C1q-TNF-Related Protein-9 Promotes Cardiac Hypertrophy and Failure


Rationale: Myocardial endothelial cells promote cardiomyocyte hypertrophy, possibly through the release of growth factors. The identity of these factors, however, remains largely unknown, and we hypothesized here that the secreted CTRP9 (C1q-tumor necrosis factor–related protein-9) might act as endothelial-derived protein to modulate heart remodeling in response to pressure overload.

Objective: To examine the source of cardiac CTRP9 and its function during pressure overload.

Methods and Results: CTRP9 was mainly derived from myocardial capillary endothelial cells. CTRP9 mRNA expression was enhanced in hypertrophied human hearts and in mouse hearts after transverse aortic constriction (TAC). CTRP9 protein was more abundant in the serum of patients with severe aortic stenosis and in murine hearts after TAC. Interestingly, heterozygous and especially homozygous knock-out C1qtnf9 (CTRP9) gene-deleted mice were protected from the development of cardiac hypertrophy, left ventricular dilatation, and dysfunction during TAC. CTRP9 overexpression, in turn, promoted hypertrophic cardiac remodeling and dysfunction after TAC in mice and induced hypertrophy in isolated adult cardiomyocytes. Mechanistically, CTRP9 knock-out mice showed strongly reduced levels of activated prohypertrophic ERK5 (extracellular signal-regulated kinase 5) during TAC compared with wild-type mice, while CTRP9 overexpression entailed increased ERK5 activation in response to pressure overload. Inhibition of ERK5 by a dominant negative MEK5 mutant or by the ERK5/MEK5 inhibitor BIX02189 blunted CTRP9 triggered hypertrophy in isolated adult cardiomyocytes in vitro and attenuated mouse cardiomyocyte hypertrophy and cardiac dysfunction in vivo, respectively. Downstream of ERK5, we identified the prohypertrophic transcription factor GATA4, which was directly activated through ERK5-dependent phosphorylation.

Conclusions: The upregulation of CTRP9 during hypertrophic heart disease facilitates maladaptive cardiac remodeling and left ventricular dysfunction and might constitute a therapeutic target in the future. (Circ Res. 2017;120:66-77. DOI: 10.1161/CIRCRESAHA.116.309398.)

Key Words: aortic valve stenosis ■ endothelial cells ■ hypertrophy ■ phosphorylation ■ signal transduction
to their role to enable myocardial tissue perfusion—regulate cardiomyocyte growth and function.4,5 In this regard, inhibition of myocardial angiogenesis during ventricular pressure overload because of experimental transverse aortic constriction (TAC) reduced, while increased angiogenesis promoted cardiac hypertrophy.6,9 Endothelial cells are thought to induce cardiomyocyte growth through the release of paracrine factors, although the identity of these factors remains largely unknown.4,5

Here, we describe that the secreted glycoprotein CTRP9 (C1q-tumor necrosis factor–related protein-9), which is abundantly produced in the heart,10 is mainly expressed in cardiac endothelial cells. CTRP9 belongs to the recently discovered family of CTRPs, which are paralogs of adiponectin.11,12 Besides adiponectin, 15 additional family members have so far been identified (CTRP1-15). Among this protein family, CTRP9 exerts by far the highest expression in the heart and can also be found in serum and adipose tissue.10,12,13 Interestingly, CTRP9 abundance in serum was reported to be reduced in mice with diabetes mellitus and after cardiac ischemia/reperfusion injury.14,15 CTRP9-deficient animals developed obesity and insulin resistance at older age, indicating a systemic role for the protein, but they are indistinguishable from wild-type (WT) littermates in the first 3 to 4 months of life.16 With regard to its local function in the heart, CTRP9 was to date reported as cell survival molecule, preventing cardiomyocyte death during ischemia/reperfusion injury and in remodeling after experimental myocardial infarction through binding of the adiponectin receptor 1 (AdipoR1), activation of the AMP-dependent kinase (AMPK), and the protein kinase A (PKA).14,17,18 We demonstrate here a maladaptive role for CTRP9 during cardiac pressure overload, where CTRP9 becomes upregulated in the heart and serum and drives cardiac hypertrophy by a previously unrecognized ERK5 (extracellular signal-regulated kinase 5)-GATA4 signaling axis.

Methods

An expanded Method section is provided in the Online Data Supplement.

Experimental Animals

C1qtnf9 (encoding for CTRP9) gene-deleted mice were obtained from the Davis campus of the University of California and were created under the National Institute of Health Knock-Out Mouse Program (project number VG12588). Constriction of the transverse aortic arch (TAC) was performed in 8- to 10-week-old gene-deleted or WT mice around a 25-gauge needle, as previously described.19,20 For the mice after adeno-associated virus serotype 9 (AAV9) injection, TAC was conducted using a 26-gauge needle. All procedures involving the use and care of animals were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Research Council (NIH Publication No. 85-23, revised 1996) and the German animal protection code. Approval was granted by the local state authorities (33.14-42502-04-11/0608), and institutional guidelines were followed.

Human Heart Samples

Analysis of human heart tissue was permitted by the Massachusetts General Hospital Institutional Review Board (United States) and by the Ethical Committee of the Hannover Medical School, Germany (Az: Z 14.06-A 1871–30724/98).21

Generation and Administration of AAV9 Vectors

The entire coding region of the CTRP9 cDNA was subcloned into the pdsCMV-MLC260-EGFP vector downstream of a CMV (cytomegalovirus)-enhanced 260-bp myosin light chain promoter (MLC260). The resulting plasmid was used to produce AAV9-CTRP9 vectors as previously described.22 AAV9 vectors that express renilla luciferase downstream of the MLC260 promoter were generated and used as control (AAV9-control).

Echocardiography

Echocardiography was performed with a linear 30 MHz transducer (Vevo770; Visualsonics, Toronto, Canada).

Quantification of CTRP9 in Serum

The CTRP9 concentration was determined by the human CTRP9 ELISA (enzyme-linked immunosorbent assay; Biovendor, Heidelberg, Germany) according to manufacturer’s instructions in the serum of male healthy blood donors or male patients experiencing severe aortic stenosis before aortic valve replacement was conducted (the patient characteristics are shown in Online Table I). This study was approved by the Ethical Committee of the Hannover Medical School, Germany. All individuals and patients gave written informed consent.

Cell Culture and Isolation of Cardiac Cells

Adult ventricular cardiomyocytes were prepared from adult Sprague-Dawley rats or mice as previously described using a Langendorff system.23,24 Cell size was determined by planimetry. The luciferase activity from adult cardiomyocytes was measured as previously described and normalized to total protein content.25,26 C166 mouse embryonic yolk sac or mouse heart endothelial cells in cell culture plate inserts (Millipore Millicell) with 0.4-µm-sized pores on top of rat adult cardiomyocytes plated in regular cell culture plates were used as 2-chambered system for endothelial/cardiomyocyte coculture.

Cardiac endothelial cells and fibroblasts were isolated from hearts of adult mice using CD146 microbeads and feeder removal microbeads with MACS (magnetic cell separation) technology from Miltenyi Biotec. The isolated cells were directly used for RNA extraction.

In Vitro Kinase Assay

A GST (glutathione S-transferase)-GATA4 fusion construct containing the amino acids 2 to 207 or GST alone was incubated in the absence or presence of recombinant ERK5.

Statistical Analysis

All values are presented as means±SEM. The unpaired, 2-tailed t test was used for comparisons between 2 groups. Differences between >3 groups were analyzed by 1-way analysis of variance followed by Sidak multiple comparisons test. A 2-tailed P value of <0.05 was considered significant. All statistics were calculated with the Graph Pad Prism 6 software.

Results

CTRP9 Is Upregulated in Hypertrophic Heart Disease

We first investigated CTRP9 abundance in mouse and human hypertrophic heart disease. Quantitative real-time polymerase chain reaction (PCR) revealed a 3-fold upregulation of CTRP9 mRNA in mouse hearts 2 weeks after the induction of pressure overload by TAC (Figure 1A). Interestingly, CTRP9 mRNA was induced
more than 20-fold in human myocardial samples from individuals with proven cardiac hypertrophy, but without signs of heart failure, while myocardial CTRP9 mRNA levels were not significantly elevated in patients with terminal cardiac failure compared with healthy hearts (Figure 1B). Immunoblotting from mouse heart tissue showed increased CTRP9 protein abundance after 2 weeks of TAC, but unchanged or even slightly reduced CTRP9 protein levels 6 and 12 weeks after TAC compared with sham (Figure 1C). An investigation of serum CTRP9 levels revealed increased CTRP9 concentrations in patients with cardiac hypertrophy because of aortic valve stenosis in comparison to healthy blood donors (Figure 1D; Online Table I).

To assess the cellular source of CTRP9 within the heart, we visualized β-galactosidase activity in the myocardium of heterozygous C1qtnf9 gene-deleted mice (CTRP9 heterozygous) that possess a lacZ expression cassette within the C1qtnf9 gene locus. As shown in Figure 1E and 1F, β-galactosidase activity or expression was found mainly in isolectin B4–positive endothelial cells, although another noncardiomyocyte cell type showed some level of expression, as evident from the presence of scattered β-galactosidase positive, but isolectin B4–negative cells (Figure 1F). Immunofluorescence staining for CTRP9 in conjunction with isolectin B4 labeling of cardiac tissue revealed large areas of colocalization between both (Figure 1G). Homozygous

Figure 1. CTRP9 (C1q-tumor necrosis factor–related protein-9) is upregulated in the heart and serum during cardiac hypertrophy and is mainly expressed in cardiac endothelial cells. A, Cardiac CTRP9 mRNA (normalized to GAPDH mRNA) measured by quantitative real-time polymerase chain reaction (qPCR) in mouse hearts 2 weeks after sham surgery or transverse aortic constriction (TAC). B, qPCR for CTRP9 mRNA (normalized to GAPDH mRNA) from myocardium of healthy (Con), hypertrophic (Hyp), or failing (Fail) human hearts. C, Immunoblot for CTRP9 from mouse hearts after sham surgery or 2, 6, 12 wk after TAC. (-) denotes negative control from a CTRP9 knock-out (KO) heart. (+) denotes positive control from adenoviral CTRP9 overexpression. GAPDH was the loading control. The quantification of the blot is shown below. D, CTRP9 abundance in serum of healthy individuals (Con) and patients with cardiac hypertrophy because of aortic stenosis (Hyp). E, LacZ staining in the heart of a heterozygous CTRP9 KO mouse. Scale bar: 30 µm. F, Cardiac immunofluorescence staining with the indicated reagents. Isolectin B4 marks endothelial cells. Scale bar: 30 µm. G, Cardiac immunofluorescence staining for CTRP9 and endothelial cells (with isolectin B4) in wild-type (WT) or homozygous CTRP9 KO mice as indicated. Scale bar: 50 µm. H, qPCR to quantify CTRP9 mRNA (normalized to GAPDH mRNA) from endothelial cells (ECs), cardiomyocytes (CMs), and fibroblasts (Fibs) isolated from mouse hearts 1 week after sham (Sh) or TAC (T) surgery. I, Absolute quantification of CTRP9 mRNA expression in mouse heart vs subcutaneous fat tissue. J, Immunoblot for CTRP9 from supernatant of cultured primary mouse cardiac endothelial cells from WT or KO mice. The CTRP9-specific band runs around 37 kDa but is lacking in KO mice. All lanes were run on the same gel but were noncontiguous where indicated by the grey line. The number within bars indicates the number of individuals or mice analyzed. *P<0.05, **P<0.01, ****P<0.0001.
C1qtnf9 gene-deleted (CTRP9 knock-out, KO) mice did not display specific immunostaining for CTRP9, indicating the specificity of the antibody used (Figure 1G). Verification of the predominant endothelial origin of CTRP9 came from a quantitative real-time PCR analysis of endothelial cells, cardiomyocytes, and fibroblasts that were isolated from mouse hearts 1 week after sham or TAC surgery. By this approach, primary cardiac endothelial cells showed several fold higher levels of CTRP9 mRNA compared with cardiomyocytes or fibroblasts, both after sham or TAC surgery (Figure 1H). Surprisingly, we found a slight, but significant, downregulation of CTRP9 mRNA after TAC in endothelial cells, while no significant regulation by TAC was found in the other cell types. The relative purity of the obtained cell preparations was verified by quantitative real-time PCR, which demonstrated strongly elevated expression of the endothelial cell marker Cd31 in endothelial cells, αMHC (alpha myosin heavy chain) in cardiomyocytes, and collagen-1 (Col1a) in fibroblasts versus the other cell types (Online Figure 1). Although CTRP9 was originally described as derived mainly from adipose tissue, we found a 2- to 3-fold higher abundance of CTRP9 mRNA molecules in the heart compared with subcutaneous fat tissue (Figure 1I). We also demonstrate that CTRP9 is secreted into the supernatant from primary WT cardiac endothelial cells but is not found from the respective KO cells (Figure 1J). Thus, CTRP9 is mainly derived from endothelial cells in the myocardium, and its abundance is increased in human and murine hypertrophic hearts.

**CTRP9 Is Necessary for the Development of Cardiac Hypertrophy and Dysfunction After TAC**

Next, we assessed the functional consequence of increased CTRP9 levels during TAC using CTRP9 heterozygous or KO mice, which exerted ≈50% reduced or completely ablated cardi-ac CTRP9 protein and mRNA levels compared with WT mice, respectively (Figure 2A). While the hearts were not different
between KO and WT after sham surgery, heterozygous and even more clearly KO mice showed less cardiac hypertrophy (ie, a reduced heart weight/body weight ratio), less pulmonary congestion (ie, reduced lung weight/body weight), a better systolic heart function (ejection fraction), and reduced cardiac dilation (left ventricular end-diastolic area) compared with WT mice after TAC (Figure 2B through 2E). The body weight was not significantly different between WT, heterozygous, or KO mice after TAC or sham surgery (Online Figure IIA). A reduced size of cardiomyocytes was found in histological heart sections of KO versus WT mice after TAC (Figure 2F and 2G). The expression of the hypertrophic marker genes ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptide) was strongly induced in heterozygous mice after TAC, but this response was significantly blunted in KO mice (Figure 2H and 2I). In addition, myocardial fibrosis was reduced after TAC in KO compared with WT mice, and this was accompanied by reduced cardiac expression of Col1a (collagen-1) mRNA (Figure 2J and 2K).

CTRP9 Overexpression Aggravates Cardiac Dysfunction After TAC

Because ablation of CTRP9 protected from maladaptive remodeling during TAC, we wanted to analyze the effects of cardiac CTRP9 overexpression. We injected mice intravenously with a CTRP9 encoding AAV9 vector (AAV-CTRP9), which led to robust overexpression of CTRP9 in the heart (Figure 3A). Although overexpression of CTRP9 had no effect after sham operation, it led to aggravated cardiac dysfunction, hypertrophic remodeling with increased ventricular wall thickness and increased ventricular dilation compared with AAV-control–treated mice 8 weeks after TAC (Figure 3B through 3D). This was accompanied by increased cardiomyocyte size and enhanced fibrosis after TAC in AAV-CTRP9–treated mice (Figure 3E and 3F). Despite these changes, overall heart/body weight ratio was not different after TAC between AAV-control and AAV-CTRP9–treated mice (Online Figure IIIA). The ratio of capillaries/cardiomyocyte was significantly increased by TAC in AAV-control–treated mice versus sham, and AAV9-CTRP9 treatment produced an even higher capillary/cardiomyocyte ratio in mice after TAC (Online Figure IIIB). We also identified increased CTRP9 levels in the serum of mice treated with AAV-CTRP9, indicating that the CTRP9 expressed from this vector is indeed secreted (Online Figure IIIC).

Paracrine and Autocrine CTRP9 Increases Cardiomyocyte Size

Because we found CTRP9 to be expressed mainly in cardiac endothelial cells, we tested in an in vitro coculture system whether endothelial CTRP9 could induce hypertrophy in cardiomyocytes in a paracrine manner. We used a 2-chamber culture system, in which adult rat cardiomyocytes were cultured in the bottom chamber, and C166 mouse endothelial cells transduced either with control adenovirus (Ad.Control)
or CTRP9 overexpressing adenovirus (Ad.CTRP9) in the top chamber. Fluids and soluble proteins could freely path between both chambers (Figure 4A). Adult cardiomyocytes that were cocultured with CTRP9 overexpressing endothelial cells were larger compared with cardiomyocytes cocultured with endothelial cells without CTRP9 overexpression (Figure 4B and 4C). Phenylephrine had no significant effect on cardiomyocyte hypertrophy in this system. In contrast, coincubation with a CTRP9 neutralizing antibody (but not control IgG) inhibited the prohypertrophic action of endothelial CTRP9 overexpression on cardiomyocytes, indicating that secreted CTRP9 is responsible for hypertrophy induction (Figure 4B and 4C). Cardiomyocytes did not change in size when no C166 endothelial cells were cocultured in this system (first bar in Figure 4C). Because cardiomyocytes also express CTRP9 mRNA, albeit at a much lower level (see above), we tested whether CTRP9 overexpression from cardiomyocytes could trigger hypertrophy in an autocrine manner. Direct infection of adult rat cardiomyocytes with Ad.CTRP9 (immunoblot in Online Figure IIIE) also induced enhanced cardiomyocyte growth compared with Ad.Control-transduced cells, and this effect was almost completely ablated by the addition of the CTRP9 neutralizing antibody (but not control IgG), suggesting that CTRP9 can trigger cardiomyocyte hypertrophy in an autocrine manner (Figure 4D). To analyze the effects of endogenous (ie, not overexpressed) endothelial CTRP9,
cocultured cardiac endothelial cells derived from WT versus CTRP9 KO mice with rat cardiomyocytes in the presence of phenylephrine to simulate a prohypertrophic environment (Figure 4E). As shown in Figure 4F and 4G, cardiomyocytes cocultured with WT cardiac endothelial cells were larger and exerted increased ANP and BNP mRNA expression compared with those cocultured with CTRP9 KO endothelial cells. Together, these results indicate a prohypertrophic role of endothelial-derived paracrine CTRP9 on cardiomyocytes.

CTRP9 Induces Cardiomyocyte Hypertrophy and Cardiac Dysfunction Through the Activation of ERK5
We analyzed the activation of multiple different hypertrophy-related signaling pathways in WT and KO hearts 2 weeks after sham or TAC surgery. As demonstrated in Figure 5A (with quantification in Figures 5B and 5C), the MAP kinase ERK5 and also its phosphorylated (activated) form were strongly upregulated after TAC in WT mice, but this was not seen in the hearts of CTRP9 KO mice. Decreased ERK5 abundance after TAC in KO mice might be the consequence of decreased ERK5 activity, because we found that ERK5 mRNA expression in isolated adult rat cardiomyocytes was markedly suppressed by overexpression of a previously described dominant negative variant of MEK5 (Ad.MEK5KM), which acts as a potent suppressor of ERK5 activity (Online Figure IV). Neither the activation nor the expression of ERK1/2, Akt/Proteinkinase B, BAD, or the AMPK was changed in the hearts of KO versus WT hearts (Figure 5A; Online Figure VA

Figure 5. CTRP9 (C1q-tumor necrosis factor–related protein-9) induces cardiac hypertrophy and cardiomyopathy via the activation of the protein kinase ERK5 (extracellular signal–regulated kinase 5). A, Immunoblot for the indicated proteins from hearts of sham or transverse aortic constriction (TAC) operated wild-type (WT) or CTRP9 knock-out (KO) mice 2 weeks after surgery. B–C, Quantification of the indicated protein ratios from the immunoblots in A as shown. P indicates the phosphorylated form of the protein, while T indicates the total amount of protein (ie, phosphorylated and unphosphorylated form). D, Immunoblot for the indicated proteins from adult cardiomyocytes treated with recombinant (rec.) CTRP9 for the indicated time points. Min denotes minutes. The quantification is shown in E, F. Quantification of the cell area of isolated adult rat cardiomyocytes cultured in media containing phenylephrine (PE), fetal bovine serum (FBS), and treated with Ad.Control (Con), Ad.CTRP9 (CT9), and Ad.MEK5KM (KM, a dominant negative MEK5 variant) as indicated. G, Immunoblot for the indicated proteins of hearts from mice 2 weeks after TAC surgery and 4 weeks after the application of AAV-control (Con) or AAV-CTRP9 (CT9). Treatment with BIX02189 (BIX, a selective inhibitor of MEK5 and ERK5) or DMSO (dimethyl sulfoxide; as control) was conducted for 2 weeks after TAC as indicated. H, Quantification of the immunoblot from G. I, Left ventricular ejection fraction and left ventricular end-diastolic ventricular area (LVEDA) measured by echocardiography in the mice described in G. J, Cardiomyocyte cell size from mice treated as described in G. The numbers in the graphs indicate the number of mice or cell culture dishes analyzed in 3 independent experiments. *P<0.05, **P<0.01, ****P<0.0001.
through VD and VF). An unchanged level of cleaved caspase 3 argued against changes in myocardial cell death between both groups of mice after TAC (Online Figure VA and VE). To more directly assess the impact of CTRP9 on ERK5, recombinant full-length CTRP9 was added to cultured rat adult cardiomyocytes. We detected enhanced activation of ERK5 10 and 20 minutes after stimulation with CTRP9 (Figure 5D and 5E). ERK5 mRNA expression was not increased by CTRP9 overexpression in isolated cardiomyocytes (Online Figure IV). To analyze whether the prohypertrophic effects of CTRP9 were dependent on the activation of ERK5, we overexpressed MEK5KM in rat adult cardiomyocytes to inhibit ERK5 activation.27 Indeed, MEK5KM blocked the increase in cell size induced by CTRP9 (Figure 5F). As the next step, we wanted to test whether inhibition of ERK5 could also ameliorate the development of cardiomyopathy after CTRP9 overexpression during TAC in vivo. For this purpose, we treated mice with AA V-control or AA V-CTRP9 and induced pressure overload by TAC (CTRP9 overexpression is shown in Online Figure IIID). This treatment resulted in increased cardiac ERK5 phosphorylation (but not increased ERK5 protein abundance) in AA V-CTRP9–treated versus AA V-control–treated animals, and this effect was markedly reduced by the MEK5/ERK5 specific inhibitor BIX02189 (Figure 5G and 5H). Interestingly, BIX02189 did not inhibit cardiac ERK5 activation in AA V-control–treated mice, perhaps because the dose was not high enough to inhibit basal TAC-triggered ERK5 activation, although AA V-CTRP9–triggered overactivation of ERK5 could be effectively reduced. ERK1/2 activation was unaffected by CTRP9 or BIX02189 (Figure 5G; Online Figure VIA), as was the rate of cardiac apoptosis (measured as caspase 3 cleavage, shown in Online Figure IIID). The activation or expression of Akt, ERK1/2, BAD, cleaved caspase 3, AMPK, or p70S6K in the myocardium was also not significantly changed by AA V-CTRP9 after sham or TAC surgery, although a minor decrease in Akt phosphorylation and a nonsignificant increase in caspase 3 cleavage were observed because of CTRP9 overexpression after sham treatment (Online Figure VIB through VII). Importantly, CTRP9 overexpression led to reduced cardiac function and enhanced ventricular dilation after 2 weeks of TAC, but this was reversed to the levels of AA V-control mice by administration of BIX02189 (Figure 5I). Cardiomyocyte cell size, which was increased by CTRP9 overexpression, was also significantly reduced by BIX02189 treatment in AA V-CTRP9–treated mice, but no effect was seen in AA V-control mice (Figure 5J).

**GATA4 Acts Downstream of CTRP9-ERK5 in Cardiomyocytes**

To interrogate the downstream targets of ERK5 in response to activation by CTRP9 in cardiomyocytes, we assessed the activation of different transcription factors by luciferase reporter assay in rat adult cardiomyocytes. The known prohypertrophic ERK5 target MEF2 was activated by phenylephrine and fetal bovine serum in cardiomyocytes, and this effect was partially dependent on ERK5 because it could be reduced by MEK5KM, but MEF2 activity was reduced by CTRP9 overexpression (Online Figure VII). The activation of GATA transcription factors was dramatically increased by prohypertrophic stimulation in adult cardiomyocytes, and this effect was significantly enhanced by CTRP9 overexpression during stimulation with phenylephrine and fetal bovine serum (Figure 6A). In turn, GATA activation was strongly inhibited by MEK5KM and by the chemical MEK5/ERK5 inhibitor BIX02189. Importantly, CTRP9 overexpression could no longer enhance GATA activation, when MEK5/ERK5 was inhibited (Figure 6A). These data indicate that GATA activation occurs to a large extent through ERK5 in response to phenylephrine, fetal bovine serum, and CTRP9 in adult cardiomyocytes. Because the prohypertrophic transcription factor GATA4 can be activated through kinase-dependent phosphorylation by ERK1/2 or p38 at its serine residue 105,24,29 which is also a possible target site of ERK5, we assessed GATA4 phosphorylation in nuclear cardiac protein lysates of WT and KO mice and found a marked reduction of GATA4 serine 105 phosphorylation in KO mice after TAC (Figure 6B and 6C). In turn, stimulation with recombinant full-length CTRP9 induced GATA4 phosphorylation at serine 105 in a time-dependent manner (Figure 6D and 6E). Overexpression of CTRP9 via AA V-CTRP9 during TAC stimulation also increased GATA4 phosphorylation in the heart in vivo (Figure 6F and 6G). Finally, coinubcation of recombinant ERK5 with GATA4 in vitro resulted in direct phosphorylation of GATA4 (Figure 6H). Thus, although additional pathophysiological relevant targets of ERK5 are likely, we found CTRP9-ERK5-GATA4 as possible prohypertrophic pathway in cardiomyocytes, and we demonstrate that CTRP9 contributes to cardiac dysfunction during pressure overload at least in part through the activation of ERK5.

**Discussion**

We demonstrate in this study that CTRP9 is induced in the mouse myocardium during cardiac pressure overload, in human hearts with pathological hypertrophy and in serum of patients experiencing aortic stenosis. This is in contrast to ischemic cardiac injury and diabetes mellitus in mice, where CTRP9 abundance was reported to be reduced in serum and adipose tissue, as well as in the heart during high-fat diet.10,14,15,18

The distinct upregulation of CTRP9 in the myocardium suggested a specific function of this protein during pressure overload. Indeed, homozygous and heterozygous genetic ablation of CTRP9 led to a gene dosage–dependent reduction in pathological cardiac hypertrophy versus WT mice in response to TAC. On the cellular level, depletion of CTRP9 during pressure overload led to reduced cardiomyocyte size and reduced cardiac fibrosis. As observed in various other mutant mouse models, reduced cardiac growth and fibrosis during pressure overload was accompanied by improved left ventricular function in CTRP9 KO mice.26,30 In turn, AA V vector–mediated overexpression of CTRP9 in the heart induced hypertrophic cardiac remodeling and fibrosis, increased ventricular dilation, and reduced cardiac function after TAC, indicating that the elevated CTRP9 levels in mice and patients with hypertrophy might facilitate adverse cardiac remodeling. Our AA V–based approach (in which CTRP9 expression is driven by the cytomegalovirus-enhanced myosin light chain promoter) will
lead to expression of CTRP9 in the whole heart, mainly in cardiomyocytes and in the liver. This is different from the physiological situation where CTRP9 in the heart is mainly derived from endothelial cells (discussed in detail below) and where no expression in the liver is observed. However, given the technical difficulty to target endothelial cells by an AA V9 approach and in the light of the following facts that (1) CTRP9 is also expressed in cardiomyocytes (although at a much lower level), (2) it can act in these cells in an autocrine manner, and (3) in general, for a secreted factor, its net functional effects rather than its source might be more important, our approach still reveals important information at least as proof of concept.

According to our data, CTRP9 is maladaptive during pressure overload, but previous data showed that it is adaptive during cardiac ischemic injury, when endogenous CTRP9 is downregulated. It was demonstrated in these studies that CTRP9 reduces infarct size (by inhibiting cell death) after ischemia/reperfusion injury and that it inhibits adverse remodeling after myocardial infarction. Although the different disease models per se might play the biggest role to explain these diverse CTRP9 effects, it is possible that in particular pathologically increased endogenous CTRP9 levels (like we observe in mice early after TAC and in hypertrophic human heart disease) are harmful. One should also consider that the protective effects of CTRP9 administration on cardiac remodeling and survival after myocardial infarction were inferred from administration of recombinant globular CTRP9 (lacking large parts at the N-terminal domain),

![Figure 6. CTRP9 (C1q-tumor necrosis factor-related protein-9) triggers ERK5 (extracellular signal-regulated kinase 5)-dependent phosphorylation and activation of the transcription factor GATA4.](image-url)
but endogenous full-length CTRP9 might be functionally different, for example, because of binding of different receptor or coreceptor proteins. In addition, the prohypertrophic role of CTRP9 that we describe here is in principal not incompatible with its antiapoptotic function during cardiac ischemia because many survival factors (eg, neuregulin-1 or endothelin-1) also trigger cardiomyocyte growth, although we did not find evidence for a cell protective role of CTRP9 during pressure overload as indicated by unchanged cleaved caspase 3 levels (an apoptosis marker) after ablation or overexpression of CTRP9.

We detected CTRP9 expression mainly in endothelial cells of the heart, indicating that it might act on cardiomyocytes in a paracrine manner. Indeed, overexpression of CTRP9 in endothelial cells induced hypertrophy in cocultured adult cardiomyocytes, and this effect was blocked by a neutralizing antibody. More importantly, endogenous CTRP9 derived from cardiac endothelial cells promoted cardiomyocyte hypertrophy in our coculture system. Thus, paracrine CTRP9 from endothelial cells is at least partially responsible for the prohypertrophic role that has been ascribed to these cells during pressure overload. Interestingly, although a higher total abundance of CTRP9 mRNA and protein levels was noted in mouse hypertrophic hearts after TAC, we still detected a small, but significant, decrease in CTRP9 mRNA in cardiac endothelial cells under these circumstances. Because the number of total endothelial cells markedly increases early after TAC surgery by angiogenesis, endothelial cells might still contribute to the increased cardiac CTRP9 levels 2 weeks after surgery, although their continuous decrease thereafter could lead to the reduction of myocardial CTRP9 6 and 12 weeks after the induction of pressure overload. Alternatively, or in addition, a currently unknown cardiac cell type might contribute to cardiac CTRP9 early during pressure overload. The exact clarification of the relative roles of CTRP9 derived from endothelial cells versus that from cardiomyocytes for the development of cardiac hypertrophy will require an endothelial-specific KO mouse.

Which signaling pathways are activated in cardiomyocytes by CTRP9 to induce cardiac hypertrophy? During ischemic injury and proinflammatory stimulation, CTRP9 promotes activation of AMPK and PKA, and both molecules contribute to the protective role of CTRP9 under these circumstances. During pressure overload, in contrast, we did not find evidence for differential activation of cardiac AMPK or PKA (measured as BAD Ser155 phosphorylation) in response to deletion or overexpression of CTRP9. Instead, we observed enhanced activation of ERK5 during stimulation of isolated cardiomyocytes with recombinant CTRP9 and cardiac AAV-CTRP9 treatment, while in CTRP9 KO mice the induction of ERK5 protein levels in response to pressure overload was markedly blunted compared with what is seen in WT mice. The reduced ERK5 expression in CTRP9 KO mice during TAC might be secondary to reduced ERK5 activity because we found that inhibition of ERK5 kinase leads to reduced ERK5 mRNA expression in cardiomyocytes. Cardiomyocyte ERK5 is a known inducer of cardiac hypertrophy and cardiac dysfunction when overactivated in the heart of MEK5 transgenic mice, and it also has been deemed necessary for pressure overload–induced hypertrophy and fibrosis in cardiomyocyte-specific ERK5 KO mice, which exerted less cardiomyocyte hypertrophy and interstitial fibrosis after TAC, similar to what we observed in CTRP9 KO mice. As evidence for the contribution of ERK5 in transducing CTRP9-dependent prohypertrophic and disease triggering signals within cardiomyocytes, inhibition of ERK5 blunted CTRP9 induced hypertrophy in isolated adult cardiomyocytes and improved cardiac dysfunction, hypertrophic remodeling, and cardiac dilatation during CTRP9 overexpression after TAC in mice. In contrast to the CTRP9 KO mice, cardiomyocyte-specific ERK5 KO mice developed cardiomyocyte cell death and cardiac dysfunction after TAC despite decreased cardiomyocyte hypertrophy and decreased fibrosis. These differences could be the consequence of partial ERK5 inhibition in our model versus virtually complete cardiomyocyte-specific ERK5 deletion in the other. In this regard, some ERK5 might be needed for the prevention of cell death, although its overactivation promotes disease during cardiac pressure overload. More work will be needed in the future to define in more detail the exact signaling mechanisms of CTRP9-triggered cardiac dysfunction during pressure overload.

Furthermore, we identified GATA4 as a downstream target of ERK5, which directly phosphorylates the transcription factor in response to CTRP9 stimulation. Phosphorylation at serine 105 is known to activate GATA4, to promote cardiomyocyte hypertrophy, and to maintain heart function. Because enhanced GATA4 activation could therefore account for increased hypertrophy during CTRP9-ERK5 signaling, but likely not for cardiac dysfunction, additional downstream targets of the CTRP9-ERK5 circuit will probably contribute and will need to be identified. Moreover, future studies should clarify how CTRP9 can trigger the activation of different signaling pathways in response to distinct disease stimuli and thereby induce either antiapoptotic or growth-promoting effects. Because this is likely the consequence of differential receptor activation, a genuine CTRP9 receptor or coreceptor protein, in addition to the AdipoR1, which is bound and activated by CTRP9 during ischemia, will likely exist and will have to be identified. In fact, because the AdipoR1 and AdipoR2 both belong to the 11-member family of PAQR (progestin and adipoQ receptors) proteins, one could speculate that other PAQR members might function as CTRP9 receptors.

The identification of a CTRP9 receptor protein in the future is especially important because this and other studies show clear functional divergence and nonredundant function between adiponectin and CTRP9.

Acknowledgments
We are grateful to Eric N. Olson, PhD, University of Texas Southwestern Medical Center, United States, for providing the MEK5KM adenovirus to us.

Sources of Funding
This study was supported by the Deutsche Forschungsgemeinschaft through the Cluster of Excellence Rebirth (EXC 62/1 to K.C. Wollert, J. Bausersch, and J. Heineke), the Heisenberg Program (HE 3658/6-1 and HE 3658/6-2 to J. Heineke) and additional research grants (HE3658/8-1 to J. Heineke and MU1654/9-1 to O.J. Müller).
Disclosures

None.

References

1. Heinke J, Molkentin JD. Regulation of cardiac hypertrophy by intracel-


Novelty and Significance

What Is Known?
- The secreted protein CTRP9 (C1q-tumor necrosis factor–related protein-9) is a paralog of adiponectin that is expressed at high levels in adipose tissue and in the heart and that can also be found in serum.
- The abundance of CTRP9 is reduced in diabetic mice, during high-fat diet and in response to ischemic cardiac injury.
- CTRP9 protects the myocardium during ischemia through inhibition of cardiomyocyte cell death by activating AMP-dependent kinase and protein kinase A signaling.

What New Information Does This Article Contribute?
- CTRP9 is upregulated in the mouse myocardium during pressure overload and in serum and hearts from patients with cardiac hypertrophy (ie, pathological cardiac growth).
- CTRP9 is mainly derived from endothelial cells in the heart and influences cardiomyocytes in a paracrine manner.
- During pressure overload, CTRP9 promotes pathological myocardial hypertrophy, fibrosis, and cardiac dysfunction at least in part via the activation of the protein kinase ERK5, which in turn can activate the hypertrophy-promoting transcription factor GATA4.

This study was designed to examine the role of CTRP9 in cardiac pressure overload, for example in aortic valve stenosis. Cardiac CTRP9 was upregulated in mice after 2 weeks of pressure overload and in the myocardium and serum of patients with cardiac hypertrophy. During experimental pressure overload, CTRP9 is maladaptive because CTRP9 knock-out mice were protected from cardiac hypertrophy, fibrosis, and cardiac dysfunction, while CTRP9 overexpression aggravated these changes. In the heart, CTRP9 is mainly expressed in endothelial cells, which also secrete this protein. We show in coculture experiments in vitro that endothelial cells promote hypertrophy in cardiomyocytes in a paracrine manner. Mechanistically, CTRP9 exerts its hypertrophy-inducing effects by activating the protein kinase ERK5, which in turn activates the prohypertrophic transcription factor GATA4. Therefore, we show for the first time a maladaptive role for CTRP9 during pressure overload and identified a previously unrecognized signaling axis. Future research should examine whether a CTRP9-specific receptor exists, and how this connects to ERK5 and to reduced cardiac function. According to our findings, although CTRP9 has beneficial effects on metabolism and during ischemia, therapeutic use of the protein might be limited by its detrimental effects during cardiac pressure overload.
C1q-TNF-Related Protein-9 Promotes Cardiac Hypertrophy and Failure

_Circ Res._ 2017;120:66-77; originally published online November 7, 2016; doi: 10.1161/CIRCRESAHA.116.309398

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/120/1/66

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/11/07/CIRCRESAHA.116.309398.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Detailed Methods

Experimental Animals

C1qtnf9 (encoding for CTRP9) gene-deleted mice were obtained from the Davis campus of the University of California and were created under the National Institute of Health Knock-Out Mouse Program (KOMP, project number VG12588). In these mice 7725 bp of the mouse C1qtnf9 gene were replaced with a lacZ reporter and a neomycin resistance cassette, which resulted in deletion of the entire coding region of this gene (large parts of exon2, all of exon3 and around half of exon 4). Male littermate homozygous and heterozygous CTRP9 knock-out (KO) on a C57Bl6N background as well as male wild-type mice on a C57Bl6N background (for the AAV9 experiments) were analyzed in this study. Constriction of the transverse aortic arch (TAC) was performed in 8- to 10 week old KO or wild-type mice around a 25-gauge needle, as previously described1, 2. For the mice after AAV9 injection TAC was conducted using a 26-gauge needle. BIX02189 (Selleckchem, Houston, Texas, USA) was administered to a group of mice as indicated at 5mg/kg/mouse every second day, starting on the second day after TAC surgery. Tail cuff blood pressure was measured in awake mice (BP 2000 Blood Pressure Analysis System, Visitech). All procedures involving the use and care of animals were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Research Council (NIH Publication No. 85-23, revised 1996) and the German animal protection code. Approval was granted by the local state authorities (33.14-42502-04-11/0608) and institutional guidelines were followed.

Human heart samples

Analysis of human heart tissue was permitted by the Massachusetts General Hospital Institutional Review Board (US), and by the Ethical Committee of the Hannover Medical School, Germany (Az. Z 14.06-A 1871-30724/98). Control tissue was taken from victims of traffic accidents or from healthy heart organ donors, when the organ was not eligible for transplantation. Hypertrophic human hearts were reported to be taken from patients initially considered as organ donors with known arterial hypertension without any history of congestive heart failure, but with marked cardiac hypertrophy as revealed by echocardiography and/or post-explant examination 3. Samples from failing hearts were from patients with end-stage heart failure undergoing cardiac transplantation 3.

Generation and administration of AAV9 vectors

The entire coding region of the CTRP9 cDNA was subcloned as KpnI/BsrGI fragment into the pdsCMV-MLC260-EGFP vector downstream of a CMV enhanced 260-bp myosin light chain promoter (MLC260). The resulting plasmid was used to produce AAV9-CTRP9 vectors as previously described 4. AAV9 vectors that express renilla luciferase downstream of the MLC260 promoter were generated and used as control (AAV9-control). 1 x 10^{12} vg of AAV9-control or AAV9-CTRP9 were injected intravenously into tail veins of 8 weeks old male C57Bl6N mice (Charles River, Sulzfeld, Germany). Sham or TAC surgeries were conducted 2 weeks after AAV9 injection.

Echocardiography

Echocardiography was performed with a linear 30MHz transducer (Vevo770, Visualsonics, Toronto, Canada) in mice that were sedated with 1-1.5% isoflurane and placed on a heating pad to maintain body temperature. LV end-diastolic area (LVEDA), enddiastolic average wall thickness (Wth) and enddiastolic volume (EDV) as well as end-systolic area (LVESA) and
endsystolic volume (ESV) were recorded or calculated from the long axis parasternal view. Ejection fraction was calculated as [(EDV-ESV)/EDV] x 100.

**Histological analysis**

After removal from the chest cavity, the hearts were rinsed in PBS and 0.5M KCL. Transversal frozen sections (7µm in thickness) of the myocardium were generated. To measure cardiomyocyte dimensions, the cardiomyocyte cell membranes were stained with tetramethyl rhodamine isothiocyanate-conjugated wheat-germ agglutinin (TRITC-WGA, Sigma Aldrich) and the nuclei with 4’,6-diamidino-2-phenylindole (DAPI, Vector Laboratories). The cellular size of cardiomyocytes in situ was determined at the level of the nucleus in longitudinal cut sections with Image J software. Fluorescein-labelled GSL I-isoelectin B4 (Vector Laboratories, Burlingame, CA) was used to visualize cardiac endothelial cells. β-galactosidase protein was visualized with an antibody from Merck/Millipore (AB1211; Darmstadt, Germany) and an Alexa Fluor 633-labeled secondary antibody from Invitrogen (Carlsbad, CA). The rabbit polyclonal antibody against the globular domain of CTRP9 was a kind gift of G. William Wong (John Hopkins University School of Medicine, Baltimore, Maryland). Fibrosis was quantified with the Sirius Red staining method in 12µm thick cryosections. Subsequently, the fraction of the fibrotic area (stained in red) from the total myocardial area was determined using Adobe Photoshop Imaging Software. To assess cardiac β-galactosidase activity, the hearts were fixed for 12 hours at 4°C in PBS containing 0.4% glutaraldehyde, 0.01% Na deoxycholate, 0.1% NP40, 0.1 M MgCl₂, and 5 mM EGTA. Subsequently, they were embedded in OCT and transversal frozen sections were generated. The frozen sections were incubated for 24 hours at 37°C in a staining solution [1 mM MgCl₂, 20 mM K₃Fe(CN)₆, 20 mM K₄Fe(CN)₆, 1 mg/ml X-gal in PBS] and mounted. Images were acquired with an Axiovert microscope (Carl Zeiss, Jena, Germany).

**RNA isolation and real-time PCR**

RNA from mouse hearts or human hearts was isolated with the Trifast reagent (Peqlab, Erlangen, Germany). cDNA was generated from RNA with the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). Quantitative PCR was performed using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and the MX4000 multiplex QPCR system from Stratagene. Gene-expression was normalized to Gapdh mRNA expression or quantified in comparison to a plasmid based DNA standard using a calibration curve. The PCR primers are listed in the Supplemental Table S2.

**Immunoblotting**

Immunoblot analysis was performed with antibodies to the following proteins (with catalog number and producing company in parenthesis): Akt (9272), phospho-Akt (on Ser473, 9271), AMPK-α (2532), phospho-AMPK-α (on Thr 172, 2525), ERK 1/2 (9102), phospho-ERK1/2 (on Thr202 and Tyr204, 9101), ERK5 (3552), phospho-Bad (on Ser155, 9297), Bad (9292), cleaved caspase 3 (9661), p70S6 Kinase (9202), phospho-p70S6 Kinase (on Ser371, 9208) (all from Cell Signaling Technology), phospho-ERK5 (Thr218/Tyr220, Santa Cruz, sc-16564), phospho-GATA4 (S105, abcam, ab5245), GATA4 (Santa Cruz, C20) and actin (2066, Sigma Aldrich). The antibody against CTRP9 is described under Histological analysis.

**Cell culture and cardiac cell isolation**

Adult ventricular cardiomyocytes were prepared from adult Sprague-Dawley rats and mice. In brief, as described in detail elsewhere, the hearts are removed from the rat chest cavity and retrograde perfusion was conducted with digestion buffer (containing Liberase DH Blendzyme, Roche Diagnostics, Mannheim, Germany) using a Langendorff system. For RNA isolation
(mouse cardiomyocytes), the cells were directly transferred to the Trifast reagent. For culturing, calcium was re-introduced and the cells were plated on laminin (BD Biosciences, Franklin Lakes, NJ) coated culture dishes. After one hour of plating, the medium was changed to maintenance medium containing blebbistatin (25µM). On the next day, adenoviral vectors encoding for either β-galactosidase (Ad.Control), mouse CTRP9 (Ad.CTRP9), MEK5KM (Ad.MEK5KM) or a GATA or MEF2 dependent luciferase construct (Ad.GATA-Luc, Ad.MEF2-Luc) were added to the cells for two hours, and stimulation with FBS (2%), phenylephrine (20µM), or BIX02189 (10µM) was subsequently conducted for 48 hours as indicated. The CTRP9 antibody (described above) was added with a dilution of 1:500 to the media to neutralize the action of CTRP9. Recombinant human CTRP9A (full length) was purchased from BioVendor and was used at a concentration of 4µg/µl. Cell size was determined by planimetry. The luciferase activity from adult cardiomyocytes was measured as previously described and normalized to total protein content 7, 8.

Primary juvenile endothelial cells were isolated from the hearts of 7-11 days old mice as previously described by Lim and Luscinskas 9. In short, the mouse hearts were digested by collagenase with subsequent purification of endothelial cells by Dynabeads (Invitrogen) coated with CD31 antibody (BD Pharmingen). The cells were plated on gelatine coated cell culture plates. When grown to confluency, the cells were purified with Dynabeads coated with CD102 antibody (BD Pharmingen) and plated for experiments.

For the analysis of cell culture supernatant primary cardiac endothelial cells were seeded in independent plates with media containing FBS. Three hours after plating, the cells were washed three times with serum-free media. The endothelial cells were cultured for 18 hours without serum, before the media was collected and pooled from three 10-cm dishes per sample and then concentrated with centrifugal filters (Amicon® Ultra-4) by centrifuging at 7,500 x g at 4°C.

Adult heart endothelial cells and fibroblasts were isolated from hearts of adult mice by digestion with collagenase I (Worthington) and subsequently using CD146 coated micro beads (for endothelial cells) and then feeder removal micro beads (for fibroblasts) with MACS technology from Miltenyi Biotec. The isolated cells were directly used for RNA extraction.

Co-culture system

Cultured C166 mouse embryonic yolk sac endothelial cells were transduced with Ad.Control or Ad.CTRP9. After 24 hours the endothelial cells were trypsinized and seeded on top of cell culture plate inserts (Millipore® Millicell®) with 0.4 µm sized pores and cultivated in 6-well culture dishes for 24 hours. Adult rat cardiomyocytes were subsequently cultured for 24 hours at the bottom of 6-well culture dishes (with the endothelial cells on the culture plate insert on top). The CTRP9 neutralizing antibody (at 1:500 dilution) or control rabbit IgG (Santa Cruz, sc-2027, 4µg/ml final concentration) as well as phenylephrine (20µM) were added to the media as indicated. In separate experiments, mouse heart endothelial cells from WT or CTRP9 KO mice were plated on the cell culture inserts and co-cultured with rat adult cardiomyocytes (on the bottom of a 6-well culture dish) for 48 hours.

In vitro kinase assay

A GST-GATA4 fusion construct containing the amino acids 2-207 or GST alone was incubated in the absence or presence of recombinant ERK5 (Sigma, SRP5242) in kinase assay buffer (25mM MOPS, pH7.2, 12.5mM glycerol 2-phosphate, 25mM MgCl₂, 5mM EGTA, 2mM EDTA, 0.25mM DTT) in the presence of 5µCi γ³³P ATP for 30 min at 30°C before samples were subjected to SDS-PAGE and visualized by Phosphor-Imager analysis (Amersham Pharmacia Biotech).
Supplemental References


5. Louch WE, Sheehan KA, Wolska BM. Methods in cardiomyocyte isolation, culture, and gene transfer. *J Mol Cell Cardiol*. 2011;51:288-298


Online Figure I: Specificity of cardiac cell isolation demonstrated by qPCR. qPCR to quantify Cd31, αMHC and Col1a mRNA (normalized to Gapdh mRNA) from endothelial cells (ECs), cardiomyocytes (CMs) and fibroblasts isolated from mouse hearts one week after sham (Sh) or TAC (T) surgery.
Online Figure II: Further characterization of CTRP9 knock-out mice (A) Body weight of wild-type (WT), heterozygous (HET) or homozygous CTRP9 knock-out mice (KO) two weeks after sham surgery or transverse aortic constriction (TAC). (B) Systolic and diastolic blood pressure in 10 weeks old WT and KO mice. (C) Capillaries per cardiomyocyte in the myocardium of mice as indicated. The numbers in the graphs indicate the number of mice analyzed. ***p<0.001
Online Figure III: Further characterization of mice and rat cardiomyocytes during CTRP9 overexpression. (A) Heart/weight body weight (HW/BW) ratio 10 weeks after the application of AAV-control (Con) or AAV-CTRP9 (CT9) and 8 weeks after sham or TAC surgery. (B) Capillaries/cardiomyocyte ratio in the myocardium of mice as indicated. The numbers in the graphs indicate the number of mice analyzed. (C) Immunoblot for CTRP9 from serum of mice as indicated four weeks after administration of AAV-control or AAV-CTRP9. (D) Immunoblot for the indicated proteins of hearts from mice two weeks after TAC surgery and four weeks after the application of AAV-control (Con) or AAV-CTRP9 (CT9). Treatment with BIX02189 (BIX, a selective inhibitor of MEK5 and ERK5) or DMSO (as control) was conducted for two weeks after TAC as indicated. The quantification of the blots is shown below. (E) Immunoblot for the indicated proteins from adult rat cardiomyocytes infected with Ad.Con or Ad.CT9 and treated with PE and FBS as indicated. **p<0.01
Online Figure IV: ERK5 mRNA expression is dependent on ERK5 activation. ERK5 mRNA expression (determined by qPCR, normalized to Gapdh mRNA) in isolated adult cardiomyocytes treated with Ad.Control (Con), Ad.CTRP9 (CT9) or Ad.MEK5KM (KM, a dominant negative MEK5 variant) as indicated. The numbers in the bar indicates the number of samples analyzed. ***p<0.001
Online Figure V: Further characterization of signal transduction in mouse hearts lacking CTRP9. (A) Immunoblot for the indicated proteins from hearts of sham or TAC operated wild-type (WT) or CTRP9 knock-out (KO) mice two weeks after surgery. (B-F) Quantification of the indicated protein ratios from the immunoblot in (A) as shown. P- indicates the phosphorylated form of the protein, while T- indicates the total amount of protein (i.e. phosphorylated and unphosphorylated form). The numbers in the bar indicates the number of samples analyzed by immunoblot.
Online Figure VI: Further characterization of signal transduction in mouse hearts with CTRP9 overexpression. (A) Quantification of the P-ERK1/2/T-ERK1/2 levels from the immunoblot shown in Figure 5G. (B) Immunoblot for the indicated proteins in mice 10 weeks after application of AAV-control (Con) or AAV-CTRP9 (CT9) and 8 weeks after sham or TAC surgery. (C-H) Quantification of the indicated protein ratios from the immunoblot in (B) as shown. P- indicates the phosphorylated form of the protein, while T- indicates the total amount of protein (i.e. phosphorylated and unphosphorylated form). The numbers in the bar indicates the number of samples analyzed by immunoblot.
Online Figure VII: Further characterization of signal transduction in response to CTRP9. Relative MEF-luciferase activity of isolated adult cardiomyocytes treated with Ad.Control (Con) or Ad.CTRP9 (CT9), phenylephrine (PE), fetal bovine serum (FBS), Ad.MEK5KM (KM, a dominant negative MEK5 variant) or with BIX02189 (BIX), as indicated. The numbers in the bar indicates the number of samples analyzed. ****p<0.0001
Online Table I:

Clinical characteristics of the patients (all male) with aortic stenosis. Data are displayed as mean ± SEM or in % of all patients as indicated. BMI denotes body mass index. NYHA indicates New York Heart Association Class.

<table>
<thead>
<tr>
<th></th>
<th>Aortic stenosis N=20</th>
<th>Normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>78.3±1.64</td>
<td></td>
</tr>
<tr>
<td>BMI (g/m²)</td>
<td>26.02±0.56</td>
<td>18.5-25</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>42.48±4.4</td>
<td>&gt;55</td>
</tr>
<tr>
<td>Septum thickness (mm)</td>
<td>14.15±0.69</td>
<td>≤11</td>
</tr>
<tr>
<td>Aortic valve gradient (mmHg)</td>
<td>34.07±3.4</td>
<td>≤20</td>
</tr>
<tr>
<td>Aortic valve area (cm²)</td>
<td>0.75±0.05</td>
<td>&gt;1.5</td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>135.7±13.5</td>
<td>59-104</td>
</tr>
<tr>
<td><strong>Clinical Classification</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NYHA I [number of patients]</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>NYHA II [number of patients]</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>NYHA III [number of patients]</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>NYHA IV [number of patients]</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Online Table II:

Primer information for qRT PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>species</th>
<th>5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh-forward</td>
<td>mouse</td>
<td>CCGCATTTCTTGTGCAGT</td>
</tr>
<tr>
<td>Gapdh-reverse</td>
<td>mouse</td>
<td>CATCACCTGGCCTACAGGAT</td>
</tr>
<tr>
<td>CTRP9-forward</td>
<td>mouse</td>
<td>AATCCAGGTCACAATGGCCTACCT</td>
</tr>
<tr>
<td>CTRP9-reverse</td>
<td>mouse</td>
<td>ATTCCATCCTTTCCCTGGGACCACCA</td>
</tr>
<tr>
<td>CTRP9-forward</td>
<td>human</td>
<td>ATGAGGATCTGGTGCTTTCTGCTTT</td>
</tr>
<tr>
<td>CTRP9-reverse</td>
<td>human</td>
<td>CCTCGTTCTCCCTTTCTCTCC</td>
</tr>
<tr>
<td>ANP-forward</td>
<td>mouse</td>
<td>ATTTGACAGGATTGGAGCCCAAGAGT</td>
</tr>
<tr>
<td>ANP-reverse</td>
<td>mouse</td>
<td>TGACACACCAAAAGGCTTCTAGAT</td>
</tr>
<tr>
<td>BNP-forward</td>
<td>mouse</td>
<td>CTCGGCTCCCTTCCAGCTTT</td>
</tr>
<tr>
<td>BNP-reverse</td>
<td>mouse</td>
<td>AGCCAGGAGTCTTTTCCTACAACAA</td>
</tr>
<tr>
<td>Col1a-forward</td>
<td>mouse</td>
<td>CCGCTGCTCAAGATGTC</td>
</tr>
<tr>
<td>Col1a-reverse</td>
<td>mouse</td>
<td>CCTCGCTCCTCCAGCTTT</td>
</tr>
<tr>
<td>Cd31-forward</td>
<td>mouse</td>
<td>CACTCCGGGAAATACAAATGCACA</td>
</tr>
<tr>
<td>Cd31-reverse</td>
<td>mouse</td>
<td>GCAAGGAACAAATGGACCAGGTCAGGA</td>
</tr>
<tr>
<td>αMHC-forward</td>
<td>mouse</td>
<td>ACTGTGGTGCTCCTCGTCC</td>
</tr>
<tr>
<td>αMHC-reverse</td>
<td>mouse</td>
<td>GCCCTGCTGCTTCCCTTCT</td>
</tr>
<tr>
<td>ERK5-forward</td>
<td>rat</td>
<td>CTCTACTGACCTTTGGCTG</td>
</tr>
<tr>
<td>ERK5-reverse</td>
<td>rat</td>
<td>CTGTGTTGTTGGTGTTGGGTGTTG</td>
</tr>
<tr>
<td>ANP-forward</td>
<td>rat</td>
<td>ATCTGCCCCCTTGGAAAAGCA</td>
</tr>
<tr>
<td>ANP-reverse</td>
<td>rat</td>
<td>GGATTTGCTCTGGCGATCTGGTC</td>
</tr>
<tr>
<td>BNP-forward</td>
<td>rat</td>
<td>ATCGGCGCAGTCTCATCGCTT</td>
</tr>
<tr>
<td>BNP-reverse</td>
<td>rat</td>
<td>GGTGGTCCAGAGCTTGAGGGA</td>
</tr>
</tbody>
</table>