Endothelial Bmx tyrosine kinase activity is essential for myocardial hypertrophy and remodeling

Tanja Holopainen[a], Markus Räsänen[a], Andrey Anisimov[a], Tomi Tuomainen[b], Wei Zheng[a], Denis Tvorogov[a], Juha J. Hulmi[c], Leif C. Andersson[d], Bruno Cenni[e], Pasi Tavi[b], Eero Mervaala[f], Riikka Kivelä[a,1], and Kari Alitalo[a,1,2]

[a]Wihuri Research Institute and Translational Cancer Biology Program, Biomedicum Helsinki, University of Helsinki, FI-00290 Helsinki, Finland; [b]Department of Biotechnology and Molecular Medicine, A. I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, FI-70211 Kuopio, Finland; [c]Department of Biology of Physical Activity, University of Jyväskylä, FI-40700 Jyväskylä, Finland; [d]Department of Pathology, Haartman Institute, University of Helsinki, FI-00014 Helsinki, Finland; [e]Novartis Institutes for Biomedical Research, Basel CH-4002, Switzerland; and [f]Department of Pharmacology, Institute of Biomedicine, Biomedicum Helsinki, University of Helsinki, FI-00014 Helsinki, Finland

Contributed by Kari Alitalo, September 8, 2015 (sent for review May 13, 2015; reviewed by Bernd Groner and Christian Kupatt)

Cardiac hypertrophy accompanies many forms of heart disease, including ischemic disease, hypertension, heart failure, and valvular disease, and it is a strong predictor of increased cardiovascular morbidity and mortality. Deletion of bone marrow kinase in chromosome X (Bmx), an arterial nonreceptor tyrosine kinase, has been shown to inhibit cardiac hypertrophy in mice. This finding raised the possibility of therapeutic use of Bmx tyrosine kinase inhibitors, which we have addressed here by analyzing cardiac hypertrophy in gene-targeted mice deficient in Bmx tyrosine kinase activity. We found that angiotensin II (Ang II)-induced cardiac hypertrophy is significantly reduced in mice deficient in Bmx and in mice with inactivated Bmx tyrosine kinase compared with WT mice. Genome-wide transcriptomic profiling showed that Bmx inactivation suppresses myocardial expression of genes related to Ang II-induced inflammatory and extracellular matrix responses whereas expression of RNAs encoding mitochondrial proteins after Ang II administration was maintained in Bmx-inactivated hearts. Very little or no Bmx mRNA was expressed in human cardiomyocytes whereas human cardiac endothelial cells expressed abundant amounts. Ang II stimulation of endothelial cells increased Bmx phosphorylation, and Bmx gene silencing inhibited downstream STAT3 signaling, which has been implicated in cardiac hypertrophy. Furthermore, activation of the mechanistic target of rapamycin complex 1 pathway by Ang II treatment was decreased in the Bmx-deficient hearts. Our results demonstrate that inhibition of the cross-talk between endothelial cells and cardiomyocytes by Bmx inactivation suppresses Ang II-induced signals for cardiac hypertrophy. These results suggest that the endothelial Bmx tyrosine kinase could provide a target to attenuate the development of cardiac hypertrophy.

Etk | endothelium | signaling | heart | cardiomyocyte

Heart failure is a continuously increasing global problem in aging populations. Despite some progress in treatment options, the prognosis of heart failure is worse than that for most cancers (1). Cardiac hypertrophy due to exercise training is referred to as physiological hypertrophy, in which the architecture and contractile function of the heart are maintained or enhanced, and it is reversible if the training is discontinued. Numerous diseases, such as myocardial infarction, aortic stenosis, hypertension and metabolic stress, induce pathological hypertrophy, which is related to activation of maladaptive cellular events in the heart and progressively leads to heart failure (2). Understanding the underlying regulatory processes in the development of pathological hypertrophy has provided effective therapeutic targets to attenuate disease progression, but there are currently no treatments to reverse cardiac hypertrophy.

The *Bmx* (*Etk*) gene in the chromosome region Xp22.2 encodes a tyrosine kinase that was originally identified and cloned from hematopoietic cells (3). Striking new evidence indicates that Bmx phosphorylates a phosphotyrosine-primed motif mediating the activation of multiple receptor tyrosine kinases (4). Bmx is highly expressed in the endocardium and in the endothelium of large arteries, starting between embryonic days 10.5–12.5 (5), indicating that Bmx is a signal transducer mainly in the arterial endothelium. However, Bmx deletion does not result in any obvious developmental phenotype in mice (5), suggesting that it has a redundant function during embryogenesis. In contrast, Bmx has been shown to be important in a variety of pathological states, including tumor growth (6–8), and its overexpression promotes ischemia-induced arteriogenesis and inflammatory angiogenesis (9).

Previous reports have indicated that Bmx deficiency attenuates pressure overload-induced cardiac hypertrophy in response to thoracic aortic constriction or vascular endothelial growth factor-B (VEGF-B) overexpression (10, 11), but the mechanisms of how this regulation occurs are as yet not known. We have here analyzed the molecular mechanisms of how Bmx regulates cardiac hypertrophy by using angiotensin II (Ang II) treatment that leads to cardiac hypertrophy and remodeling (12). Importantly, we show that pathological cardiac growth is suppressed in a mouse model where the Bmx kinase is inactivated by a missense point mutation. Our findings provide therapeutic proof of principle indicating that the blocking of Bmx tyrosine kinase activity could be used to inhibit pathological cardiac hypertrophy.

Significance

During the last decades, heart failure has developed into a major burden in the western world, increasingly affecting millions. Cardiac hypertrophy is an adaptive response to myocardial infarction or increased blood pressure, and it often leads to heart failure. Understanding the underlying regulatory processes in the development of pathological hypertrophy is needed for the development of effective therapies. Our results show that the kinase activity of the endothelial bone marrow kinase in chromosome X (Bmx) protein is necessary for the development of pathological cardiac hypertrophy. This finding could provide significant therapeutic applications when specific Bmx kinase inhibitors become available in the clinics.

Author contributions: T.H., P.T., E.M., R.K., and K.A. designed research; T.H., M.R., A.A., T.T., W.Z., D.T., J.J.H., and R.K. performed research; T.T., B.C., P.T., and E.M. contributed new reagents/analytic tools; T.H., M.R., J.J.H., L.C.A., E.M., R.K., and K.A. analyzed data; and T.H., R.K., and K.A. wrote the paper.

Reviewers: B.G., Georg Speyer Haus; and C.K., Klinikum Grosshadern Munich.

Conflict of interest statement: B.C. is an employee of Novartis.

Freely available online through the PNAS open access option.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE47420).

[1]R.K. and K.A. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: kari.alitalo@helsinki.fi.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1517810112/-/DCSupplemental.

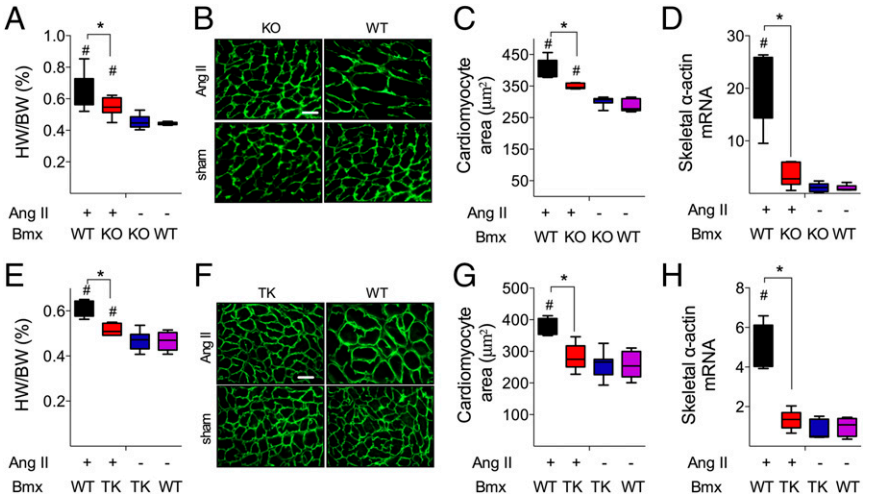

Fig. 1. Deletion of Bmx protein or inactivation of its tyrosine kinase activity suppresses Ang II-induced cardiac hypertrophy. (*A*) Heart weight (HW)-to-body weight (BW) ratios in the indicated experimental groups (Ang II/WT, $n = 17$; Ang II/KO, $n = 16$; sham/KO, $n = 14$; sham/WT $n = 10$) at 2 wk. Data are pooled from two independent experiments. (*B*) Dystrophin-stained cardiac sections. (*C*) Quantification of the cardiomyocyte cross-sectional areas. (*D*) Relative levels of skeletal α-actin mRNA in Bmx KO mice and respective control WT mice. (*E*) Heart weight-to-body weight ratios in the indicated experimental groups (Ang II/WT, $n = 4$; Ang II/TK-, $n = 5$; sham/TK-, $n = 7$; sham/WT $n = 8$) at 2 wk. (*F*) Dystrophin-stained cardiac sections. (*G*) Quantification of the cardiomyocyte cross-sectional areas. (*H*) Relative levels of skeletal α-actin mRNA in Bmx TK mice and respective control WT mice. $^{*}P < 0.05$. $^{\#}P < 0.05$ vs. WT sham. Data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA. (Scale bar: 20 μm.)

Results

Bmx Tyrosine Kinase Activity Is Necessary for Ang II-Induced Cardiac Hypertrophy. Previous studies have shown that cardiac hypertrophy in response to aortic coarctation or VEGF-B overexpression is greatly attenuated in mice deficient of the Bmx protein (10, 11). Because of the continuing development of increasingly specific Bmx tyrosine kinase inhibitors, it would be important to know whether specific inactivation of the Bmx tyrosine kinase activity would be enough to inhibit cardiac hypertrophy. Thus, our aim was to determine whether attenuation of cardiac hypertrophy detected in Bmx knockout (KO) mice is dependent on the tyrosine kinase activity of the Bmx protein. We analyzed cardiac growth upon Ang II administration to mice in which the Bmx gene had been deleted, or replaced with a K421R mutant allele (equivalent to human mutation K445R) (13), rendering the kinase catalytically inactive (referred to as TK mice) (14).

To compare the effects of Bmx gene deletion or tyrosine kinase inactivation on Ang II-induced cardiac hypertrophy, we first implanted Ang II minipumps into Bmx KO and WT mice for 2 wk. The WT mice developed cardiac hypertrophy, but, as expected, the hypertrophic changes were significantly milder in KO mice than in WT mice (Fig. 1*A*). Immunostaining for dystrophin demonstrated that the cardiomyocyte size was increased in both groups after Ang II infusion; however, the increase in Bmx KO mice was significantly smaller than in WT mice (Fig. 1 *B* and *C*). Importantly, the Ang II-induced cardiac growth and cardiomyocyte size were reduced also in mice expressing the kinase-inactive Bmx allele (Fig. 1 *E*–*G*). The mRNA level of skeletal α-actin, a marker for pathological cardiac hypertrophy, was significantly increased in WT mice, but not in Bmx KO mice or in TK mice (Fig. 1 *D* and *H*). Accordingly, after a prolonged Ang II infusion for 6 wk, the extent of cardiac hypertrophy was also less in Bmx KO mice than in WT mice (Fig. S1). However, there were no significant differences in the blood pressure between KO and WT mice before or 2 or 6 wk after Ang II infusion measured by the tail cuff method. Similar results were obtained when a catheter was inserted into the carotid artery to monitor blood pressure more accurately. These results confirmed that Bmx deficiency does not affect blood pressure (Fig. S2). Taken together; these data indicate that the development of cardiac hypertrophy is dependent on Bmx tyrosine kinase activity and that the difference in the cardiac Ang II response is not due to differences in blood pressure.

The Bmx Tyrosine Kinase Is Important for Endothelial Cell–Cardiomyocyte Cross-Talk. A previous report has suggested that Bmx is expressed also in cardiomyocytes in addition to its expression in the arterial endothelium, in endocardium, and in cells of the hematopoietic myeloid lineage (15). Thus, we compared Bmx mRNA levels in isolated arterial and microvascular endothelial cells as well as in fibroblasts and cardiomyocytes from human heart. As shown in Fig. 2*A*, Bmx expression was highest in arterial endothelial

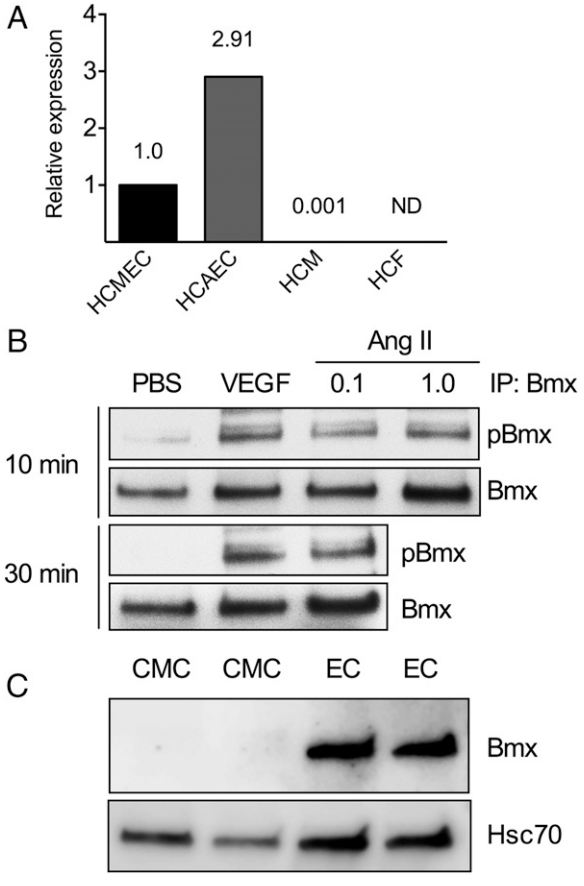

Fig. 2. Bmx is expressed mainly in cardiac endothelial cells and phosphorylated upon Ang II treatment. (*A*) Bmx mRNA expression in human cardiac cells. HCMEC, human cardiac microvascular endothelial cells; HCAEC, human cardiac arterial endothelial cells; HCM, human cardiomyocytes; HCF, human cardiac fibroblasts; ND, not detected. (*B*) Phosphorylation of Bmx (pBmx) immunoprecipitated (IP) from HUVECs and stimulated with 0.1 and 1.0 nM Ang II for 10 min or 30 min. VEGF was used as a positive control. (*C*) Western blot showing Bmx expression in isolated adult cardiomyocytes (CMC) and HUVECs (EC). An equal amount of total protein was loaded in each lane. Hsc70, heat shock cognate 70.

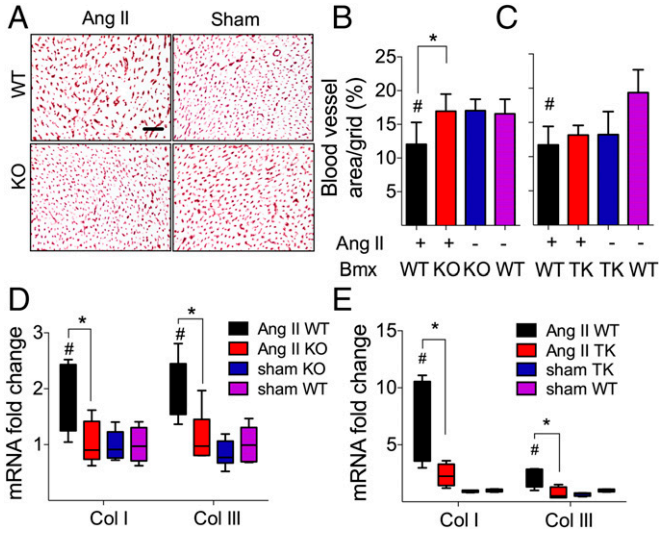

Fig. 3. Attenuated Ang II-induced cardiac responses in Bmx KO and TK mice. (*A*) Myocardial blood vessels stained for CD31 antigen. (*B* and *C*) Blood vessel area/grid. (*D* and *E*) Collagen I (Col I) and collagen III (Col III) mRNA expression in Bmx KO (*D*) and TK (*E*) mice. $^{*}P < 0.05$. $^{\#}P < 0.05$ vs. WT sham. Data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA. (Scale bar: 100 μm.)

cells. The microvascular endothelial cells contained about 35% of this amount whereas expression in the cardiomyocytes was about 0.1% of that found in microvascular endothelial cells, and no Bmx RNA could be detected in cardiac fibroblasts. These results strongly suggest that the effects of Bmx on cardiac hypertrophy are mediated via the endothelial cells of the coronary vasculature.

Next, we analyzed whether Ang II can induce Bmx phosphorylation in endothelial cells. Human umbilical venous endothelial cells (HUVECs) were stimulated with different concentrations of Ang II, and vascular endothelial growth factor (VEGF) was used as a positive control. Similarly to VEGF, Ang II induced strong Bmx phosphorylation in HUVECs after 10 min and 30 min of stimulation (Fig. 2*B*). In contrast, Bmx was not detected in isolated adult mouse cardiomyocytes even after Ang II stimulation, further indicating that Bmx is specific for cardiac endothelial cells (Fig. 2*C*).

Reduced Myocardial Capillary Density in Ang II-Treated WT Mice but Not in Bmx-Deleted Mice. Pathological pressure overload-induced cardiac growth is often associated with a decrease of capillary density, especially in the left ventricular myocardium, because vessel growth does not match the cardiomyocyte growth (16). Staining for the vascular endothelial marker CD31 showed decreased blood vessel density and area in the hearts of Ang II-treated WT mice compared with sham-operated control WT mice whereas the capillary density in the Bmx-deleted hearts was not altered by Ang II treatment (Fig. 3 *A* and *B*). Similar results were obtained when the TK mice were used for the analysis (Fig. 3*C*). This difference was due to the inhibition of cardiomyocyte growth because there was no statistical difference in the capillary-to-cardiomyocyte ratio between the groups (WT sham, 1.68 ± 0.05; WT Ang II, 1.75 ± 0.03; KO sham, 1.60 ± 0.04; KO Ang II, 1.77 ± 0.06; $P > 0.05$).

To analyze how the loss of Bmx kinase activity regulates the pathogenesis of Ang II-triggered hypertrophy, cardiac samples from Bmx KO, TK, and WT mice with and without preceding systemic Ang II infusion were subjected to genome-wide RNA expression analysis. Without Ang II treatment, only a few genes were differentially expressed between Bmx KO and WT hearts (Table S1). Ang II-treated WT hearts had alterations in several gene clusters attributable to cardiac hypertrophic response, including increased expression of genes encoding extracellular matrix proteins. Expression of this gene cluster was significantly lower in Bmx KO or TK hearts than in WT hearts after the Ang II treatment (Tables S2 and S3). To confirm microarray findings, we analyzed a set of mRNAs with quantitative PCR (qPCR). For example, procollagen I and III mRNAs were up-regulated in Ang II-treated WT mice but not in Bmx KO or TK mice (Fig. 3 *D* and *E*). Of note, Ang II treatment did not affect the mRNA expression of Bmx in WT hearts in any of the experiments.

Mitochondrial Content Is Maintained in Bmx-Deficient Hearts After Ang II Treatment. Whole-genome gene expression analyses showed that Ang II induces markedly fewer changes in KO and TK mice than in WT mice (Fig. 4*A* and Tables S2 and S3). Interestingly, genes encoding components of the inner mitochondrial membrane and the electron transport chain were more abundantly expressed in Ang II-treated Bmx KO or TK hearts than in Ang II-treated WT hearts. PGC-1-α is a major regulator of mitochondrial biosynthesis, and decreased levels of PGC-1-α and citrate synthase are associated with the development of pathological cardiac hypertrophy (reviewed in ref. 17). Decreased PGC-1-α RNA level and decreased citrate synthase activity were detected in the Ang II-treated WT hearts but not in the Bmx KO and TK hearts (Fig. 4 *B*–*E*). Together with the microarray data, these results indicate a better sustained mitochondrial content in the hearts of Bmx KO and TK mice than in WT mice upon Ang II-induced cardiac stress.

Bmx Regulates Ang II-Induced Inflammatory Cytokine Responses. Increased expression of inflammatory cytokines has also been implicated in cardiac hypertrophy. Because Bmx has been shown to regulate inflammatory responses (9, 14, 18), we examined whether Bmx affects the expression of inflammatory genes upon Ang II induction. Indeed, mRNA levels of interleukin-6 (IL-6), IL-8, tumor necrosis factor receptor-2 (Tnfr2), and matrix metalloproteinase 2 (Mmp2) were significantly lower in Bmx KO and TK mice than in WT mice after Ang II induction (Fig. 5 *A*–*D*). These data indicate that Bmx regulates at least a subset of inflammation-related cytokine responses in Ang II-induced hypertrophy.

Tissue inhibitor of matrix metalloproteinases-1 (Timp1) mRNA was markedly induced upon Ang II treatment in WT, but not in Bmx KO or TK mice (Fig. 5 *B* and *E*). Heat shock protein 70 (Hsp70) has been shown to be up-regulated in hypertrophic cardiomyopathy (19). Interestingly, a clear trend of Hsp1a mRNA induction was detected in the hypertrophic WT hearts whereas lack of Bmx abolished this response (Fig. 5 *C* and *F*).

Involvement of Bmx Tyrosine Kinase Activity in Ang II-Induced Downstream Signaling. To elucidate the influence of Bmx inactivation on signaling pathways mediating cardiomyocyte growth, we analyzed activation of the mechanistic target of rapamycin complex 1 (mTORC1) pathway, which is a major regulator of protein synthetic pathways during cell growth (20). Consistent with the smaller cardiomyocyte size, we found that the mTORC1 downstream target ribosomal protein S6 (rpS6) phosphorylation at Ser-240/244 was less activated in Bmx KO hearts than in WT hearts after the 2-wk Ang II infusion (Fig. 6 *A* and *B*). To further analyze the in vivo signaling induced by Ang II, we injected Ang II into the tail vein of TK and WT mice and analyzed the phosphorylation of rpS6, Akt, and STAT3 in the heart after 10 min of stimulation. Ang II induced phosphorylation of all these three signaling proteins in WT mice, but the response was blunted in TK mice (Fig. 6 *C*–*F*). In addition, microarray data indicated that Ang II treatment increased Stat3 [false discovery rate (FDR) = 0.005] and decreased Stat1 (FDR = 0.00001) mRNA expression in WT hearts, but there was no effect of Ang II on either gene in KO hearts.

To study the effect of Bmx on Ang II signal transduction in more detail, we silenced Bmx in HUVECs with three different shRNA constructs and analyzed the effect of Ang II stimulation on STAT3 activation. Ang II induced STAT3 phosphorylation in control cells transduced with shScr, but not in Bmx-silenced cells (Fig. 6*G*), indicating that Bmx is needed for Ang II-induced STAT3 signaling in endothelial cells.

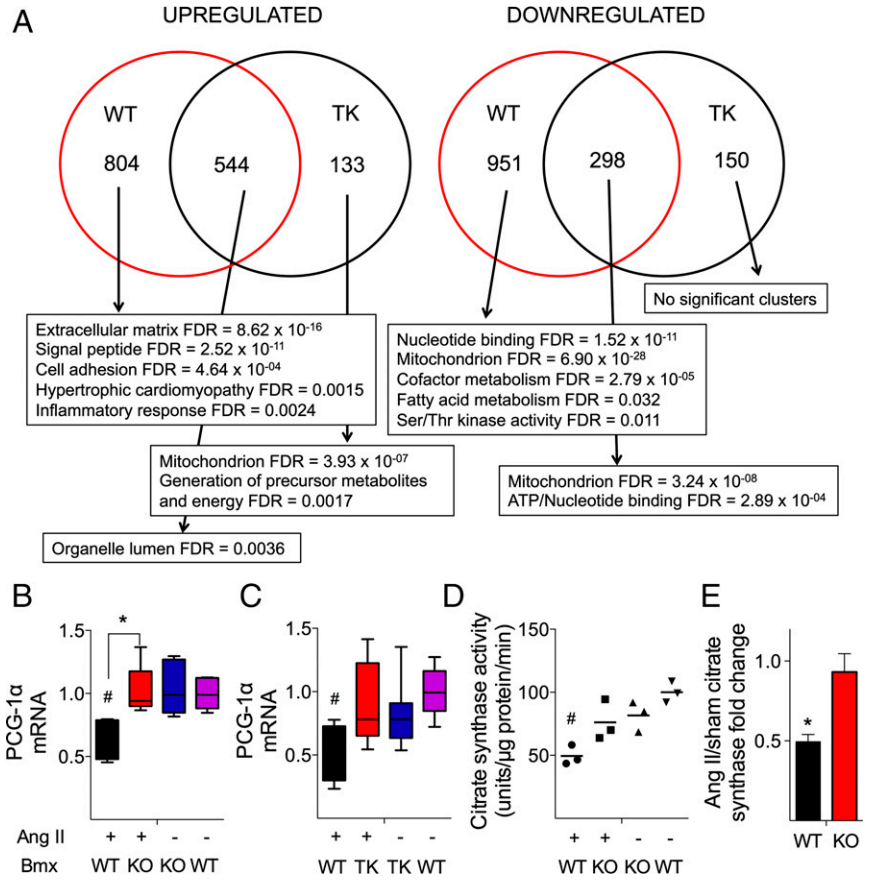

Fig. 4. Comparison of global and mitochondrial gene expression in Bmx TK and WT hearts in response to Ang II treatment. (*A*) An overview of the total number of significantly changed genes and functional gene clusters from Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis showing markedly fewer changes in Bmx TK mice compared with WT mice after Ang II treatment. Statistical values are presented in Table S3. (*B* and *C*) PGC-1α mRNA expression in cardiac samples of Bmx WT, KO and TK mice after 14 d of Ang II infusion. The values shown indicate fold change in comparison with sham WT (set to 1). (*D*) Citrate synthase activity in the hearts from Ang II- or sham-treated Bmx WT and KO mice after 14 d of infusion ($n = 3$ in each group). Note that citrate synthase activity is statistically significantly reduced after Ang II infusion in WT mice but not in the Bmx KO mice. (*E*) Relative change of citrate synthase activity after Ang II treatment. Data are presented as mean± SEM. Statistical analysis was performed with one-way ANOVA. $^{*}P < 0.05$. $^{\#}P < 0.05$ vs. WT sham.

Discussion

Our results show that Bmx tyrosine kinase activity is necessary for the cardiac hypertrophy induced by angiotensin II. This finding contrasts with results from a macrophage-driven inflammatory arthritis model where Bmx protein, but not tyrosine kinase activity, was found to be essential (14). In the Ang II hypertrophy model, both the deletion and tyrosine kinase inactivation of Bmx prevented increased inflammatory cytokine and matrix gene activation, stimulation of the mTORC1 pathway, and the decrease of mitochondrial gene expression and activity that characterize the hypertrophic and dysfunctional myocardium in the Ang II hypertension model. The present results on Bmx expression in human and mouse cardiac cells, together with our previous findings (5), indicate that Bmx is expressed mainly in the

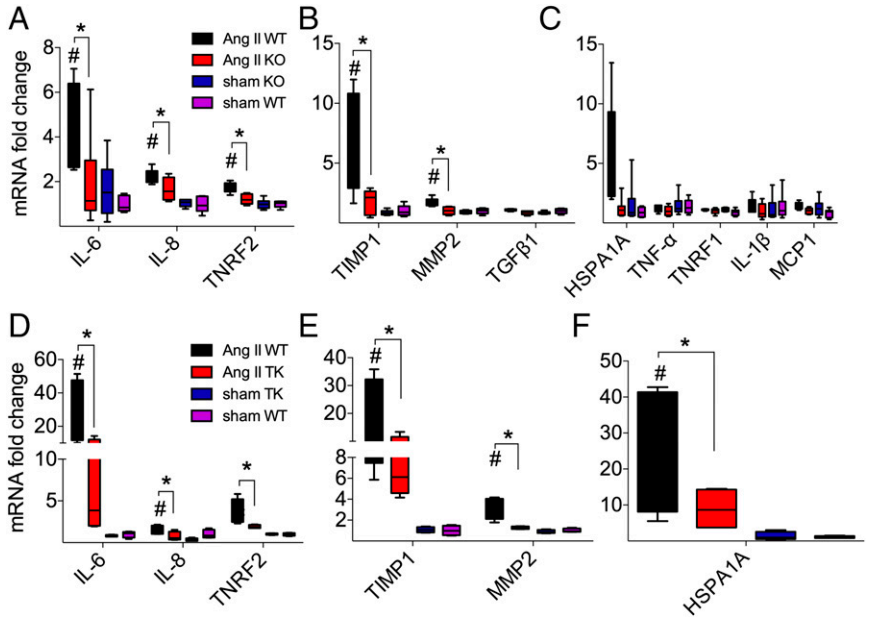

Fig. 5. Bmx inactivation abolishes the increased expression of genes encoding inflammatory and matrix remodeling proteins in Ang II-treated hearts. (*A–F*) qPCR analysis of the indicated genes. Bmx WT and KO mice (*A–C*) and Bmx WT and TK mice (*D–F*) after 14 d of Ang II or sham treatment. Values are shown as fold of change in comparison with sham-operated WT hearts. Data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA. $^{*}P < 0.05$. $^{\#}P < 0.05$ vs. WT sham.

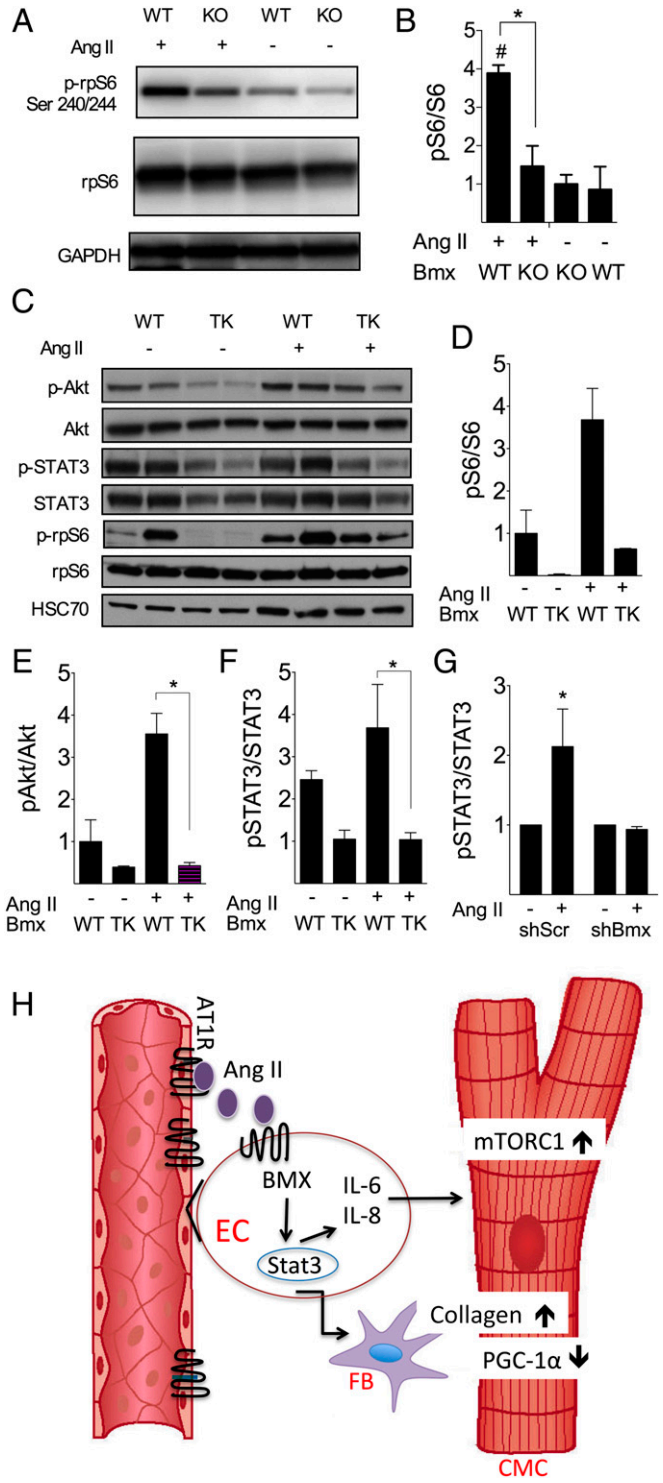

Fig. 6. Lack of Bmx tyrosine kinase activity alters intracellular signaling responses to Ang II treatment. (*A*) Representative Western blots from cardiac samples of Bmx WT and KO mice after 14 d of Ang II or sham treatment. The blots were probed with antibodies against phosphorylated rpS6 (Ser-240/244), rpS6, and GAPDH. The uppermost and lowermost signals are from the same blot; the middle blot contains the same samples. (*B*) Quantitative analysis of phosphorylated rpS6 Ser-240/244 normalized to GAPDH from three samples per treatment group. (*C*) Western blots from WT and TK hearts after 10 min of Ang II stimulation in vivo. (*D–F*) Quantification of the Western blots for phospho-Akt (Ser-473), phospho-rpS6, and phospho-STAT3. (*G*) Quantification of STAT3 phosphorylation in HUVECs transduced with shScr or shBmx and stimulated with Ang II. The data are pooled from three experiments using three different Bmx silencing constructs, and shScr is set to a relative expression of 1. (*H*) Schematic summary of the suggested mechanism by which endothelial Bmx regulates cardiomyocyte growth. EC, endothelial cell; CMC, cardiomyocyte; FB, fibroblast; AT1R, angiotensin type 1 receptor. Data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA or two-tailed Student's *t* test. $^{*}P < 0.05$, $^{\#}P < 0.05$ vs. sham/WT.

arterial and microvascular endothelium in the heart. Very little, if any, Bmx mRNA was detected in primary human cardiomyocytes, and no Bmx protein was detected in adult mouse cardiomyocytes. This observation highlights the importance of the cross-talk between vascular endothelial cells and cardiomyocytes in the development of pathological hypertrophy. Furthermore, these mouse models show that deletion of Bmx does not affect normal cardiac homeostasis, which provides a wide therapeutic window for possible use of Bmx tyrosine kinase inhibitors in heart disease.

Angiotensin II activates the angiotensin type 1 receptor (AT1R), which then transactivates the epidermal growth factor receptor (EGFR) to mediate cellular growth. Knockdown of Bmx was shown to attenuate tyrosine phosphorylation of the EGFR by angiotensin II stimulation in mammary epithelial cells, but Bmx did not have an effect on direct stimulation of the EGFR with EGF, indicating that Bmx functions between the activated AT1R and EGFR (21). Bmx also induces the tyrosine phosphorylation and DNA binding activity of STAT1, STAT3, and STAT5 (22). Of these proteins, STAT3 has been shown to be important for physiological homeostasis and stress-induced remodeling of the heart (23). In our experiments, Bmx silencing completely abrogated STAT3 activation by Ang II in endothelial cells and also significantly attenuated phosphorylation in vivo. Although we cannot fully exclude the possibility that Bmx functions also in cardiomyocytes, our results and those of others strongly suggest that Bmx mainly acts in endothelial cells (24). Thus, the present results highlight the importance of endothelial cell-to-cardiomyocyte signaling in cardiac remodeling (see the schematic summary in Fig. 6*H*).

Our transcriptomic analyses of the Ang II-treated hearts indicated a better preserved mitochondrial content in Bmx kinase-deficient mice upon pathological cardiac growth. Mitochondrial and NADPH oxidase dysfunction and the resulting oxygen radicals have been shown to induce cellular inflammatory responses leading to increased cytokine expression (17, 25–28). Ang II also activates proinflammatory and profibrotic pathways. In this regard, it is interesting that Bmx kinase activity contributed to the regulation of a range of inflammatory cytokine responses associated with pathological cardiac hypertrophy. Inflammatory markers have not been previously analyzed in the context of cardiac hypertrophy in Bmx-deficient mice. In other models, such as in ischemia-mediated arteriogenesis in the hind limb, Bmx has been shown to interact with the TNFR2 pathway to promote adaptive arteriogenesis and angiogenesis (29). In a model of rheumatoid arthritis, Bmx was shown to act in a kinase-independent manner downstream of, or at the same level as, TGF-β activated kinase 1 (TAK1) to mediate inflammatory cytokine signaling, including IL-8 (14). Recently, it was shown that deletion of IL-6 prevents cardiac inflammation, fibrosis, and dysfunction after Ang II (30). Our results showed that inactivation of Bmx abolished the Ang II-induced increase in IL-6 and IL-8, placing Bmx upstream of the inflammatory cascade.

Our data on the prevention of Ang II-induced cardiac hypertrophy when Bmx tyrosine kinase is rendered inactive provide a proof of principle that inhibitors of Bmx tyrosine kinase activity could do the same. Bmx-deficient, as well as tyrosine kinase-inactivated, mice are healthy and fertile, suggesting that Bmx-specific tyrosine kinase inhibitors could provide a wide therapeutic window. This finding could turn out to be significant for therapeutic applications when Bmx kinase inhibitors become available in clinics (31–33). At the moment, many Bmx inhibitors also inhibit other kinases, such as EGFR, which may cause cardiotoxicity, whereas the more specific inhibitors have not yet been tested in vivo. Additional studies will be necessary

to explore the possible benefits of modulating Bmx activity as a therapeutic target.

Materials and Methods

A detailed description of all materials and methods can be found in *SI Materials and Methods*.

Ang II-Induced Cardiac Hypertrophy. Bmx KO mice (described in ref. 5), backcrossed to the C57BL/6J background at least eight times, and age- and gender-matched WT C57BL/6J mice were used in the present studies. Ang II infusion at 0.1 $mg{\cdot}kg^{-1}{\cdot}h^{-1}$ for 14 d was induced by s.c. implantation of osmotic minipumps (Alzet model 1002; Durect Corporation). Sham-operated KO mice ($n = 14$) and WT mice ($n = 10$) were used as controls. At 2 or 6 wk, the mice were weighed and killed, and then the hearts were excised, weighed, and processed for further analysis as described in *SI Materials and Methods*. We also studied mice with the kinase-deficient Bmx K421R mutation (equivalent to the human K445R mutation) (13) in the BALB/c background, referred to here as Bmx TK mice (14).

The National Animal Board for Animal Experiments at the Provincial State Office of Southern Finland approved all animal experiments, which were performed in accordance with Finnish legislation regarding the humane care and use of laboratory animals.

Ang II–Bmx Signal Transduction, RT-qPCR, and Western Blot Analysis. Ang II–Bmx signal transduction, RT-qPCR, and Western blot analysis are detailed in *SI Materials and Methods*. Primers used are listed in Table S4.

Whole-Genome Microarray and Data Analysis. RNA samples were analyzed with genome-wide Illumina MouseWG-6 v2 microarrays (Illumina Inc.). Detailed microarray data analysis is described in *SI Materials and Methods*. The data have been deposited in the Gene Expression Omnibus (GEO) data repository with accession number GSE47420.

Immunohistochemical Analyses. Five- to 7-μm frozen sections were fixed with acetone, immunostained, and analyzed as detailed in *SI Materials and Methods*.

Statistical Analysis. Values are indicated as mean ± SEM in the figures. Statistical analysis of multiple groups was performed with one-way ANOVA, followed by Tukey's post hoc test for groups with equal variances and by Games–Howell's post hoc test for groups with unequal variances. Statistical analysis of two groups was performed with unpaired *t* test. All statistical tests were two-tailed. Differences were considered statistically significant at $P < 0.05$.

ACKNOWLEDGMENTS. We thank Dr. Thomas M. Vondriska for helpful comments and Dr. Ulrich Pohl, Dr. Seppo Kaijalainen, and Dr. Jaana Rysä for the help with experiments. Katja Palonen, Miia Taavitsainen, Salli Antila, Tanja Laakkonen, Katja Salo, Kirsi Lintula, Päivi Leinikka, Tapio Tainola, and the Biomedicum Imaging Unit are acknowledged for excellent technical assistance. This study was supported by the Antti and Jenny Wihuri Foundation, Academy of Finland Grants 262976 (to K.A.), 131711 (to R.K.), and 267637 (to P.T.), European Research Council (TX-FACTORS) Grant ERC-2010-AdG-268804, the Sigrid Juselius Foundation, the Finnish Foundation for Cardiovascular Research, the Leducq Foundation, Biocenter Finland, and the Helsinki University Central Hospital. T.H. and R.K. have been personally supported by the Finnish Foundation for Cardiovascular Research, and T.H. has also been supported by Finska Läkaresällskapet, the Instrumentarium Science Foundation, the Maud Kuistila Memorial Foundation, the Paulo Foundation, the Ida Montin Foundation, and the Finnish Cultural Foundation. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of European Union Seventh Framework Programme FP7/2007-2013 under Research Executive Agency Grant 317250.

1. Braunwald E (2015) The war against heart failure: The Lancet lecture. *Lancet* 385(9970):812–824.
2. Oka T, Akazawa H, Naito AT, Komuro I (2014) Angiogenesis and cardiac hypertrophy: Maintenance of cardiac function and causative roles in heart failure. *Circ Res* 114(3):565–571.
3. Tamagnone L, et al. (1994) BMX, a novel nonreceptor tyrosine kinase gene of the BTK/ITK/TEC/TXK family located in chromosome Xp22.2. *Oncogene* 9(12):3683–3688.
4. Chen S, et al. (2013) Tyrosine kinase BMX phosphorylates phosphotyrosine-primed motif mediating the activation of multiple receptor tyrosine kinases. *Sci Signal* 6(277):ra40.
5. Rajantie I, et al. (2001) Bmx tyrosine kinase has a redundant function downstream of angiopoietin and vascular endothelial growth factor receptors in arterial endothelium. *Mol Cell Biol* 21(14):4647–4655.
6. Holopainen T, et al. (2012) Deletion of the endothelial Bmx tyrosine kinase decreases tumor angiogenesis and growth. *Cancer Res* 72(14):3512–3521.
7. Guryanova OA, et al. (2011) Nonreceptor tyrosine kinase BMX maintains self-renewal and tumorigenic potential of glioblastoma stem cells by activating STAT3. *Cancer Cell* 19(4):498–511.
8. Cohen I, et al. (2010) Etk/Bmx regulates proteinase-activated-receptor1 (PAR1) in breast cancer invasion: Signaling partners, hierarchy and physiological significance. *PLoS One* 5(6):e11135.
9. Paavonen K, et al. (2004) Bmx tyrosine kinase transgene induces skin hyperplasia, inflammatory angiogenesis, and accelerated wound healing. *Mol Biol Cell* 15(9):4226–4233.
10. Mitchell-Jordan SA, et al. (2008) Loss of Bmx nonreceptor tyrosine kinase prevents pressure overload-induced cardiac hypertrophy. *Circ Res* 103(12):1359–1362.
11. Bry M, et al. (2010) Vascular endothelial growth factor-B acts as a coronary growth factor in transgenic rats without inducing angiogenesis, vascular leak, or inflammation. *Circulation* 122(17):1725–1733.
12. Yamazaki T, Komuro I, Yazaki Y (1999) Role of the renin-angiotensin system in cardiac hypertrophy. *Am J Cardiol* 83(12A):53H–57H.
13. Ekman N, et al. (2000) The Bmx tyrosine kinase is activated by IL-3 and G-CSF in a PI-3K dependent manner. *Oncogene* 19(36):4151–4158.
14. Gottar-Guillier M, et al. (2011) The tyrosine kinase BMX is an essential mediator of inflammatory arthritis in a kinase-independent manner. *J Immunol* 186(10):6014–6023.
15. Willey CD, et al. (2008) STAT3 activation in pressure-overloaded feline myocardium: Role for integrins and the tyrosine kinase BMX. *Int J Biol Sci* 4(3):184–199.
16. Shiojima I, et al. (2005) Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J Clin Invest* 115(8):2108–2118.
17. Rosca MG, Tandler B, Hoppel CL (2013) Mitochondria in cardiac hypertrophy and heart failure. *J Mol Cell Cardiol* 55:31–41.
18. Pan S, et al. (2002) Etk/Bmx as a tumor necrosis factor receptor type 2-specific kinase: Role in endothelial cell migration and angiogenesis. *Mol Cell Biol* 22(21):7512–7523.
19. Kee HJ, et al. (2008) Activation of histone deacetylase 2 by inducible heat shock protein 70 in cardiac hypertrophy. *Circ Res* 103(11):1259–1269.
20. Ma XM, Blenis J (2009) Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 10(5):307–318.
21. George AJ, et al. (2013) A functional siRNA screen identifies genes modulating angiotensin II-mediated EGFR transactivation. *J Cell Sci* 126(Pt 23):5377–5390.
22. Saharinen P, et al. (1997) The Bmx tyrosine kinase induces activation of the Stat signaling pathway, which is specifically inhibited by protein kinase Cdelta. *Blood* 90(11):4341–4353.
23. Haghikia A, Ricke-Hoch M, Stapel B, Gorst I, Hilfiker-Kleiner D (2014) STAT3, a key regulator of cell-to-cell communication in the heart. *Cardiovasc Res* 102(2):281–289.
24. Noels H, et al. (2014) Deficiency of endothelial CXCR4 reduces reendothelialization and enhances neointimal hyperplasia after vascular injury in atherosclerosis-prone mice. *Arterioscler Thromb Vasc Biol* 34(6):1209–1220.
25. Abel ED, Doenst T (2011) Mitochondrial adaptations to physiological vs. pathological cardiac hypertrophy. *Cardiovasc Res* 90(2):234–242.
26. Maejima Y, Kuroda J, Matsushima S, Ago T, Sadoshima J (2011) Regulation of myocardial growth and death by NADPH oxidase. *J Mol Cell Cardiol* 50(3):408–416.
27. Lassègue B, San Martín A, Griendling KK (2012) Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ Res* 110(10):1364–1390.
28. Burgoyne JR, Mongue-Din H, Eaton P, Shah AM (2012) Redox signaling in cardiac physiology and pathology. *Circ Res* 111(8):1091–1106.
29. Luo Y, et al. (2010) Endothelial-specific transgenesis of TNFR2 promotes adaptive arteriogenesis and angiogenesis. *Arterioscler Thromb Vasc Biol* 30(7):1307–1314.
30. González GE, et al. (2015) Deletion of interleukin-6 prevents cardiac inflammation, fibrosis and dysfunction without affecting blood pressure in angiotensin II-high salt-induced hypertension. *J Hypertens* 33(1):144–152.
31. Hur W, et al. (2008) Clinical stage EGFR inhibitors irreversibly alkylate Bmx kinase. *Bioorg Med Chem Lett* 18(22):5916–5919.
32. Jarboe JS, Dutta S, Velu SE, Willey CD (2013) Mini-review: Bmx kinase inhibitors for cancer therapy. *Recent Patents Anticancer Drug Discov* 8(3):228–238.
33. Liu F, et al. (2013) Discovery of a selective irreversible BMX inhibitor for prostate cancer. *ACS Chem Biol* 8(7):1423–1428.
34. O'Connell T, Ni Y (2002) *Isolation of Adult Mouse Cardiac Myocytes from One Heart*. AfCS Procedure Protocol ID PP00000125. Available at www.signaling-gateway.org/data/cgi-bin/ProtocolFile.cgi?pid=PP00000125. Accessed September 22, 2015.
35. Schefe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H (2006) Quantitative real-time RT-PCR data analysis: Current concepts and the novel "gene expression's CT difference" formula. *J Mol Med (Berl)* 84(11):901–910.
36. Huang W, Sherman BT, Lempicki RA (2009) Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 37(1):1–13.
37. Huang W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1):44–57.
38. Subramanian A, et al. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102(43):15545–15550.
39. Mootha VK, et al. (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34(3):267–273.