Puma Deletion Delays Cardiac Dysfunction in Murine Heart Failure Models Through Attenuation of Apoptosis

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Background—Puma (p53-upregulated modulator of apoptosis) is a proapoptotic Bcl-2 family protein that serves as a general sensor in response to pathological apoptotic stimuli. In previous work, we demonstrated that puma ablation protects the heart from reperfusion injury in a Langendorff setting. Consistent with this, downregulation of Puma in isolated cardiac myocytes prevented apoptosis induced by different proapoptotic agents. Here, we extended our research to investigate the role of Puma, a downstream mediator of p53, in the development of heart failure using Puma−/− mice.

Methods and Results—Mice underwent transverse aortic constriction, and the characteristics of cardiac remodeling were analyzed by echocardiography, histology, and gene expression at multiple time points after surgery. Four weeks after the operation, puma deletion attenuated pressure overload–induced apoptosis and fibrosis; however, it did not affect hypertrophy and angiogenesis and maintained functional performance (fractional shortening, 39% versus 25.2% in Puma−/− versus WT mice, respectively). Even at 12 weeks after transverse aortic constriction, Puma−/− mice displayed only slightly reduced contractility. In addition, transverse aortic constriction induced puma expression in a partially p53-dependent manner. To corroborate these findings, we studied another heart failure model in which heart-specific mdm4 deletion leads to p53 activation and dilated cardiomyopathy. In these mice, Puma was upregulated and its deletion rescued the cardiomyopathy phenotype.

Conclusions—Our data indicate that Puma might be a critical component of the apoptotic signaling pathways that contribute to ventricular remodeling and heart failure. Therefore, Puma inactivation may serve as a preferential target to prevent heart failure induced by cellular stress. (Circulation. 2011;124:00-00.)

Key Words: apoptosis • hypertrophy • heart failure • remodeling

It is estimated that at least 5 million people in the United States suffer from chronic heart failure, which is associated with progressive myocardial dysfunction accompanied by cardiac remodeling. Recent studies identified the p53 molecular pathways underlying cardiac remodeling during pathophysiological stimulation such as pressure overload. P53 is a tumor suppressor protein with proapoptotic and antiproliferative activities achieved through transcriptional activation of a large number of target genes, including different proapoptotic proteins such as Puma (p53-upregulated modulator of apoptosis), Noxa, and Bax.

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Puma, which contains only 1 Bcl-2–like homology domain (BH3-only), is a unique member of the Bcl-2 family because it integrates and implements most signals mediated by different apoptosis inducers, including doxorubicin, hypoxia/reoxygenation, kinase inhibitors, phorbol esters, cytokines, growth factor deprivation, and gamma irradiation. Similar to other BH3-only proteins, Puma serves as a proximal signaling molecule that transduces death signals to the mitochondria, where it acts through multidomain Bcl-2 family members such as Bcl-2, Bcl-xL, and Mcl-1. Puma-induced apoptosis proceeds through a typical mitochondrial pathway, involving the depolarization and permeabilization of the mitochondrial membrane and the release of mitochondrial proteins, including cytochrome c, Smac, and apoptosis-inducing factor, as well as activation of caspases.

In general, Puma is expressed at a very low level in different tissues, and its level is regulated primarily by transcriptional mechanisms. Among the relevant transcription factors (p53, p73, Foxo3a, and E2F1), the regulation by p53 is the most widely investigated. In vivo expression of puma and its association with apoptosis have been demonstrated for hematopoietic, neuronal, cardiac, intestinal, and immune cells, underscoring a potentially universal role of Puma in the development of different diseases.

It thus appears that Puma as a target of p53 represents a general sensor of cell death stimuli and therefore a promising...
drug target to prevent tissue injury in many organs such as the heart, brain, and intestine and in AIDS and many other conditions.10

On the other hand, the level of p53 is regulated primarily by ubiquitination through the E3 ubiquitin ligase family of Mdm proteins (Mdm2 and Mdm4).11–13 Both Mdm2 and Mdm4 are negative regulators of p53 activity, although their mechanisms are different. In contrast to Mdm2, Mdm4 does not regulate p53 level but downregulates its transcriptional activity through direct binding.11–13 The role and the regulatory connection between the Mdm proteins and p53 have been extensively investigated in cancers. In the heart, our understanding is much more limited in vivo because mdm4 loss of function results in embryonic lethality with cardiac abnormalities. In another model of Mdm deficiency using the heart-specific deletion of mdm4, mice develop dilated cardiomyopathy in association with increased myocyte apoptosis.14 Both models underpin the importance of the p53-Mdm system in myocardial homeostasis. Other investigators also reported that p53 plays a role in cardiomyocyte apoptosis; however, the direct molecular delineation of this pathway has not been explored.2–6 Our work fills this gap by analyzing the p53-Puma axis in vivo first by using a constriction (TAC) and second by monitoring the development mouse model combined with transverse aortic constriction.11–13 The role and the regulatory connection between the Mdm proteins and p53 have been extensively investigated in cancers. In the heart, our understanding is much more limited in vivo because mdm4 loss of function results in embryonic lethality with cardiac abnormalities. In another model of Mdm deficiency using the heart-specific deletion of mdm4, mice develop dilated cardiomyopathy in association with increased myocyte apoptosis.14 Both models underpin the importance of the p53-Mdm system in myocardial homeostasis. Other investigators also reported that p53 plays a role in cardiomyocyte apoptosis; however, the direct molecular delineation of this pathway has not been explored.2–6 Our work fills this gap by analyzing the p53-Puma axis in vivo first by using a constriction (TAC) and second by monitoring the development of heart failure in double Puma−/− and conditional Mdm4−/− animals. Here, we demonstrate that ablation of puma curbs excessive apoptosis associated with cardiac tissue damage and helps preserve cardiac function, providing rationale for Puma as a potential therapeutic target in cardiovascular diseases.

Methods

Experimental procedures are provided in the online-only Data Supplement for the generation of Puma−/−, conditional Mdm4−/−, double-knockout, and wild-type (WT) mice; TAC surgery; echocardiography; quantitative reverse-transcription polymerase chain reaction; histology; and immunohistochemistry. Results are shown as mean±SEM. For statistical analysis of data from multiple groups, 2-way ANOVA or 2-way repeated-measures ANOVA was performed, followed by Holm-Sidak posthoc analysis with SigmaStat 3.5 software. If the normality test failed, log transformation was performed before the test. Survival of mice was plotted on a Kaplan-Meier curve. A value of P<0.05 was considered statistically significant.

Results

Targeted Deletion of Puma Delays the Development of Cardiac Dysfunction After Transverse Aortic Constriction

Initially, we evaluated Puma−/− and WT age-matched control mice and confirmed that puma was dispensable for normal embryonic and postnatal development of the heart (see the Results section and Table I in the online-only Data Supplement). To investigate the effect of Puma deletion on stressed hearts, 8-week-old mice received TAC surgery to induce left ventricular pressure overload. These studies usually included sham- and TAC-operated Puma−/− and WT age-matched controls. Cardiac function was monitored at multiple time points by echocardiography: before TAC (day 0), at the early adaptation time (1 week), at the time of development of cardiac dysfunction (4 weeks), and during the progression of the disease (12 weeks). As expected, fractional shortening (FS) was maintained during the first week in WT mice and then declined at 4 weeks and further decreased by 12 weeks (Figure 1A and 1B). In contrast, Puma−/− mice maintained their function at 1 and 4 weeks after TAC and remained similar to sham-operated controls (Figure 1A and 1B). At 12 weeks, however, cardiac function declined slightly in this study group as well. Significant increases in diastolic and systolic left ventricular internal diameters in WT but not in Puma−/− mice were measured at 4 weeks after induction of pressure overload compared with sham-operated animals (Figure 1C and 1D). Similar changes in the Puma−/− animals began to develop by 12 weeks after TAC surgery (not shown), leading to changes in FS. In the present study, cardiac functional performance was not monitored beyond 12 weeks owing to animal welfare concerns in the WT TAC group; therefore, further functional deterioration in the Puma−/− TAC animals cannot be excluded. Together, these data indicate that Puma−/− animals maintained cardiac function in response to pressure overload for a longer period of time than their WT controls. Therefore, puma deletion slowed but did not eliminate the progression of pressure overload–induced cardiac dysfunction.

Puma Ablation Does Not Prevent Transverse Aortic Constriction–Induced Hypertrophy and Angiogenesis

To better understand the potential role of Puma in the development of cardiac hypertrophy, we measured 3 different parameters of hypertrophy after TAC surgery: the ratio of heart weight to body weight, posterior wall thickness at diastole, and myocyte cross-sectional area (Figure 2A through D).3,15

We observed significant TAC-induced increases in the ratio of heart weight to body weight, posterior wall thickness at diastole, and cross-sectional area in both animal types (WT and Puma−/−) compared with their sham controls but did not observe any significant alterations between WT/TAC and Puma−/−/TAC animals (Figure 2A through D). These data indicate that puma deletion did not interfere with TAC-induced early “adaptive” and late changes. Moreover, together with functional assessment (FS), these findings suggest that the hypertrophic myocardium of WT animals but not that of the Puma−/− animals is already in the heart failure stage. Because the hypertrophic response to pressure overload in Puma−/− mice is intact, it is possible that the function of Puma is not critical for hypertrophy or can be compensated for by other hypertrophic signaling molecules. In any case, puma ablation appears to slow the development of the maladaptive phase of TAC-induced cardiac hypertrophy.

We also examined the effect of Puma on angiogenesis by quantifying vascular endothelial growth factor expression and evaluating capillary density by CD-31 staining (Figure I in the online-only Data Supplement), but we found no differences between WT and Puma−/− TAC-operated animals, indicating that Puma does not play a role in pressure overload–induced angiogenesis.
Transverse Aortic Constriction–Induced Pressure Overload Activates *Puma* Transcription in the Heart in a Partially p53-Dependent Manner

Because 4 weeks after TAC operation myocardial hypertrophy is still associated with normal cardiac function in *Puma*−/− animals (Figure 1), we hypothesized that *puma* activation accelerates the development of TAC-induced heart failure. To test this, we first measured time-dependent expression of *puma* in the TAC model. Because *puma* is transcriptionally regulated, its activity correlates with *puma*

**Figure 1.** Cardiac function of *Puma*-ablated animals in a pressure-overload transverse aortic constriction (TAC) model. A, Representative M-mode echocardiographic recordings are shown from sham- and TAC-operated animals after 1 and 4 weeks. B, Changes in fractional shortening (FS) in wild-type (WT) and *Puma* knockout (KO) mice for up to 12 weeks after TAC or sham surgery. Two-way repeated-measures ANOVA of FS in the same study groups revealed no time dependence in the WT sham and *Puma* KO sham groups, whereas the WT TAC group showed significant decline starting at 3 weeks (P<0.05 at 3 and 4 weeks, P<0.01 at 12 weeks vs day 0). Fractional shortening in the *Puma* KO TAC group remained stable for most of the study duration and decreased only at 12 weeks (P<0.05 vs day 0). Group comparisons: *P*<0.05, †P<0.01 vs corresponding sham; ‡P<0.05 vs corresponding WT TAC. There was a statistically significant interaction between genotype and operation vs time (P<0.001). Diastolic (LVIDd; C) and systolic (LVIDs; D) left ventricular internal diameter 4 weeks after surgery. *P*<0.05 vs WT sham; †P<0.05 vs WT TAC (n=12 to 15 per group). There was a statistically significant interaction between genotype and operation in both C and D (P<0.005).

**Figure 2.** Effect of *Puma* ablation on transverse aortic constriction (TAC)-induced hypertrophy. A, Ratio of heart weight to body weight (HW/BW) after sham or TAC surgery. *P*<0.05, †P<0.01 vs corresponding sham (n=6 to 10 per group). B, Diastolic posterior wall thickness at 4 weeks after TAC or sham operation. *P*<0.05 vs corresponding sham (n=12 to 15/group). C, Representative histological images of TAC-induced cardiomyocyte hypertrophy at the 4-week time point with FITC-tagged WGA labeling (green). D, Quantification of cardiomyocyte cross-sectional area by National Institutes of Health Image J software at 4 weeks. *P*<0.01 vs corresponding sham (n=4 hearts per group).
mRNA levels (Figure 3A), which was detectable in sham-operated WT animals but not in puma-deleted heart samples. Importantly, puma expression did not change 1 week after TAC in the WT animals but showed a 2.75-fold increase at 4 weeks after surgery (Figure 3A). Because p53 is a critical upstream regulator of this molecule, we also tested p53<sup>−/−</sup> animals in the TAC model to assess whether puma upregulation is p53-dependent. Although puma levels were similar at baseline in p53<sup>−/−</sup> and WT sham animals, TAC did not induce puma in p53<sup>−/−</sup> mice, indicating that its transcriptional activation is driven, at least in part, by p53 in response to cellular stress. Puma expression was also detected by immunohistochemistry in TAC-operated WT animals showing a characteristic cytoplasmic staining (Figure 3B). We noticed that Puma staining was easily detectable in TAC-operated but not in sham-operated animals (data not shown), suggesting that cellular stress is an important inducer of puma expression in the heart. The precise location of Puma in these TAC-operated mice cannot be identified because fluorescence antibody costaining was not successful with the anti-Puma antibody. Guided by tissue morphology, we concluded that Puma could be upregulated in both cardiac and noncardiac cells in this model.16

Together, these data indicate that upregulation of puma coincides with a decline in heart function, suggesting that this molecule may participate in TAC-induced functional decompensation of WT animals.

**Puma Deletion Inhibits Pressure Overload–Induced Apoptosis**

To determine whether Puma is involved in apoptosis of heart cells, we compared apoptosis in WT and Puma<sup>−/−</sup> mice after TAC using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (Figure 4A). The apoptotic rate was negligible in sham-operated WT and Puma<sup>−/−</sup> hearts and did not change significantly 1 week after TAC either (data not shown). However, the level of apoptosis was strikingly
Transverse Aortic Constriction–Induced Fibrosis Is Attenuated in Puma Knockout Hearts

Long-term exposure to TAC triggers myocardial cell loss and, in turn, replacement fibrosis. To determine the effect of puma deletion on interstitial fibrosis, sections of hearts from WT and Puma−/− mice were stained with Masson’s trichrome. Sham-operated mice had normal tissue morphology with no collagen deposition (Figure 5A). In line with previous reports using pressure overload models, fibrosis became prominent in the myocardium from WT mice 4 weeks after TAC, characterized by scattered lesions in the myocardium at multiple foci (Figure 5A). In contrast, Puma−/− animals had minimal accumulation of fibrous tissue in the myocardial space, suggesting lower remodeling in response to pressure overload. Some collagen accumulation in Puma−/− animals rather appeared perivascular in mid-sized and large arteries. Quantitative analysis of trichrome staining showed a 2- to 3-fold increase in Puma−/− mice over sham-operated controls and an ∼10-fold increase in WT hearts at this time point (Figure 5B). Extracellular matrix turnover was further evaluated by analyzing collagen III and matrix metalloproteinase-2 and 9 expressions (Figure II in the online-only Data Supplement). Although these markers were elevated in Puma−/− mice after TAC, their levels remained significantly lower than their wild-type counterparts. In summary, pressure overload–induced myocardial fibrosis was mitigated by puma ablation, an effect that could potentially contribute to the preservation of cardiac function in this heart failure model.

Deletion of Puma Rescues the Dilated Cardiomyopathy Phenotype in Conditional Mdm4 Knockout Mice

To further investigate whether puma expression through p53 activation can lead to the development of heart failure, we tested whether puma ablation can rescue the cardiomyopathy phenotype of heart-specific Mdm4−/− mice. Although heart-specific Mdm4-null mice are viable and fertile, they spontaneously develop dilated cardiomyopathy in a p53-dependent manner.14 Therefore, this model offers a reasonable tool for in vivo analysis of the p53-Puma pathway in heart failure. Because of the low frequency of homozygotes for both alleles (Puma−/−/Mdm4−/−), mice from different litters participated in this 2-year study. Mice were monitored for heart function and body weight by echocardiography every month up to 8 months of age, together with single-knockout (Mdm4−/−/Puma−/− or Mdm4+/−/Puma−/−) and WT (Mdm4+/+/Puma−/−) animals, starting at the age of 2 months (Figure 6A and 6B). At this point, sham-operated mice of all genotypes were not different in body weight and heart function. Mdm4−/−/Puma−/− and Mdm4+/−/Puma−/− groups had a 2-month delay in the onset of heart failure compared with WT mice. However, Mdm4−/−/Puma−/− and Mdm4+/−/Puma−/− mice exhibited a significant increase in heart weight and left ventricular mass compared with WT mice and Mdm4−/−/Puma+/− mice (Figure 6C and 6D). TAC mice of all genotypes were euthanized at 8 months of age and hearts were harvested for further analysis. As shown in Figure 6E and 6F, TAC surgery was associated with marked left ventricular dilatation, which was significantly higher in WT mice compared with sham-operated mice. In contrast, TAC surgery induced a significantly smaller increase in left ventricular mass in Mdm4−/−/Puma−/− and Mdm4+/−/Puma−/− mice compared with WT mice and Mdm4−/−/Puma+/− mice. Furthermore, TAC surgery induced a significant increase in heart weight in WT, Mdm4−/−/Puma−/−, and Mdm4+/−/Puma−/− mice, whereas Mdm4−/−/Puma+/− mice showed a significant reduction in heart weight compared with WT mice. These findings support a protective role for puma in the development of cardiac hypertrophy in the Mdm4−/−/Puma−/− and Mdm4+/−/Puma−/− genotypes.
initial time, minimal and nonsignificant differences were measured between WT and Puma−/− animals in FS (42% and 39%), and no change occurred between these groups during the entire study duration. In the Mdm4−/− study group, FS was slightly lower (35%) at 2 months than in age-matched controls, and as expected, it steadily declined with time as wall thinning occurred, in line with the development of dilated cardiomyopathy (Figure 6A and 6B). Interestingly, double-knockout animals (Mdm4−/−/Puma−/−) presented with normal FS at 2 months of age, and myocardial function was maintained at a significantly higher level compared with mice deficient in only mdm4. Most importantly, double-knockout mice had a longer lifespan than Mdm4−/− animals. Although most Mdm4−/− mice died by 7 to 9 months of age, double knockouts looked healthier, and most of the animals survived into their first year (The Kaplan-Meier survival curve is shown in Figure III in the online-only Data Supplement).

Puma mRNA expression was measured on a monthly basis and increased progressively in Mdm4−/− mice from 3 months of age, coinciding with the deterioration in cardiac function (Figure 6C). This increase, however, was not detectable in age-matched WT controls, providing a further link between puma expression and heart failure. Sections stained for apoptotic events with kinetics similar to apoptosis of the same samples also showed elevation in puma expression and heart failure. Sections stained for apoptotic nuclei were quantified on 3 sections from each animal at 20 different areas of ×200 magnification with the Apoptag Peroxidase In Situ Apoptosis Detection kit. Two-way ANOVA in the same study groups revealed a strong time dependence in Mdm4 KO/Puma WT mice (P<0.01 at 4 to 5 months and P<0.01 at 6 to 8 months vs 2 months); no time dependence was found in the WT group. Group comparisons: *P<0.01 vs corresponding WT (n=3 to 5 per group). D, Apoptotic nuclei were quantified on 3 sections from each animal at 20 different areas of ×200 magnification with the Apoptag Peroxidase In Situ Apoptosis Detection kit. Two-way ANOVA in the same study groups revealed a strong time dependence in Mdm4 KO/Puma WT mice (P<0.05 at 5 and 8 months and P<0.01 at 6 to 7 months vs 2 months). Mdm4 KO/Puma KO mice showed time dependence at 6 months only (P<0.05 vs 2 months); no time dependence was found in the WT and Mdm4 WT/Puma KO groups. Group comparisons: *P<0.05, †P<0.01 vs corresponding WT (n=3 to 5 per group). TUNEL indicates terminal deoxynucleotidyl transferase dUTP nick-end labeling.

Discussion

Role of the BH3-Only Protein Puma in Ventricular Remodeling and Heart Failure

In the present study, we investigated the role of Puma in the development of ventricular remodeling and cardiac failure using a pressure overload model (TAC) and genetically modified mice (Mdm4−/− and Puma−/−). Our data indicated that puma expression was upregulated in the myocardium in response to mechanical stress and induced apoptosis. In contrast, puma ablation attenuated cardiac

Figure 6. Impact of Puma deletion on the progression of dilated cardiomyopathy in heart-specific mdm4-null mice. Wild-type (WT), Mdm4 WT/Puma knockout (KO), Mdm4 KO/Puma WT, and Mdm4 KO/Puma KO mice were aged, and their cardiac function was monitored by echocardiography between 2 and 8 months. Time course of (A) fractional shortening (FS) and (B) posterior wall thickness at diastole (PWTd) is shown in all genotypes. Two-way repeated-measures ANOVA of FS and PWTd in the same study groups revealed no time dependence in the WT and Mdm4 WT/Puma KO groups, whereas the Mdm4 KO/Puma WT group showed significant decline for FS (P<0.01 at 3 to 6 months and P<0.01 at 7 to 8 months vs 2 months) and for PWTd (P<0.05 at 5 to 6 months and P<0.01 at 7 to 8 months vs 2 months). The Mdm4 KO/Puma KO group exhibited reduced FS at 6 to 8 months and reduced PWTd at 7 to 8 months (P<0.05 vs 2 months). Group comparisons: for both FS and PWTd, †P<0.05, †P<0.01 vs corresponding WT; ‡P<0.05 vs Mdm4 KO/Puma WT group (n=6 to 8 per group). There was a statistically significant interaction between genotype and time in both A and B (P<0.001). C, Puma mRNA expression with quantitative polymerase chain reaction in hearts from Mdm4 KO/Puma WT and WT mice from the age of 2 to 8 months. Two-way ANOVA in the same study groups revealed a strong time dependence in Mdm4 KO/Puma WT mice (P<0.05 at 4 to 5 months and P<0.01 at 6 to 8 months vs 2 months); no time dependence was found in the WT group. Group comparisons: *P<0.01 vs corresponding WT (n=3 to 5 per group). D, Apoptotic nuclei were quantified on 3 sections from each animal at 20 different areas of ×200 magnification with the Apoptag Peroxidase In Situ Apoptosis Detection kit. Two-way ANOVA in the same study groups revealed a strong time dependence in Mdm4 KO/Puma WT mice (P<0.05 at 5 and 8 months and P<0.01 at 6 to 7 months vs 2 months). Mdm4 KO/Puma KO mice showed time dependence at 6 months only (P<0.05 vs 2 months); no time dependence was found in the WT and Mdm4 WT/Puma KO groups. Group comparisons: *P<0.05, †P<0.01 vs corresponding WT (n=3 to 5 per group). TUNEL indicates terminal deoxynucleotidyl transferase dUTP nick-end labeling.
remodeling by reducing apoptosis and fibrosis and delayed the development of heart failure without affecting volumetric changes of cardiac myocytes or angiogenesis. In this model, we also demonstrated that *puma* expression is at least partially p53 dependent. Moreover, *Mdm4*−/− mice could be rescued from heart failure by the lack of *puma*, underlining the importance of apoptosis in the pathomechanism of heart failure.

Recent studies described that p53 may exert its effects via multiple mechanisms. First, in this study, *mdm4* deletion led to Puma-dependent apoptosis. Second, p53 activation by doxorubicin treatment initiated apoptosis and acute toxicity by blocking the mammalian target of rapamycin signaling cascade. Finally, p53 promoted the ubiquitination and proteasomal degradation of hypoxia-inducible factor-1α. Interestingly, the identity of the specific E3 ubiquitin ligase has not yet been revealed. Further studies are required to elucidate whether both the Puma-regulated apoptosis pathway and the hypoxia-inducible factor-1α-regulated angiogenesis pathway need to be targeted for successful therapy.

**Role of Other BH3-Only Proteins in Heart Failure**
Puma is not the only BH3-only protein that has been shown to be activated by TAC or other heart failure models. As described earlier, the effect of Nix is rather specific for TAC-induced myocyte apoptosis, whereas the effect of BNIP3 is more similar to ischemia-induced necrosis. In our earlier work, we reported the involvement of Puma in ischemic myocardial damage in isolated hearts. The present heart failure data warrant the extension of our research to in vivo ischemic conditions. These experiments are currently in preparation in our laboratory. If Puma expression is elevated in both ischemia and heart failure (Figure 3), it could present a unique therapeutic opportunity for both ischemic and nonischemic myocardial impairments. However, it remains to be elucidated whether any hierarchical relationship exists among the BH3-only family members and whether other BH3-only proteins, not yet studied in detail in the heart (eg, Bim or Bid), play a significant role in the development of heart failure. Interestingly, individual knockouts of all 3 genes (Nix, BNIP3, and Puma) are protective, indicating that their function may be overlapping and not redundant.

In the present study, we applied 2 murine heart failure models. We used the TAC model to induce pressure overload and a genetic model of dilated cardiomyopathy based on *mdm4* deletion. Our studies demonstrated that in both models Puma-dependent apoptosis may be sufficient for the development of cardiac failure. In future studies, it would also be interesting to examine the effect of knocking out Nix and/or BNIP3 in our currently investigated mouse models.

**Impact of p53 and Puma Deletion on the Development of Cancer and Heart Failure**
In response to pathological stimuli, p53 undergoes stabilization and activation, thereby promoting apoptosis and growth arrest, leading to tumor suppression. Although p53 inactivation protects against cardiac injury, p53 knockout mice develop tumors with a high frequency, and their lifespan is shortened. In contrast, Puma knockout mice are not tumor prone, indicating that inhibition of apoptosis by itself is not sufficient for cancer generation, but they are resistant to heart tissue damage. It thus appears that Puma antagonists might be developed to provide cardioprotection without increasing tumor susceptibility.

**ASK1 Is a Potential Activator of Puma in Cardiomyocytes**
The complex nature of apoptosis regulation during pressure overload is further emphasized by studies on apoptosis signal-regulating kinase 1 (ASK1), which is a proapoptotic mitogen-activated protein kinase kinase. Interestingly, we found that Puma knockout mice possess the same characteristic features as the ASK1 knockout mice when the different elements of cardiac remodeling are examined by echocardiography and histology. These data raise the possibility that ASK1 and Puma belong to the same nodal regulator factor for cardiac apoptosis. Consequently, Puma might be either a phosphorylation target or a binding partner for ASK1. These possibilities are to be tested to identify a potential link between Puma and ASK1.

The non–drug-related activation mechanism of the p53 pathway in the heart under pathological conditions is not well explored. However, recent data demonstrated the activation of the p53-Puma pathway by reactive oxygen species, providing a potential connection between the development of heart failure and aging (Sirt1) or chronic inflammation such as atherosclerosis (monocyte chemoattractant protein-1). Therefore, the comparison of mitochondrial function in *Puma*−/− and wild-type hearts could provide additional details about the mechanism of apoptosis and the link between cytoplasmic and mitochondrial pathways.

**Anti-Puma Therapy for Heart Diseases?**
The Puma protein is located at the crossroads of major signal transduction pathways serving as an apoptotic effector. Therefore, manipulation of Puma activity, directly or indirectly, may provide a potential target in both cancer and heart failure treatment and has been at the center of current drug research.

One group of related drugs includes Mdm2 antagonists, which reactivate the p53 pathway in p53-positive cancers. Importantly, these drugs (the prototype is called Nutlin) may also provoke severe cardiac side effects. Puma is regulated primarily by transcription, and although it can be activated by several transcription factors such as p53, p73, FOXO3a, and E2F1, only the role of p53 was analyzed in detail. At the same time, Mdm2 may also be activated by Akt-driven phosphorylation, which is protective against heart failure. In a reverse case, however, if oncogenes (such as Akt or Mdm2) are activated, that activation may lead to tumor formation, which is not the case when the downstream target of p53, ie, Puma, is inactivated.
Finally, a more specific and direct regulation of Puma levels may be achieved by a \textit{puma}-specific miRNA recently described in the Epstein-Barr virus, being potentially suitable for downregulation of \textit{puma} levels even for the treatment of human heart failure.\textsuperscript{36} These data together indicate that attenuation of Puma activity may provide a preferential target for heart disease without inducing cancer as a side effect.

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Disclosures
None.

References


CLINICAL PERSPECTIVE

p53 is a tumor suppressor gene that exerts its effect through transcriptional activation. Puma (p53-upregulated modulator of apoptosis) and Mdm4 represent 2 critical p53 targets; although Puma serves as the major downstream proapoptotic effector of p53, Mdm4 inhibits p53-mediated apoptosis by direct binding. Puma is a unique member of the Bcl-2 family because it integrates and implements most signals mediated by different apoptosis inducers. In the present study, we used Puma knockout mice in 2 heart failure models, triggered by pressure overload and heart-specific Mdm4-ablation, and found that Puma-induced apoptosis may play a role in the progression of heart failure. Interestingly, Puma can be activated not only by pressure overload but also by myocardial ischemia. Our results thus provide insight into the function of the Mdm4-p53-Puma axis in cardiomyocytes, which could ultimately aid in the development of antiapoptotic drug candidates to treat various cardiac diseases. Moreover, lack of Puma does not recapitulate the tumor-prone phenotype observed in p53-negative mice. Because the Mdm4-p53-Puma interface has also been the focus of recent drug discovery efforts in cancer, additional functional analysis of this system in the heart could contribute to the development of safer anticancer therapies with minimal cardiotoxic side effects. Of note, Puma is not the only contributor to cardiac signaling during heart failure, and further studies are required to elucidate its connection with other apoptotic pathways. In conclusion, Puma antagonists might be developed in the future to provide cardiac protection without increasing susceptibility to tumors, an adverse effect of therapies based on p53 inactivation.
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals: The investigation conformed to the use of the US National Institute of Health Guideline for the Care and Use of Laboratory Animals. All in vivo protocols were approved by the IACUC committee of Harvard University. Both puma−/− and heart-specific mdm4−/− mice had a C57BL/6J genetic background and they were kind gifts from Dr. Zambetti1 and Dr. Lozano2, respectively. Colonies were propagated at the Animal Facility of Harvard University. A conditional allele of Mdm4, designated as Mdm4FX, had 2 loxP sites that surround exon 2 (which contains the ATG start codon)2. Deletion of exon 2 results in a null allele, designated as Mdm4Δ2. First, Mdm4+/Δ2-αMyHC-Cre mice were crossed with Puma−/− mice to generate Mdm4+/Δ2-αMyHC-Cre-Puma+/− litters and Mdm4+/FX-αMyHC-Cre mice were bred with Puma−/− mice to generate Mdm4+/FX-αMyHC-Cre-Puma+/− litters. Subsequently, these mice were crossed to generate the following study cohorts: Mdm4+/+αMyHC-Cre-Puma+/+ (designated as Mdm4 wt/Puma wt), Mdm4Δ2/FX-αMyHC-Cre-Puma+/+ (Mdm4 KO/Puma wt), Mdm4+/+αMyHC-Cre-Puma−/− (Mdm4 wt/Puma KO), and Mdm4Δ2/FX-αMyHC-Cre-Puma−/− (Mdm4 KO/Puma KO). Genotyping of these mice was typically performed at the age of 3 weeks for puma, mdm4, and Cre alleles.

Minimally invasive transverse aortic constriction:3,4 Minimally invasive transverse aortic constriction (TAC) was performed in mice weighing 24-26g to trigger pressure overload of the left ventricle. Animals were anesthetized with 1-2% Isoflurane in 100% oxygen delivered through a vaporizer. Body temperature was controlled by a heat pad, equipped with a circulating water bath throughout the entire procedure. After the left parasternal midline skin incision, the aorta was exposed and a 6-0 braided silk suture was placed around the transverse aortic arch. Aortic constriction was carried out by ligating the silk suture against a 28G needle, followed by
quick removal of the needle. The chest wound was closed in layers (muscle and skin) with a 6-0 absorbable suture. Sham-operated mice underwent a similar surgical procedure without constriction of the aorta. Mice were allowed to recover from anesthesia under warm conditions. Mice received Buprenorphine (0.1 mg/kg s.c.) as analgesic twice daily for three days. Mice were sacrificed 1 or 4 weeks after operation. Mortality during and immediately following the procedure was approximately 5%.

**Echocardiography:** Echocardiography was performed preoperatively (baseline) and at the indicated time points after TAC operation. Animals which did not meet the study inclusion criteria at baseline level were removed from the study. Data acquisition was performed on lightly anesthetized animals using inhalation of 0.75-1% Isoflurane in 100% oxygen mixture through a nose cone. Typically, heart rate was 500-550 beats/minute. Two-dimensional (2D) guided M-mode echocardiography was performed by a Sonos 5500 echocardiograph equipped with a 15-16 MHz pediatric linear transducer. Cardiac dimensions such as left ventricular systolic and diastolic diameters and posterior wall thickness (LVIDs, LVIDd, and PWTd) were obtained from M-mode tracings. Typically, three separate cardiac cycles were averaged for analysis. Cardiac contractile function represented by LV fractional shortening (FS%) was calculated as \[
\frac{[(LV \text{ diastolic diameter} - LV \text{ systolic diameter}) / LV \text{ diastolic diameter}] \times 100.}
\] All measurements were done from leading edge to leading edge according to the American Society of Echocardiography guidelines.

**Quantitative RT-PCR:** Total RNA was extracted from snap-frozen mouse myocardial tissue by Trizol reagent. Real-time qRT-PCR was performed with a Bio-Rad Opticon II instrument. For relative quantification of mRNA expression, cDNA was produced with reverse transcriptase (Applied Biosystems) from total RNA and was subjected to RT-PCR using SYBR green ready mix (New England BioLabs) and gene specific primers. Copy numbers were normalized to GAPDH. All reactions were performed in triplicates; triplicates were from the same cDNA reaction and hence represent technical replicates.
**Histology:** After perfusion with physiological saline, hearts were trimmed from the aorta then immersed in 10% formalin, embedded in paraffin and cut into 6-µm sections. Masson’s Trichrome staining was used for fibrosis visualization. The fibrotic area was calculated as percentage of total LV myocardial area with the use of Metamorph software. Myocyte cross-sectional area was determined with the use of FITC-tagged wheat germ agglutinin (WGA) labeling. Cardiomyocyte cross-sectional area was calculated by taking 15 pictures of each section and measuring the cardiomyocyte diameter of 50 cardiomyocytes/picture using NIH Image J software. Assessment of apoptosis from paraffin sections was performed with Apoptag Peroxidase In Situ Apoptosis Detection kit (Chemicon) according to the manufacturer’s instructions. Briefly, deparaffinized sections were treated with Proteinase K for 30 min and DNA fragments labeled with biotin-conjugated dUTP and terminal deoxynucleotidyl transferase for 1 hour at 37°C. Nuclei were counterstained with hematoxylin then visualized with light microscopy. Twenty pictures of each section were taken at 200× magnification.

**Immunohistochemistry:** Frozen heart sections were cut into 8-µm sections then stained with Puma (Imgenex), cleaved caspase-3 (Cell Signaling Technology), or CD-31 (BD Pharmingen) antibody and counterstained with WGA and DAPI.

**Statistical analysis:** Results are shown as mean ± SEM. For statistical analysis of data from multiple groups, two-way ANOVA or two-way repeated-measures ANOVA was performed followed by Holm-Sidak posthoc analysis using the SigmaStat 3.5 software. In case the normality test failed, a log transformation was performed before the test. Survival of puma−/− and/or mdm4−/− animals were plotted on a Kaplan-Meier curve. A value of $P<0.05$ was considered statistically significant.
Supplemental Results

Characterization of the heart in *puma* knockout mice

To elucidate the role of Puma in the development of heart failure, we utilized a previously established *puma* knockout murine model (*Puma*<sup>−/−</sup>). *Puma*<sup>−/−</sup> mice were fertile and propagated as heterozygotes. They were born according to the Mendelian frequency and proved indistinguishable in appearance from age-matched WT controls. We found no significant difference in body weight and heart weight between age-matched *Puma*<sup>−/−</sup> and WT mice (Supplemental Table 1S). Similarly, *Puma*<sup>−/−</sup> hearts exhibited no evidence of any morphological defects, nor did histological examination of the hearts demonstrate any signs of cardiomyopathy, necrosis, or ventricular fibrosis (data not shown).

To determine whether *puma* ablation affects baseline cardiac function, echocardiography was performed on 8-week-old mice that showed no significant difference in end-diastolic (LVIDd) and end-systolic (LVIDs) internal dimensions of the left ventricle, posterior wall thickness (PWTd), and fractional shortening (FS) between *Puma*<sup>−/−</sup> and WT (Supplemental Table 1S), indicating that *Puma*<sup>−/−</sup> hearts have normal global cardiac structure and function. Some *Puma*<sup>−/−</sup> mice were followed until 1 year of age. These animals also had normal heart function, indicating that lack of *puma* does not result in defect even at older age. These results together confirmed that *puma* was dispensable for normal embryonic and postnatal development of the heart.
Supplemental Table 1S. Physiological parameters and echocardiography data at baseline level in *Puma*⁻/⁻ and WT mice at 8 weeks of age

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Puma⁻/⁻</th>
<th>Wild-type</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>5</td>
<td>8</td>
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<tr>
<td>Body weight</td>
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<td>25.83 ± 0.9</td>
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<td>Heart weight</td>
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<td>Lung weight</td>
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<td>HW/BW</td>
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<td>LW/BW</td>
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<td>6.23 ± 0.12</td>
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<tr>
<td>LVIDd</td>
<td>cm</td>
<td>0.353 ± 0.004</td>
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<tr>
<td>LVIDs</td>
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<td>FS</td>
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<tr>
<td>LVPWd</td>
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<tr>
<td>Heart rate</td>
<td>beats/min</td>
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<td>521 ± 8</td>
</tr>
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</table>

HW/BW indicates heart weight to body weight ratio; LW/BW, lung weight to body weight ratio; LVIDd and LVIDs, diastolic and systolic left ventricular internal diameter, respectively; FS, fractional shortening; LVPWd, diastolic left ventricular posterior wall thickness. Data are expressed as mean ± SEM. There are no significant differences in any listed parameters between *Puma* knockout and wild-type mice.
Supplemental Figure 1S: Effect of Puma ablation on TAC-induced angiogenesis. 

A. VEGF (vascular endothelial growth factor) mRNA expression was analyzed by qPCR. *, p<0.05 vs. corresponding sham (N=5-6/group).

B. Representative histological images of the myocardium indicate no differences in capillary density (4 weeks after TAC or sham operation). Sections were stained by FITC-tagged WGA (green), CD-31 antibody (red), and nuclear DAPI (blue) (400× magnification).
Supplemental Figure 2S. Impact of Puma deletion on TAC-induced matrix metalloproteinase expression. A. MMP-2 and B. MMP-9 mRNA expression was analyzed using qPCR; *, p<0.05 vs. corresponding sham; †, p<0.05 vs. WT TAC 4 weeks (N=4-5/group). There was a statistically significant interaction between operation and time vs. genotype in both A and B (p<0.001).
Supplemental Figure 3S. Kaplan-Meier survival curve of mice with different genotypes.

Group comparisons: *, p<0.001, †, p<0.01 vs. WT; ‡, p<0.01 vs. Mdm4 KO/Puma wt (N=18-33/group).
Supplemental References