cFB were isolated from adult f/f mice and co-cultured as previously described.\textsuperscript{10,14} CMyo-cFB co-cultures were transfected with Silencer® Select Negative Control siRNA (Life Technologies) or Silencer® Select ADAM17 siRNA (Life Technologies) for 48 hours by using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer’s instructions. The cells were then randomized into 2 groups, control or ischemia, for 24 hours: (1) Control, cells were changed to fresh Eagle’s MEM with 0.1% BSA, incubated under normoxic conditions (21% oxygen); (2) Ischemia, cell were incubated in Kreb’s modified buffer (120.4mM NaCl, 14.7mM KCl, 0.6mM KH\textsubscript{2}PO\textsubscript{4}, 0.6mM Na\textsubscript{2}HPO\textsubscript{4}, 1.2mM MgSO\textsubscript{4}-7H\textsubscript{2}O, 10mM Na-HEPES, 4.6mM NaHCO\textsubscript{3} and 1mM CaCl\textsubscript{2}) and incubated under hypoxic conditions (1% oxygen).\textsuperscript{5} At the end of the protocol, conditioned media were collected to analyze shedding levels of different proteins (ELISA), and cells were harvested for mRNA analyses as before.\textsuperscript{10}
REFERENCES


Supplemental Table I - Taqman RT-PCR primers and probe for the indicated genes.

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<td>ADAM10</td>
<td>5’- GTGCCAGTACAGGCTTTGC-3’&lt;br&gt;5’'- CACAGTAGCCTCTGAAGTCATTACATG -3’&lt;br&gt;5’'- FAM- ACTATCACTCTGAGCCGGCTCTCC-TAMRA-3’</td>
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<td>5’-ATCAGTGCTTCGGCGGTCA-3’&lt;br&gt;5’'-GGCAATTCTTCCTGTTGTTACATACC-3’&lt;br&gt;5’'-FAM-CCATGCAGTGCCACGGCCG-TAMRA-3’</td>
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<td>ADAM17</td>
<td>5’-AAGTGCAAGGCTGGAAATG-3’&lt;br&gt;5’'-CACACGGGCCAGAAAGGTT -3’&lt;br&gt;5’'-FAM-CCTGCGCATGCATTGACACTGACAAC-TAMRA-3’</td>
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**Assay ID**

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<td>ADAM9</td>
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<td>VEGFR2</td>
<td>Mm01178819_m1</td>
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Supplemental Figure I-R1. ADAM17 and wheat germ agglutinin (WGA) staining in ADAM17flox/flox heart at 3 days post-MI. Five micrometer sections from paraffin-embedded hearts were stained with anti-ADAM17 and Alexa Fluor® 594-conjugated secondary antibody (red), and double stained with Oregon Green 488 conjugated wheat germ agglutinin (WGA) (green) to stain the cell membranes. Overlay shows superimposed ADAM17 and WGA stainings.
Supplemental Figure II-R1. Confirmation of cardiomyocytes (CM)-specific ADAM17 downregulation. 

A, mRNA levels of A Disintegrin and Metalloprotease 17 (ADAM17), ADAM9, ADAM10 and ADAM12 in freshly isolated CM and non-CM (NCM) from ADAM17flox/flox (f/f) and f/f/αMHC-Cre (f/f/Cre) mice. ADAM17 protein (B) and activity (C) in f/f/Cre CM and NCM compared to parallel f/f cells.

n=4-7 mice/genotype; t-test A to C, Graphs represent mean±SEM; # p<0.05 vs. parallel f/f group.
Supplemental Figure III-R2. No functional or structural difference was detected between ADAM17 flox/flox (f/f) and f/f/αMHC-Cre (f/f/Cre) mice at 3 days post-MI. A) Trichrome staining images of heart cross-sections from indicated genotypes. B) Heart function was assessed by echocardiography. EF: ejection fraction, FAC: fractional area change. n=7-11/group/genotype; bar graphs represent mean±SEM. 2-way ANOVA with Bonferroni multiple comparison test. *p<0.05 vs. corresponding sham.
Supplemental Figure IV-R1. Knockdown of ADAM17 in cardiomyocytes does not affect cardiac recovery from ischemia-reperfusion in isolated hearts ex vivo. A-F, In a Langendorff isolated heart perfusion, 30 minutes of ischemia followed by 45 minutes of reperfusion resulted in similar changes in the rate of LV contraction and relaxation (+dp/dt) (A), the left ventricular developed pressure (LVDP) (B), LV end-systolic pressure (LVESP) (C), LV end-diastolic pressure (LVEDP) (D), heart rate (beats per minute, BPM) (E), and flow rate (F) in ADAM17floxflox (ff) and ff/αMHC-Cre (ff/Cre) hearts. n=10/genotype. Graphs represent means±SEM.
A. overlay images of p-VEGFR2, lectin, and DAPI staining

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B. overlay of p-VEGFR2 and DAPI staining on bright field images

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**Supplemental Figure V- R1.** Disperse localization of phosphorylated vascular endothelial growth factor receptor-2 (p-VEGFR2) post-MI. 

A) Superimposed images of p-VEGFR2 (activated VEGFR2), lectin (outlining microvessels) and DAPI (identifying nucleated cells) show that p-VEGFR2 is detected in vascular walls (yellow: overlapping red and green), which could be endothelial cells. However, they are also detected in other cell types including cardiomyocytes as shown by the bright field overlay (B) that show the morphometry of the cardiomyocytes. Images were obtained from sham-operated hearts, as well as infarct, Peri-infarct (peri) and non-infarct (non) hearts at 1 week post-MI. f/f: ADAM17flox/flox, f/f/Cre: ADAM17flox/flox/αMHC-Cre.
Supplemental Figure VI-R1. Assessment of the efficiency and specificity of ADAM17 siRNA in cardiac fibroblasts (cFB). A) The ADAM17 protein level in cFB after the cells were transfected with negative control (-) siRNA or ADAM17 siRNA for 72 hours. B) ADAMs mRNA expression in cFB after the cells were transfected with negative control (-) siRNA or ADAM17 siRNA for 48 hours. n=3. T test. Graphs represent mean±SEM. *p<0.05 vs. parallel (-) siRNA.