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

Ph.D. Thesis

Author: Adel Mandl, M.D.

Program leader: Prof. Kalman Toth, M.D., D.Sc.
Project leader: Peter Erhardt, M.D., Ph.D.

First Department of Medicine
University of Pécs Medical School
Pécs, Hungary

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INTRODUCTION

Heart Failure

Heart failure is a common clinical syndrome representing the aftermath of a number of different cardiac diseases. It can result from any structural or functional cardiac disorder that impairs the ability of the left ventricle to fill with or eject blood. Aging of the population and prolongation of the lives of cardiac patients by modern therapeutic innovations has led to an increasing incidence of heart failure. A 2013 update from the American Heart Association (AHA) reported that there were an estimated 23 million people with heart failure worldwide. Congestive heart failure (CHF) is one of the most common reasons for hospital admission in the United States and most Western European countries. Despite improvements in therapy, the mortality rate in patients with heart failure has remained unacceptably high; therefore the need for further therapeutic inventions is still significant.

Heart failure is caused by any condition that reduces the efficiency of the myocardium through damage or overload. These conditions include myocardial infarction and other forms of ischemic heart disease, hypertension, valvular heart diseases, cardiomyopathy, myocarditis, and amyloidosis, among others. Although the causes of these diseases are different, they share molecular, biochemical, and cellular events to collectively change the shape of the myocardium and lead to the failing of the heart. This study investigates heart failure caused by pressure overload of the heart, mimicking the impact of hypertension or certain valvular diseases such as aortic stenosis. High blood pressure is one of the most important risk factors for heart failure; about 75% of heart failure cases have antecedent hypertension. The primary consequence of hypertension and aortic stenosis is an increased workload of the heart, which leads to increased pressure in the left ventricle. Initially, the left ventricle (LV) compensates by myocardial hypertrophy in order to maintain adequate pumping pressure. Although short-term hypertrophy may be adaptive, serving to normalize wall stress and oxygen demand, persistent, long-term activation of this response is detrimental. Compensatory mechanisms eventually fail to increase stroke volume and lead to end-stage heart failure. In the later stages, the left ventricle dilates, the wall thins, and the systolic function deteriorates.

At this stage, patients suffer from serious symptoms, such as dyspnea during exertion or when lying down, fatigue and weakness, edema in legs, ankles and feet, rapid or irregular heartbeat, reduced ability to exercise, persistent cough or wheezing with white

or pink blood-tinged phlegm, ascites, sudden weight gain from fluid retention, lack of appetite and nausea, difficulty concentrating or decreased alertness and eventually death.

Our understanding of the mechanisms underlying the transition from adaptive hypertrophy to maladaptive failure remains incomplete. The goal of this study was to investigate the mechanism of the transition during the development of heart failure. By learning more about the pathways that lead to deterioration of cardiac function, we hope to be able to develop therapeutic interventions that can prevent the process that potentially leads to death.

Cardiac Remodeling

In the setting of disease, the LV manifests a robust plasticity response that has been called pathological remodeling. It is the key pathophysiological process that leads to heart failure. Cardiac remodeling involves molecular, cellular, and interstitial changes that manifest clinically as changes in size, shape, and function of the heart after injury or stress stimulation. The cardiac myocyte is the primary cell involved in remodeling. Disease-related left ventricular remodeling is a complex process involving cardiac myocyte growth and death. In addition, other cellular elements within the ventricle participate, including fibroblasts (promoting fibrosis), vascular smooth muscle cells (promoting vascular stiffness), vascular endothelial cells (promoting endothelial dysfunction), and leukocytes (promoting inflammation).

Cardiac hypertrophy is a common type of cardiac remodeling that occurs when the heart experiences elevated workload. Because terminally differentiated cardiac myocytes are inefficient at reentering the cell cycle, these cells respond to pressure-overload stress by enlargement, ultimately leading to ventricular wall thickening and stiffness. This response, called hypertrophy is part of a compensatory process to maintain stroke volume at first. As the heart transitions from compensated hypertrophy to dilated heart failure, these changes intensify, resulting in myocyte lengthening, chamber dilation, and impaired systolic and/or diastolic function. Microischemia due to inadequate angiogenesis contributes to hypertrophy and decompensation; in contrast, promoting angiogenesis may slow the progression of the disease.

Apoptotic loss of cardiomyocytes can increase hemodynamic stress through ventricular dilation and wall thinning and is therefore hypothesized to play an important role in the progressive deterioration of the hypertrophied left ventricle, which will eventually

precipitate in overt heart failure. During the last few years there has been increasing evidence from human and animal models suggesting that apoptosis or programmed cell death could be a key modulator especially in the transition from “compensatory” hypertrophy to heart failure. In myocardial samples from patients who underwent heart transplantation, apoptosis was increased more than 200-fold in the failing heart. This study tests the hypothesis that apoptosis is a critical pathophysiological nodal point for multiple factors that induce hypertrophy and decompensation.

Cardiac myocytes are tethered and supported by a network of connective tissue that is primarily composed of fibrillar collagen. Collagen is a major determinant of myocardial architecture by stabilizing cardiomyocytes in their proper three-dimensional alignment, in parallel, in series, and in layers. Collagen is synthesized by interstitial fibroblasts and degraded by locally produced enzymes called collagenases, including matrix metalloproteinases (MMPs). After an insult, neurohormonal changes and cytokines stimulate collagen synthesis, leading to fibrosis and remodeling of the extracellular matrix.

Cardiac remodeling is both an adaptive and a maladaptive process. The adaptive component enables the heart to maintain function in response to pressure overload in the acute phase of cardiac injury. In contrast, progressive remodeling is deleterious and associated with a poor prognosis. This underscores the need to differentiate the pathways responsible for the initial phase of compensated hypertrophic growth from those promoting decompensation, dilation, and extreme ventricular remodeling. Because ventricular remodeling is an active process that contributes to physiological deterioration long after the primary cardiac insult, blocking the transition from compensated to decompensated phase remains an attractive therapeutic target.

Cardiac Myocyte Apoptosis

Functional deterioration of the hypertrophied left ventricle, eventually culminating in heart failure, is thought to be related to progressive loss of cardiac myocytes. Cell death may occur in a destructive, uncontrolled manner via necrosis or through a highly regulated programmed cell death, called apoptosis. Apoptosis is a highly complex process, involving an energy-dependent cascade of molecular events, which lead to controlled elimination of the cell. Apoptosis is considered to be a vital component of various physiologic events, including normal cell turnover, embryonic development, and functioning of the immune system. However, inappropriate apoptosis, either too little or

too much, is a factor in many human conditions, including neurodegenerative diseases, ischemic damage, autoimmune disorders, and cancer.

A cell undergoing apoptosis can be recognized by stereotypical morphological changes that include shrinking and deformation of the cell, ultimately loosing contact with the neighboring cells; chromatin condensation; blebbing or budding of the plasma membrane; and fragmentation of the cell into compact, membrane-enclosed structures, called apoptotic bodies. These morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of the proteolytic enzymes, caspases. Caspases eventually mediate the cleavage of deoxyribonucleic acid (DNA) and various proteins that are responsible for the integrity and shape of the cytoplasm or organelles. At the end, macrophages engulf and remove the apoptotic bodies from the tissue in an orderly manner, without eliciting an inflammatory response.

Cardiac myocytes carry out the contractile function of the myocardium, and they are largely incapable of replication; hence, their survival is crucial. In other words, once cardiac myocytes are lost, they cannot be replaced. Since apoptosis is a tightly regulated cell death program, modulation of certain steps of the apoptotic pathway has immense therapeutic potential. Recent evidence from human and animal studies suggests that apoptosis might be a key modulator in the transition from “compensatory” hypertrophy to heart failure. Therefore, limiting cardiac muscle loss by inhibiting apoptosis may have therapeutic potential in heart failure. Our study focused on the role of apoptosis in the development of heart failure.

Mdm4-p53-Puma Pathway

p53, the Guardian of the Genome: The tumor suppressor, p53 has been called the "guardian of the genome" because of its ability to determine the cell’s fate upon DNA damage. The main role of p53 is the removal of compromised cells when other systems fail. Under normal circumstances, the level of p53 protein is low. In response to a variety of stress signals, especially DNA damage, p53 becomes activated and induces growth arrest and DNA repair. Alternatively, in the case of extensive damage, p53 initiates the controlled demise of the cell by apoptosis. p53 helps preventing the proliferation of cells containing abnormal DNA, either via growth arrest and repair or apoptosis. Since p53 is known to promote apoptosis, and apoptosis is thought to be involved in heart failure, the role of p53 in heart failure has been investigated for long. Recent studies found that p53

signaling may be involved in cardiac remodeling during pathophysiological stimulation, such as pressure overload. It was reported that p53 accumulation was essential for the transition from cardiac hypertrophy to heart failure. Moreover, deletion of *p53* rescued the pressure overload-induced cardiomyopathy phenotype.

Puma and p53: 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). While p53 influences many different fundamental processes in cell life, the function of Puma is restricted to apoptosis, as a proapoptotic member of the Bcl-2 family. Bcl-2 family proteins govern mitochondrial outer membrane permeabilization and can be either proapoptotic or antiapoptotic. The members of the Bcl-2 family share one or more of four characteristic domains of homology, termed Bcl-2 homology (BH) domains (BH1, BH2, BH3, and BH4). The BH domains are crucial for the functional integrity of Bcl-2 proteins, as deletion of these domains affects their pro- or antiapoptotic characteristics. The antiapoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-xL contain all four BH domains. Some of the proapoptotic Bcl-2 proteins carry several BH domains (e.g., Bax and Bak), while others have only the BH3 domain (e.g., Puma, Bid, Bim, and Bad). After apoptotic stimuli, Puma binds to the antiapoptotic Bcl-2 or Bcl-xL proteins, thereby releasing the proapoptotic Bax and Bak from inhibition. Bax and Bak can induce the permeabilization of the mitochondrial membrane, release of cytochrome-c, Smac, and apoptosis-inducing factor (AIF), activation of caspases, and ultimately cell death.

Puma, which contains only one BH domain (hence called BH3-only), is a unique member of the Bcl-2 family, because it integrates and implements signals mediated by different apoptosis inducers. These signals include doxorubicin, hypoxia/reoxygenation, kinase inhibitors, phorbol esters, cytokines, growth factor deprivation, and gamma irradiation. When activated, Puma-induced apoptosis proceeds through the typical mitochondrial pathway, as described above. Puma is one of the most potent killers among the BH3-only subgroup of Bcl-2 family members.

In general, Puma is expressed at a very low level in different tissues and its level is primarily regulated 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, it may be a promising drug target to prevent tissue injury in many organs including the heart, brain, intestine, as well as in AIDS and many other conditions.

Mdm4 and p53: Given the critical role of p53 as the guardian of the genome, its cellular concentrations must be tightly regulated. The level of p53 is primarily regulated by ubiquitination through the E3 ubiquitin ligase family of Mdm proteins, Mdm2 and Mdm4. Both Mdm2 and Mdm4 are negative regulators of p53 activity. Mdm4 inhibits p53-mediated cell cycle arrest and apoptosis by directly binding to its transcriptional activation domain.

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. Mdm4 acts in vivo as an essential, non-redundant, negative regulator of p53 during embryonic development. Loss of *mdm4* leads to death before birth, precluding the possibility of studying adult animals. Heart-specific, conditional *mdm4* knockout animals develop dilated cardiomyopathy in association with increased cardiac myocyte apoptosis, similar to that induced by pressure overload. This provided an excellent, genetically defined loss of function mouse model to investigate the role of p53, Puma, and Mdm4 in the development of pathologic hypertrophy and subsequent heart failure.

STUDY OBJECTIVES

Since Puma is a p53 target with proapoptotic functions, we hypothesized that, in response to cellular stress, p53 undergoes stabilization and activates Puma. In turn, Puma promotes apoptosis of cardiomyocytes, ultimately leading to dilated cardiomyopathy. Therefore, our current study analyzed the Mdm4-p53-Puma axis in two *in vivo* models:

- first, by utilizing a *puma* loss-of-function mouse model in combination with transverse aortic constriction (TAC)-induced pressure overload;
- second, by monitoring the development of dilated cardiomyopathy and heart failure in double *Puma*$^{-/-}$ and conditional *Mdm4*$^{-/-}$ mice.

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.

RESULTS

Establishing and Characterizing the TAC Model

To investigate if Puma plays a role in the progression of heart failure, we first established an animal heart failure model called TAC. TAC resulted in pressure overload of the left ventricle mimicking the cardiac effects of human diseases such as hypertension and aortic stenosis. Hypertrophy developed approximately 7 days after TAC. Moreover, 4 weeks after operation cardiac dilation occurred, accompanied by several characteristic signs of cardiac remodeling, including:

- enlargement of the heart, demonstrated by increased heart weight/body weight ratio (HW/BW);
- cardiac dysfunction, demonstrated by reduced fractional shortening;
- increased myocyte cross-sectional area;
- increased cardiomyocyte apoptosis; and
- increased perivascular and myocardial fibrosis.

TAC-Induced Pressure Overload Activated *puma* Transcription in the Heart

To test if *puma* was activated during the development of TAC-induced heart failure, we first measured time-dependent expression of *puma* in the TAC model. Since *puma* is transcriptionally regulated, its activity correlates with *puma* mRNA levels. *Puma* mRNA expression was detected in sham-operated wild-type animals, but not in *puma*-deleted heart samples. Importantly, *puma* expression did not change 1 week after TAC in the wild-type animals, but showed 2.75-fold increase at 4 weeks post-surgery.

Puma expression was also detected by immunohistochemistry in TAC-operated wild-type animals showing a characteristic cytoplasmic staining. We noticed that Puma staining was easily detectable in TAC-operated, but not in sham-operated animals, suggesting that cellular stress was an important inducer of *puma* expression in the heart. These data together indicate that upregulation of *puma* coincides with a decline in heart function, raising the possibility that this molecule may participate in TAC-induced functional decompensation of wild-type animals.

Since p53 is a critical upstream regulator of *puma*, we also tested $p53^{-/-}$ animals in the TAC model to assess whether *puma* upregulation was p53-dependent. Although *puma* levels were similar at baseline in $p53^{-/-}$ and wild-type sham animals, TAC did not induce

puma in *p53*$^{-/-}$ mice, indicating that its transcriptional activation is, at least in part, driven by p53 in response to cellular stress.

Characterization of the Heart in *puma* Knockout Mice

To further elucidate the role of Puma in the development of heart failure, we utilized a *puma* knockout murine model (*Puma*$^{-/-}$). *Puma*$^{-/-}$ mice were fertile and propagated as heterozygotes. They were born according to the Mendelian frequency and proved indistinguishable in appearance from age-matched wild-type controls. We found no significant difference in body weight and heart weight between age-matched *Puma*$^{-/-}$ and wild-type mice. Similarly, *Puma*$^{-/-}$ hearts exhibited no evidence of any morphological defects, nor did histological examination of the hearts demonstrate any signs of cardiomyopathy, necrosis, or ventricular fibrosis.

To determine whether *puma* ablation affects baseline cardiac function, echocardiography was performed on 8-week-old mice. Echocardiography 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*$^{-/-}$ and wild-type mice. These findings indicated that *Puma*$^{-/-}$ hearts had normal global cardiac structure and function. Some *Puma*$^{-/-}$ mice were followed until 1 year of age and they 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.

Targeted Deletion of *puma* Delays the Development of Cardiac Dysfunction after TAC

To investigate the effect of *puma* deletion on stressed hearts, 8-week-old mice were operated by TAC surgery to induce left ventricular pressure overload. These studies usually included sham and TAC-operated *Puma*$^{-/-}$ wild-type 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 was maintained during the first week in wild-type mice, then declined at 4 weeks and further decreased by 12 weeks. In contrast, *Puma*$^{-/-}$ mice

maintained their function at 1 and 4 weeks after TAC, and remained similar to sham-operated controls.

At 12 weeks, however, cardiac function slightly declined in this study group as well. Significant increases in LVIDd and LVIDs in wild-type, but not in *Puma*$^{-/-}$ mice were measured at 4 weeks after induction of pressure overload compared to sham-operated animals. Similar changes in the *Puma*$^{-/-}$ animals began to develop by 12 weeks after TAC surgery, leading to changes in FS. Together these data indicate that *Puma*$^{-/-}$ animals maintained cardiac function in response to pressure overload for a longer time than their wild-type controls. Therefore, *puma* deletion slowed down but did not completely prevent the progression of pressure overload-induced cardiac dysfunction.

Puma Ablation does not Prevent TAC-Induced Hypertrophy

To better understand the potential role of Puma in the development of cardiac hypertrophy, we measured 3 different parameters of hypertrophy after TAC surgery, including heart weight to body weight ratio (HW/BW), posterior wall thickness (PWTd), and myocyte cross-sectional area (CSA). We observed significant TAC-induced increases in HW/BW, PWTd and CSA in both animal types (wild-type and *Puma*$^{-/-}$) compared to their sham controls, but did not observe any significant alterations between WT/TAC and *Puma*$^{-/-}$/TAC animals.

These data indicate that *puma* deletion did not interfere with TAC-induced early "adaptive" as well as late changes. Moreover, together with functional assessment (FS) these findings suggest that the hypertrophic myocardium of wild-type animals, but not that of the *Puma*$^{-/-}$ animals, are already in the heart failure stage. Since the hypertrophic response to pressure overload in *Puma*$^{-/-}$ mice is intact, it is possible that Puma's function is not critical for hypertrophy or can be compensated for by other hypertrophic signaling molecules. In any case, *puma* ablation appears to slow down the development of the maladaptive phase of TAC-induced cardiac hypertrophy.

Puma Deletion does not Affect TAC-Induced Angiogenesis

We also examined the effect of Puma on angiogenesis by quantifying vascular endothelial growth factor (*VEGF*) expression and evaluating capillary density by CD-31 staining. In these experiments, we found no differences between TAC-operated wild-type and *Puma*$^{-/-}$ animals in either *VEGF* expression or capillary density. Based on these results, Puma does not seem to play a role in pressure overload–induced angiogenesis.

Puma Deletion Inhibits Pressure Overload-Induced Apoptosis

To determine whether Puma is involved in apoptosis of heart cells, we compared apoptosis in wild-type and *Puma*$^{-/-}$ mice after TAC using TUNEL assay. Apoptotic rate was negligible in sham-operated wild-type and *Puma*$^{-/-}$ hearts and did not change significantly 1 week after TAC either. However, the level of apoptosis was significantly different between wild-type and *Puma*$^{-/-}$ mice 4 weeks after surgery. Apoptotic nuclei were easily detectable in the wild-type myocardium but not in *Puma*$^{-/-}$ hearts. The number of apoptotic nuclei increased by approximately 10-fold in wild-type mice 4 weeks after TAC, while in *Puma*$^{-/-}$ hearts, the increase was only about 3-fold 4 weeks after TAC, compared to sham-operated mice. To further confirm our observations, we examined caspase-3 cleavage on histological sections and found similar changes as presented by TUNEL assay.

We also measured the expression of anti-apoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-xL and Mcl-1 by qPCR. No significant differences were observed in these factors 4 weeks after TAC surgery in wild-type mice. These data imply that the observed low levels of apoptosis in TAC-operated *Puma*$^{-/-}$ mice are not attributed to a compensatory upregulation in anti-apoptotic factors. In summary, *puma* ablation delays the development of pressure overload-induced heart failure, at least partially, via suppression of apoptosis.

TAC-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 wild-type and *Puma*$^{-/-}$ mice were stained with Masson's trichrome. Sham-operated mice had normal tissue morphology with no collagen deposition.

In line with previous reports using pressure overload models, fibrosis became prominent in the myocardium from wild-type mice 4 weeks after TAC, characterized by scattered lesions in the myocardium at multiple foci. 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 at mid and large size arteries. Quantitative analysis of trichrome staining showed 2-3-fold increase in *Puma*$^{-/-}$ mice over sham-operated controls, while about 10-fold increase in wild-type hearts at this time point. Extracellular matrix turnover

was further evaluated by analyzing *collagen III* and *matrix metalloproteinase-2* and *9* expressions. Although these markers were elevated in *Puma*$^{-/-}$ mice following 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

In our final set of experiments, we further investigated the role of *puma* expression in the development of heart failure in a p53-dependent cardiomyopathy model. 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. Therefore, this model offers a reasonable tool for *in vivo* analysis of the p53–Puma pathway in heart failure. Due to 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 by echocardiography every month up to 8 months of age, together with single knockout (*Mdm4*$^{-/-}$/*Puma*$^{+/+}$ or *Mdm4*$^{+/+}$/*Puma*$^{-/-}$) and wild-type (*Mdm4*$^{+/+}$/*Puma*$^{+/+}$) animals, starting at the age of 2 months.

At this initial time, minimal and not significant differences were measured between wild-type and *Puma*$^{-/-}$ animals in FS (42% and 39%) and no change occurred between these groups during the whole duration of the study. In the *Mdm4*$^{-/-}$ study group FS was slightly lower (35%) at 2 months than in age-matched controls and, as it was expected, it steadily declined by time as wall-thinning occurred in line with the development of dilated cardiomyopathy. 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 to *mdm4*-only deficient animals. Most importantly, double knockout mice had a longer life span than *Mdm4*$^{-/-}$ animals. While most *Mdm4*$^{-/-}$ mice died by 7-9 months of age, double knockouts looked healthier and most of the animals survived into their first year.

Puma mRNA expression was measured on a monthly basis and progressively increased in *Mdm4*$^{-/-}$ mice from 3 months of age coinciding with the deterioration in cardiac function. This increase, however, was not detectable in age-matched wild-type controls

providing a further link between *puma* expression and heart failure. Sections stained for apoptosis of the same samples also showed elevation in apoptotic events with kinetics similar to *puma* expression. All these data together in the double knockout animals confirmed Puma's involvement in the development of heart failure and documented a functional connection within the p53 pathway.

DISCUSSION

In the present study, we investigated the role of Puma in the development of ventricular remodeling and cardiac failure using *Puma* knockout mice in two animal models. We applied TAC surgery to induce pressure overload and utilized a genetic model of dilated cardiomyopathy based on *mdm4* deletion. Our studies demonstrated that in both models Puma-induced apoptosis may play a role in the progression of heart failure. 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 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* deletion led to Puma-dependent apoptosis and *Mdm4*$^{-/-}$ mice could be rescued from heart failure by the lack of *puma*.

These data demonstrate the importance of Puma upregulation in the pathomechanism of heart failure. The results we obtained also provide important basic scientific information about the function of the Mdm4-p53-Puma pathway in cardiac myocytes. Furthermore, these data support the theory observed by other investigators that cell death plays a critical role in the pathogenesis of heart failure. Apoptosis has long been recognized as a highly regulated mode of cell suicide. The regulated nature of apoptosis opens up the possibility of manipulating cell death pathways by therapeutic interventions.

SUMMARY

1. The results we obtained establish the Mdm4-p53-Puma axis as one the potential regulators of cell death in cardiac myocytes in response to pressure overload.
2. Thereby the Mdm4-p53-Puma pathway may serve as a therapeutic target for drug development efforts in heart failure or various other cardiac diseases involving cell death.
3. Furthermore, the Mdm4-p53-Puma axis is also a target of recent drug discovery efforts in cancer therapy. Lack of *puma* does not recapitulate the tumor-prone phenotype observed in *p53*-negative mice. Therefore, functional analysis of the system in the heart would contribute to the development of efficient and safe anticancer drugs or treatment protocols that have minimal cardiotoxic side effects.

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