Cardiac myocyte p38α kinase regulates angiogenesis via myocyte-endothelial cell cross-talk during stress-induced remodeling in the heart

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Stress-induced p38 mitogen-activated protein kinase (MAPK) activity is implicated in pathological remodeling in the heart. For example, constitutive p38 MAPK activation in cardiomyocytes induces pathological features, including myocyte hypertrophy, apoptosis, contractile dysfunction, and fetal gene expression. However, the physiological function of cardiomyocyte p38 MAPK activity in beneficial compensatory vascular remodeling is unclear. This report investigated the functional role and the underlying mechanisms of cardiomyocyte p38 MAPK activation in cardiac remodeling induced by chronic stress. Using both in vitro and in vivo model systems, we found that p38 MAPK activity is required for hypoxia-induced pro-angiogenic activity from cardiomyocytes and that p38 MAPK activation in cardiomyocyte is sufficient to promote paracrine signaling-mediated, pro-angiogenic activity. We further demonstrate that VEGF is a paracrine factor responsible for the p38 MAPK-mediated pro-angiogenic activity from cardiomyocytes and that p38 MAPK pathway activation is sufficient for inducing VEGF secretion from cardiomyocytes in an Sp1-dependent manner. More significantly, cardiomyocyte-specific inactivation of p38α in mouse heart impaired compensatory angiogenesis after pressure overload and promoted early onset of heart failure. In summary, p38α MAPK has a critical role in the cross-talk between cardiomyocytes and vasculature by regulating stress-induced VEGF expression and secretion in cardiomyocytes. We conclude that as part of a stress-induced signaling pathway, p38 MAPK activity significantly contributes to both pathological and compensatory remodeling in the heart.

In response to mechanical stress or loss of myocytes due to pathological injury, a network of stress-signaling pathways is activated in the myocardium, including stress-activated MAP kinase p38. In previous studies from both in vitro and in vivo, activated p38 MAPK activity in cardiomyocytes was shown to induce many aspects of pathological remodeling in heart, including hypertrophy, myocyte death, loss of contractility, interstitial fibrosis, fetal genes, and proinflammatory gene induction. Constitutive activation of the p38 pathway in mouse heart was associated with lethal cardiomyopathy, whereas inhibiting p38 MAPK activity attenuated myocardial injury to ischemia/reperfusion injury or ameliorated the onset of heart failure in experimental animals. These studies laid down the mechanistic foundation for a human clinic trial for ischemic heart failure by targeted inhibition of p38 kinase. However, p38 MAPK activity was also reported to be reduced in end-stage heart failure, and genetic inactivation of p38α in mouse heart actually accelerated the progression of heart failure under chronic stress. These observations seemingly contradict to the previous notion that the functional consequence of p38 MAPK activation in heart is largely pathological without a significant beneficial effect.

It is known that both adaptive and maladaptive remodeling occur in stressed heart. In addition to interstitial fibrosis, fetal gene induction, and myocyte hypertrophy, changes in the coronary and capillary network of the heart is also a major part of the remodeling process. In the heart, adequate perfusion is required to maintain cardiac function. Therefore, cardiac vascular function is constantly regulated by cross-communication between myocytes and endothelium. As in other organs, the cardiac vascular function can be regulated in an acute fashion by vasoconstriction or vasodilation (1) or more gradually through the growth of new vessels and angiogenesis under chronic conditions (2). Recently a number of studies demonstrate that myocyte hypertrophy is accompanied by a corre-
sponding increase in capillary density (3–6). Furthermore, they have also shown that a reduction in capillary density plays a critical role in the transition from a compensated hypertrophy to a decompensated state of heart failure. Indeed, therapeutic angiogenesis in the heart has been the focus of a number of investigations with significant potential (2).

Angiogenesis is a complex process that requires coordinated action from multiple cytokines secreted from both the surrounding tissues and the vascular cells themselves (7, 8). These cytokines act in either a paracrine or autocrine manner upon endothelial cells, vascular smooth muscle cells, and pericytes. Although the cytokines and their effect on vascular cells are well studied, the signaling pathways that promote angiogenic cytokine production for paracrine signaling, particularly in stressed cardiomyocytes, are not well understood.

In this report we employed both in vitro and in vivo model systems to interrogate the function of p38α, the dominant isoform in mouse heart, in the context of stress response in heart. We found that p38 MAPK activation in cardiomyocytes was sufficient and necessary for the induction of a pro-angiogenic activity secreted from the stressed cardiomyocytes. We further demonstrated that vascular endothelial growth factor (VEGF) was responsible for the pro-angiogenic paracrine activity induced by p38 MAPK in cardiomyocyte. VEGF was induced in stressed myocytes or intact hearts in a p38-dependent manner, and VEGF mRNA levels and its secretion was induced by p38 activation via Sp1 transcription factor in cardiomyocytes. Most relevantly, we found that the cardiomyocyte-specific inactivation of p38α led to a blunted angiogenesis after mechanical overload in intact heart associated with an accelerated progression to heart failure. Therefore, we have uncovered an unexpected beneficial role for the cardiomyocyte p38α activity in promoting cardiac angiogenesis by regulating VEGF induction in response to stress. This finding offers a mechanistic basis for the contradicting functions of stress signaling during cardiac remodeling and demonstrates a complex cell-cell communication network regulated by p38 MAPK pathway that has both beneficial and pathological consequences in a stressed heart.

Results

p38 MAPK activity regulated pro-angiogenic paracrine activity in cardiomyocyte

One of the most potent inducers of angiogenesis among different cell types is hypoxia. In neonatal rat ventricular myocyte (NRVMs) cultures (with >90% pure for cardiomyocytes; see Fig. 1, 4 h of hypoxia robustly induced p38 MAPK activation, as demonstrated by phoso-p38/total p38 (Fig. 2A). The conditioned media from the hypoxia-treated NRVMs promoted vascular tube formation compared to untreated cells (Fig. 2B). Furthermore, pretreatment with 2 μM SB202190, a p38 specific inhibitor in NRVMs, significantly reduced this hypoxia-induced pro-angiogenic activity (Fig. 2B). To test whether the p38 MAPK activity induced during hypoxia is sufficient to promote pro-angiogenic paracrine activity from myocytes, NRVMs were transfected with Adv-MKK3bE, an upstream kinase with specific activity for p38 MAPK without a significant impact on ERK or Akt activities (Fig. 3A) (9). The conditioned media from the p38-activated NRVMs were applied to porcine aortic endothelial cells to measure the pro-angiogenic ability as described (10). The conditioned media from the MKK3bE-expressing NRVMs had a potent pro-angiogenic activity (2.1-fold over control) similar to that observed from the hypoxia-treated NRVMs (Fig. 3, B and C). Co-expressing p38α dominant negative (DN) mutant in NRVMs significantly reduced MKK3bE-induced paracrine activity for endothelial cell growth (Fig. 3, B and C). These results suggest that cardiomyocyte p38α activity is both necessary and sufficient for stress-induced pro-angiogenic paracrine activity from cardiomyocytes.
Cardiomyocyte p38 MAPK activity regulated VEGF expression and secretion

To investigate the molecular basis of p38 MAPK-mediated pro-angiogenic paracrine activity in cardiomyocytes, we investigated the impact of p38 MAPK activation on pro-angiogenic factor VEGF in cardiomyocytes. As shown in Fig. 4A, activation of p38 MAPK led to a significant increase (3.9-fold) in VEGF mRNA levels in myocytes. The induction level was significantly reduced with the co-expression of p38\textsuperscript{DN} or pre-treatment with p38 inhibitor SB202190 (Fig. 4B). ERK and AKT are both implicated in VEGF regulation (11, 12). However, co-expression of p38\textsuperscript{DN} with MKK3b(E) in NRVMs actually increased the basal phospho-Akt levels modestly (Fig. 3A) but reduced VEGF mRNA induction. Similarly, inhibiting ERK activity with U0126 did not significantly affect MKK3b(E)-induced VEGF expression (Fig. 4C). Therefore, specific activation of p38\textalpha is sufficient to induce VEGF mRNA levels in myocytes. Reflecting what was seen at the mRNA level, myocytes expressing MKK3b(E) showed an increase in total intracellular VEGF protein levels (Fig. 4D), whereas co-expressing with p38\textalpha DN reduced it. Finally, the conditioned media collected over a 24-h incubation period from the NRVMs treated with Adv-MKK3b(E) had significantly higher concentrations of VEGF than those from cells treated with Adv-LacZ (2.3-fold, 3.7-fold, and 4.8-fold at 48, 72, and 96 h post-infection, respectively) or Adv-p38\textalpha-DN (2.3-fold, 3.6-fold, and 2.5-fold at 48, 72, and 96 h post-infection, respectively) (Fig. 4E). Furthermore, co-expressing p38\textalpha DN and MKK3b(E) resulted in a significant decrease in VEGF induction in the conditioned media (23.2% and 23.9% reduction at 48 and 72 h post infection, respectively), and the same trend remained at the 96-h time point where the decrease (31.0%) had \( p = 0.084 \) (Fig. 4E). Because p38 activation in cardiomyocytes can lead to cardiomyocyte hypertrophy (13, 14), we examined the effect of pro-hypertrophic treatment of phenylephrine in NRVMs but observed no significant impact on the VEGF expression (Fig. 4F). In contrast, phenylephrine (PE)-treated NRVMs showed significant hypertrophy in size and induction of ANF gene expression (Fig. 4, G–I) associated with sustained changes in MAP kinases and AKT signaling (Fig. 4, J and K). These results indicate that p38 MAPK...
activity in cardiomyocytes is both sufficient and necessary for VEGF expression and secretion. VEGF induction is not a direct consequence of cardiomyocyte hypertrophy but, rather, a specific downstream event of p38-mediated stress response.

**VEGF induction was essential to p38 MAPK-mediated pro-angiogenic paracrine activity from cardiomyocytes**

We further examined the role VEGF induction in p38 MAPK-mediated pro-angiogenic paracrine activity from cardiomyocytes. PAE-VEGFR2 cells treated with media from MKK3b(E)-expressing cardiomyocytes showed a significant level of phospho-VEGFR2 compared with the other treatment groups (Fig. 5A). Furthermore, treatment of the NRVMs media with an anti-VEGF antibody before application in the in vitro angiogenesis assay blocked the pro-angiogenic effect significantly (decreased 62.4%, Fig. 5, B and C). This result indicates that VEGF induction is essential to p38 MAPK-mediated pro-angiogenic paracrine activity from cardiomyocytes.
It is reported that SP1, HIF1α, siPGC1α, COX2, GATA4, and MEF2c are potential downstream targets of p38 MAPK, and SP-1, HIF-1α, COX-2, GATA-4, and MEF-2c are also implicated in VEGF regulation in different cell types. Using siRNAs specific for these genes, we tested their specific contribution to p38 MAPK-mediated VEGF induction in cardiomyocytes. As shown in Fig. 6, siRNA treatments specifically knocked down SP1 (79.5%), HIF1α (85.8%), PGC1α (62.5%), COX2 (69.7%), GATA4 (61.6%), and MEF2c (67.3%) expression when compared with samples treated with nonspecific siRNA in cultured NRVMs 24 h before infection with adenovirus. VEGF mRNA was measured 48 h after MKK3bE expression. As shown in Fig. 6G, knocking down SP1 reduced MKK3bE-mediated induction of VEGF expression, whereas knocking down other genes did not (Fig. 6, H–L). Although HIF1α knockdown resulted in a modest 12.6% decrease in VEGF expression, this change did not reach a significant level (p = 0.092). Therefore, p38-mediated VEGF induction is mediated at least in part by SP1-dependent transcription.

Inactivation of p38α activity in cardiomyocyte-impaired vascular remodeling in response to pressure overload

To investigate the role p38α MAPK plays in myocyte-endothelial cell cross-talk in vivo, we examined whether or not the loss of p38α would affect the angiogenic response in the intact heart both at baseline and after pressure overload induced by transverse aortic constriction (TAC). Cardiac-specific knockout (CKO) of p38α was accomplished by cardiac-specific cre/loxP-mediated recombination (15, 16). The resulting p38α CKO animals showed a significant loss of p38α protein in the myocardium (Fig. 7A) (15). Similar to what was reported earlier (16), the p38α CKO mice were phenotypically normal at baseline, showing no gross anatomical abnormalities in heart (Fig. 7B) and exhibited no baseline functional differences in heart rate (HR), ejection fraction (EF), or fractional shortening (FS) (Fig. 7C).

In agreement with earlier reports, we observed that p38α CKO hearts developed an accelerated progression to heart failure after TAC without a significant impact on hypertrophy (Fig. 8, A–C). As shown in Fig. 9, cross-sectional areas measured from WGA-stained sections showed no significant differences between p38α CKO and wild-type (WT) hearts 28 days after TAC (Fig. 9, A and B). We also measured the fibrotic area and found no significant difference between p38α CKO and WT hearts 28 days after TAC (Fig. 9, C and D). The capillary density in the myocardium showed no significant difference between p38α CKO and WT hearts at baseline (Fig. 10, A and B), and the observed ratio of capillary/myocyte in the ventricle was in good agreement with other studies (17, 18). After 28 days of TAC, capillary density in p38α WT controls was significantly lower than in WT hearts at baseline. This suggests that p38α plays a crucial role in maintaining angiogenic function in the heart during pathological states.
p38α MAP kinase regulates angiogenesis in the stressed heart

Discussion

In this study we demonstrated that p38 MAPK activation is both necessary and sufficient to a stress-induced pro-angiogenic paracrine activity from cardiomyocytes. VEGF expression is induced through p38 MAPK/SP1 pathway in cardiomyocytes and is shown to be responsible for this pro-angiogenic paracrine activity. Loss of p38α activity from adult cardiomyocytes impairs stress-induced angiogenesis and vascular remodeling in intact heart, leading to accelerated heart failure after pressure overload. Therefore, all these data provide the first in vitro and in vivo evidence that a pathologically related stress-response pathway, p38 MAPK, also has an important beneficial role in compensatory vascular remodeling by mediating the cross-talk between cardiomyocytes and endothelial cells via a VEGF-dependent paracrine signaling mechanism.

Previous studies in a number of cell types, including myocytes, have shown the importance of p38 MAPK signaling in inflammatory cytokine production. Our study provides direct evidence that the p38 MAPK pathway also plays a critical role in angiogenesis by inducing a potent paracrine factor VEGF from cardiomyocytes. This observation is consistent with similar observations in other cell types, including breast cancer cells (19), Lewis lung carcinoma cells (20), osteoblasts (21), skeletal muscle (22), and vascular smooth muscle (21, 23–25). One previous study showed that IL-1β treatment of cardiomyocytes increased VEGF in a p38-dependent manner (26), suggesting a potential synergy between these two downstream events of p38 MAPK signaling: inflammatory response and angiogenesis.

Our study established the underlying mechanism of VEGF induction via the p38 MAPK/SP1 pathway as well as the functional link between p38 MAPK activity and VEGF expression in cardiomyocyte. Previous work showed that a cardiomyocyte-enriched transcription factor GATA4 is a downstream target of p38, MAPK and its transcriptional activity is induced by activated p38 MAPK (27). GATA4 is also reported to regulate VEGF induction (29, 30). PGC1α is reported to regulate angiogenesis via VEGF induction in skeletal muscle and is a downstream target of p38 MAPK (31, 32). COX2 is a well known downstream target of p38 MAPK as well as the transcription factor factor that is activated by p38 MAPK and induces angiogenesis via VEGF induction (29, 30). PGC1α is reported to regulate angiogenesis via VEGF in skeletal muscle and is a downstream target of p38 MAPK (31, 32). COX2 is a well known downstream target of p38 MAPK in heart, and it is reported to regulate VEGF expression (33, 34). HIF1α is one of the most well known activators of VEGF expression that is also reported to be regulated by p38 MAPK activity (35–40). HIF1α expression in hearts was also reduced when treated with a p38 inhibitor in vivo (41). We found that SP1 knockdown resulted in a 20.4% decrease in p38-induced VEGF expression. However, the loss of function studies reported here indicated that GATA4, MEF2c, PGC1α, and COX2 were not required for p38-mediated VEGF induction, and loss of HIF1α during p38 activation only resulted in a modest but insignificant reduction of VEGF mRNA. Therefore, p38 MAPK-mediated VEGF regulation in stressed myocardium is most likely carried out in a SP1-dependent mechanism but in GATA4, MEF2C, PGC1α, COX2, and HIF1α independent mechanisms. Because inflammatory cytokines, including IL-1β and TNFα (42, 43), are regulated by p38 MAPK and they can function as a potent inducer of VEGF expression, p38 MAPK may function through proinflammatory cytokines and induce VEGF expression indirectly via autocrine effects of other cytokines. It is intriguing that SP1 appears to be specifically responsible for p38α MAPK-mediated VEGF induction in heart. The specific molecular basis for such a specificity remains enigmatic, and molecular mechanisms involved p38 MAPK-mediated VEGF regulation in heart should be further investigated.
Figure 6. p38-induced VEGF expression in cardiomyocytes was mediated by SP1. A–F, mRNA levels of siRNA target genes in NRVMs treated with adenoviral vectors expressing LacZ or MKK3b(E) and different siRNAs for SP1, HIF1α, PGC1α, COX2, GATA4, and MEF2c as indicated. G–L, mRNA levels of VEGF from the same samples (A–F), respectively. *, p < 0.05 (versus no treatment or siScramble); §, p < 0.05 (versus MKK3b(E) + siScramble).
In the myocyte-specific p38 MAPK knock-out mouse hearts (CKO), VEGF expression and vascular density, as measured from immunohistochemistry and mRNA levels of vascular markers (CD31, VEGFR2, and VE-CAD), were not significantly different from the wild-type controls at the basal state. Therefore, p38 MAPK activity is not essential for normal vascular homeostasis in heart. However, myocyte-specific loss of p38 kinase activity significantly blunted angiogenic growth after chronic pressure overload as supported by histological and molecular evidences. Therefore, p38 \( \text{MAPK} \) activity appears to play a specific and essential role in stress-induced compensatory vascular remodeling. The lack of compensatory vascular growth after TAC may be a potential mechanism for the accelerated heart failure observed in the p38 \( \text{MAPK} \) CKO mice. It raises an important issue that a stress-induced signaling pathway associated with cardiac pathological remodeling and deterioration of heart function also has a beneficial role in the heart by promoting cardiac angiogenesis. This insight reveals an important mechanism in the intricate regulatory network between cardiomyocytes and cardiac vasculature. Because p38 MAPK is induced by a variety of stressors, from mechanical to ischemia/reperfusion injury, its activity can serve as an important signal to translate cardiomyocyte stress into compensatory response from the surrounding endothelial cells. The p38 MAPK-mediated VEGF expression from cardiomyocytes and the paracrine effect on the surrounding cardiac vasculature offer an effective and elegant feedback loop to match blood supply with long-term metabolic demand in heart (Fig. 10E).

**Experimental procedures**

**NRVM purity by flow cytometry**

Twenty-four hours after culture, NRVMs were stained with an antibody directed against myosin heavy chain, MF-20, directly conjugate to FITC, or an isotype-matched FITC-conjugated control antibody, to assess the purity of cardiomyocytes in culture. Purity of immunostained NRVMs was determined by flow cytometry (6).

**Cell culture**

NRVMs were isolated from P1-P3 day old Sprague-Dawley rat pups of mixed gender as described previously with modifications (9). Briefly, hearts were digested with collagenase, and the resulting cell slurry was fractionated on a Percoll gradient. The myocyte-rich fraction was isolated and plated in plating media. After resting overnight in plating medium, the NRVMs were then changed to serum-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 1% insulin-transferrin-selenium (BD Bioscience) and 1% penicillin/streptomycin. Cells were incubated at 37 °C in 5% CO₂. Recombinant adenoviruses expressing constitutively activated MKK3 (MKK3b(E)) and p38α DN were generated and used as previously reported (9). p38 inhibitor SB202190 and MEK inhibitor U0126 was added 6 h after Adv infection.
NRVMs were infected with adenovirus at a multiplicity of infection of 25–50 particles/cell and allowed to incubate for 24–96 h before biochemical analysis. Hypertrophy studies were conducted by treating NRVMs with phenylephrine (10 μM, Sigma) for 48 h. Small interfering (si)RNA experiments were conducted as described previously (44). Briefly, cells were transfected with 150 pmol of GATA4, HIF1α, COX2, MEF2c (Qiagen), PGC1α (Invitrogen), or SP1 (Ambion) siRNA or nonspecific scrambled siRNA (Qiagen) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. 24 h after transfection, NRVMs were infected with adenovirus as described above and incubated for an additional 48 h. In hypoxia experiments, cells were exposed to hypoxia (1% O2, 5% CO2) with or without p38 inhibitor for 4 h. Hypoxia incubator (from Thermo Fisher; with variable O2 and CO2 regulator) was used to maintain the hypoxic conditions throughout the experiment. Each experiment using cultured cell was repeated more than three times.

**Western blotting and antibodies**

Protein analysis was done on total cell lysate obtained from cultured cells. Protein was isolated by a standard protocol with modifications as described previously (45). Briefly, cells were scraped from the plate in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM...
sodium orthovanadate, 1 mM PMSF, 10 mM NaF, and a protease Inhibitor mixture (Roche Diagnostics), then sonicated and centrifuged at 13,000 rpm. Cell lysates were snap-frozen in liquid nitrogen and stored at −80 °C until use. Protein concentration was determined by BCA Protein Assay (Thermo Scientific). 15–25 μg of protein was separated onto 4–12% bis-tris polyacrylamide gels (Invitrogen) and transferred to Hybond-ECL membrane (GE Healthcare). All immunoblots were performed using standard methods with 5% nonfat dry milk as a blocking agent and 5% BSA (Sigma) as a primary antibody diluting agent. Antigens were probed using the following primary antibodies: MKK3 (#9238), p-p38 (#9211), p38 (Thr-180/Tyr-182) (#9218), p-JNK (Thr-183/Tyr-185) (#9251), JNK (#9252), p-ERK (Thr-202/Tyr-204) (#9101), ERK (#9102), p-AKT (Thr-308) (#4056), AKT (#9272), p-VEGFR2 (#2478), VEGFR2 (sc-2479) (all from Cell Signaling), and actin (sc-1616) (Santa Cruz). VEGF antibody was provided from Dr. Luisa Iruela-Arispe (UCLA). Primary antibodies were detected using a secondary anti-rabbit horseradish peroxidase-conjugated antibody (Bio-Rad) and ECL detection (Thermo Scientific).

### Quantitative RT-PCR

Total RNA was reverse-transcribed into cDNA using the SuperScript II RT system (Invitrogen) with oligo dT primers according to the manufacturer’s instructions. mRNA levels of selected genes were determined by quantitative PCR from the generated cDNA. 50–μl reactions were used with iQ SYBR Green Supermix (Bio-Rad), 250 nM concentrations of each primer, and 1 μl of cDNA. The reactions were run on a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad), and data were collected using iQ5 software (Bio-Rad). The cycler program used was an initial denaturation at 95 °C for 5 min, 40 cycles of 45 s each of 95 °C, 60 °C, and 72 °C, another denaturation at 95 °C for 5 min, and a final product melting curve. Data were normalized to the GAPDH mRNA levels. The primer sequences are listed in Table 1.

### VEGF ELISA

To determine the amount of VEGF released from NRVMs, a standard ELISA technique was used. NRVMs were treated with adenovirus as outline above, and media were collected every 24 h for a total of 96 h. Upon collection, media were treated with protease inhibitors (aprotinin 2 mg/ml, leupeptin 10 mg/ml, PMSF 100 mM), centrifuged to remove any cell debris, snap-frozen, and stored at −80 °C until use. Media were then subjected to VEGF ELISA (R&D Systems) according to the man-
The resulting absorbance was read at 540 nm in a plate reader (BioTek PowerWave XS).

In vitro angiogenesis assay

Two independent methods were used for in vitro angiogenesis assays. First, is to use porcine aortic endothelial (PAE) cells stably expressing human VEGFR2 (PAE-VEGFR2) cultured as previously described (10). Briefly, PAE-VEGFR2 cells were cultured in F-12 medium (Cellgro) supplemented with 10% bovine growth serum and 1% penicillin/streptomycin at 37 °C in 5% CO₂. Cytodex beads (700,000 beads/ml; Sigma) were incubated with PAE-VEGFR2 cells for 4 h and were then embedded into fibrinogen/fibronectin gel (2.5 mg/ml; Sigma) containing 1 unit/μl thrombin (Sigma). 2 ml of medium from adenovirus-treated NRVMs was transferred directly from myocytes to gel-embedded PAE-VEGFR2-coated beads. NRVMs were infected for 24 h before the first transfer, and media were transferred every 24 h thereafter for 96 h. VEGF-blocking experiments were done by removing the media from the NRVMs adding either anti-VEGF, IgG control (both R&D systems, 2 μg/ml), or nothing and incubating at 37 °C for 1 h before adding to the PAE culture. Second, is to use human microvascular

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Figure 10. p38α in cardiomyocytes is necessary for compensatory vascular remodeling in response to chronic pressure overload. A, representative fluorescent microscopic images of left ventricular tissue labeled with tropomyosin for cardiomyocytes (red) and lectin for capillaries (green) at baseline (top) and after 28 days after TAC (bottom). B, quantification of capillary densities in the left ventricles from the same group as in A. *, p < 0.05. C, quantitative mRNA analysis from total left ventricular RNA for VE-CAD, VEGF receptor 2 (VEGFR2), and CD31 in samples from Sham and 28 days post-TAC hearts. *, p < 0.05. Sample size is indicated in each column. D, mRNA levels of VEGF from hearts in Sham or 28 days post-TAC hearts. *, p < 0.05; Sample size is indicated in each column. E, schematic illustration of p38α-mediated cross-talk between stressed cardiomyocytes and endothelium.
endothelial cells (HUVECs, Clonetics, Walkersville, MD) to perform endothelial cell tube formation assay as adapted from previous studies (6). Briefly, the formation of tube-like structures was assayed on tumor-derived basement membrane matrix (Millipore). HUVECs were seeded on Matrigel-coated 96-wellplate in endothelial cell growth medium (5000 HUVECs/well). After 2–3 h of culture, endothelial cell growth medium was replaced with 150 l of NRVM-conditioned medium (from hypoxia or normoxia) and then incubated for 6–8 h. After incubation, 6–8 random microscopic (Å~40 magnified) view fields were pictured for each well. ImageJ software was used to calculate tube length.

**Cell imaging**

All myocyte and endothelial cell cultures were evaluated, and images were collected using the SPOT digital camera system (Diagnostic Instruments). Endothelial cell area was quantified using the SPOT Advanced software (Diagnostic Instruments).

**Animals**

All animal handling and procedures were carried out in compliance with institutional guidelines and Institutional Animal Care and Use Committees-approved protocols. Loss of p38 activity in myocytes was achieved using a conditional knock-out approach as previously described (15). Briefly, mice containing a floxed p38α allele (Lexicon Genetics, Inc.) were crossed with mice with myocyte-specific Cre recombinase expression under control of the myosin light chain 2a (MLC-2a) promoter. MLC-2a is expressed throughout the heart during the early stages of development, but its expression is down-regulated in the ventricle at later stages of development (46). Use of this promoter has previously been established as a method to successfully knock-out a gene in a cardiomyocyte-specific manner (47). p38ααloxP/loxp Cre+ was established as a CKO animal, and their Cre− littermates (p38ααloxP/loxp Cre−) were used as WT controls. Only male mice were used for these studies.

**Transverse aortic constriction**

TAC was performed as previously described with modifications (9). Briefly, mice were anesthetized with ketamine/xylazine (80 and 20 mg/kg, respectively) by intraperitoneal injection and mechanically ventilated with 95% O2 (tidal volume 0.5 ml, 130 breaths/min). A left parasternal thoracotomy was performed to access the transverse aorta, which was tied with a 5-0 nylon suture on a 27-gauge needle. After needle removal, the transverse aortic lumen remained constricted by 65–70%. Sham mice underwent a similar procedure except the aorta was not tied. All surgeries were performed on male mice at the age of 15–18 weeks old.

**Histology**

The mouse hearts were perfused and fixed with 10% formalin before embedding in paraffin. All sections were cross-sections of the heart taken from the midpoint of the ventricle. To obtain capillary density images, mice were injected with 200 l of FITC-conjugated lectin (Vector Laboratories) diluted 1:2 with phosphate-buffered saline. The hearts were fixed as outlined above. Samples were counter-stained with anti-tropomyosin (Sigma) primary antibody and Alexa 568 (Molecular Probes) secondary. To obtain cross-sectional area image, sections were incubated with Alexa 594-conjugated WGA (Life Technologies) and anti-tropomyosin primary antibody/Alexa 488 secondary. Nuclei were counter-stained with Hoechst 33342 (Molecular Probes). Images were collected using confocal microscopy.

**Trichrome staining**

Heart sections were stained with Trichrome stain kit (Sigma) according to manufacture protocol. Images were collected using microscopy.

**Table 1**

| Gene name | Forward | Reverse | Species |
|-----------|---------|---------|---------|
| COX2      | TCAAGACAGATCAGAAGCGA | TACCTGAGTGCCTCTTGATTG | Rat |
| GATA4     | CACCGAGAGACACCCCAAT | TGGACATGGCCCCATGCTGAGT | Rat |
| HIF1α     | ACAACAGTTGAAAGGACGCA | AGCGATATCGGCCCCATGCTGAGT | Rat |
| MEF2c     | GCAGAGCAGACAGACATGCTCA | ACTGGAATGACCTGATCTGCA | Rat |
| PGC1α     | CAGGATATTGAAAGGACGCA | CGAGAGCAGACAGACATGCTCA | Rat |
| SP1       | GCAGCTCTCTCTCGACGCTTCAC | CGGAGGTCATCCATATCTGCA | Rat |
| VEGF      | ATCTCCACAGGGTCCTGATTG | CGGAGAATGACGCTGATCTGCA | Mouse |
| CD31      | ATCGGGCTGGCTCCTGCTTCAG | ATCGGATCGCCATGCTGATCTGCA | Mouse |
| VE-CAD    | TGGAGATCCCCAGCGGCTGAGT | TGGAGATCCCCAGCGGCTGAGT | Mouse |
| VEGFR2    | GAGAAGAAGATGTTATGAGATGCTCAG | GAGAGCAGAGACAGACATGCTCA | Mouse |
| GAPDH     | GGAGAATGACGCTGATCTGCA | AACCATGCTCTGGAATGTTATGAC | Mouse |
| VEGF      | GAGAAGAAGATGTTATGAGATGCTCAG | GAGAGCAGAGACAGACATGCTCA | Mouse |

**p38α MAP kinase regulates angiogenesis in the stressed heart**
p38α MAP kinase regulates angiogenesis in the stressed heart

Statistical analysis
Comparisons between more than two groups were accomplished using a factorial analysis of variance (ANOVA) with Fisher’s protected least significant difference post-hoc test. Comparisons between two groups were accomplished using an unpaired two-tailed t test. Analysis was carried out using the StatView program (Abacus Concepts, Berkeley, CA). In all cases a significant result was defined as p < 0.05.

Author contributions—B. A. R. and T. Y. contributed to the experimental design, data collection, analysis interpretation, and manuscript preparation. V. C. and A. Y. K. contributed to the hypoxia-related experiments. S. R. contributed to animal surgery. L. I.-A. and S. M. contributed to manuscript preparation and experimental design. Y. W. contributed to experimental design, data interpretation, and manuscript preparation.

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