Dilated cardiomyopathy (DCM) is one of the leading causes of heart failure in the United States, with an annual incidence estimated to be 5 to 8 cases per 100 000 population. DCM is characterized by ventricular chamber dilation with normal or decreased wall thickness and impaired systolic function.1–3 DCM has both hereditary and acquired forms. Two sarcomere structural genes have been identified to be involved with DCM, the cardiac actin and myosin heavy chain genes.4,5 Additionally, several loci for familial dilated cardiomyopathy (DCM) have been mapped, the origin of a large percentage of DCM remains unclear. Mdm2, a p53-negative regulator, protects cardiomyocytes from ischemic and reperfusion-induced cell death. Mdm4, a homolog of Mdm2, inhibits p53 activity in numerous cell types. It is unknown whether Mdm4 plays a role in the inhibition of p53 in fully differentiated tissues such as adult cardiomyocytes and whether this role is associated with DCM.

Methods and Results—The conditional knockout of Mdm4 in the heart by use of cardiomyocyte-specific Cre (αMYHC-Cre) allele does not result in any developmental defects. With time, however, mice with deletion of Mdm4 in the adult heart developed DCM and had a median survival of 234 days. More interestingly, the onset of DCM occurs significantly earlier in male mice than in female mice, which mimics human DCM disease. DCM in Mdm4 mutant mice was caused by loss of cardiomyocytes by apoptosis, and it was p53-dose dependent.

Conclusion—Activity of p53 was inhibited by Mdm4 even in the fully differentiated cardiomyocyte. Elevated apoptosis mediated by the p53 pathway in cardiomyocytes may be a mechanism for DCM. (Circulation. 2007;115:2925-2930.)

Key Words: apoptosis ■ myocytes, cardiac ■ cardiomyopathy ■ genetics ■ survival

During embryonic development, p53 activity is suppressed by both Mdm2 and Mdm4. Loss of Mdm2 in mice results in embryonic lethality by p53-dependent apoptosis.14,15 Loss of Mdm4 also causes p53-dependent embryonic lethality by initiation of cell cycle arrest and apoptosis.16–18 Additionally, Mdm2 and Mdm4 synergize to inhibit p53 in the developing central nervous system during embryogenesis.19,20 These data demonstrate that p53 activity is inhibited by Mdm2 and Mdm4 in proliferating cells. In postmitotic cells in the central nervous system, both Mdm2 and Mdm4 are also required to inhibit p53.20 However, when Mdm2 and Mdm4 are deleted in the adult smooth muscle cells, loss of Mdm2-induced p53-dependent apoptosis, but acute loss of Mdm4 does not show obvious defects,21 which suggests that, in quiescent or fully differentiated cells, Mdm4 is not required to inhibit p53 activity. It remains unclear whether Mdm4-mediated p53 inhibition in differentiated cells is tissue specific.

To determine whether Mdm4-mediated p53 inhibition is important in another differentiated cell type, we chose to delete Mdm4 in cardiomyocytes in mice. Previously, we crossed the α-myosin heavy chain promoter–driven Cre mouse (αMYHC-Cre) to a Mdm4-conditional allele to generate cardiomyocytes that lack Mdm4. Mice with deletion of Mdm4 in cardiomyocytes did not show obvious defects during development and perinatal stages,22 which provides an

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Mdm2 is an E3 ubiquitin ligase and negative regulator of p53. Mdm4 is a homolog of Mdm2, which also inhibits p53 activity by masking its transcripational activation domain.13
excellent opportunity to study whether Mdm4 is required to inhibit p53 activity in the adult cardiomyocytes. Adult mouse cardiomyocytes do not have regenerative capacity, although they do proliferate during fetal development. Shortly after birth, positive cell cycle factors such as cyclin A and cdk2 are downregulated and the cell cycle inhibitors p21 and p27 are upregulated, which allows cardiomyocytes to become quiescent. Mice with deletion of Mdm4 in cardiomyocytes showed severe edema and heart failure as early as 3 months of age. Mutant mice developed DCM and exhibited apoptosis in adult cardiomyocytes, which indicates a role for Mdm4 in differentiated cardiomyocytes.

To test whether the DCM phenotype was p53-dependent, we used p53-conditional and null alleles to generate Mdm4 mutant mice with 1 or no p53 alleles. Survival studies of these mice clearly showed the phenotype was dependent on p53 dose, which demonstrates that elevated p53 activity may be one of the causes for DCM.

Methods

Mice
A conditional allele of Mdm4, Mdm4<sup>FX</sup>, has 2 lox P sites that surround exon 2, which contains the ATG start codon. Deletion of exon 2 results in a null allele, designated Mdm4<sup>−/−</sup>. Mdm4<sup>−/−</sup>MyHC-Cre mice were crossed to Mdm4<sup>lox/lox</sup> mice to generate the cohorts of Mdm4<sup>−/−</sup>MyHC-Cre and Mdm4<sup>lox/lox</sup>MyHC-Cre mice. The breeding and maintenance of mice were performed in a specific pathogen–free mouse facility under institutional guidelines.

X-Gal Staining of Adult Heart
Frozen cross-sections of αMyHC-Cre, Rosa26-lacZ adult hearts were stained according to a previous protocol.

TUNEL Assays/Immunohistochemistry Staining
Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assays carried out on paraffin-embedded sections were performed in a specific pathogen–free mouse facility under institutional guidelines.

Statistical Analysis
Two-way ANOVA and Kaplan-Meier survival analysis were performed with Prism 4 software (GraphPad Software, San Diego, Calif). Differences were considered significant at a value of P<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Loss of Mdm4 in the Adult Heart Caused Dilated Cardiomyopathy
A conditional allele of Mdm4, Mdm4<sup>FX</sup> contains lox P sites that surround exon 2, and deletion of exon 2 in the germline results in a null allele designated Mdm4<sup>−/−</sup>. Mdm4<sup>−/−</sup> with the αMyHC-Cre transgene<sup>29</sup> were crossed to mice homozygous for the Mdm4-conditional allele (Mdm4<sup>FX/FX</sup>) to generate mice with different combinations of alleles, such as those that lack Mdm4 in cardiomyocytes. Deletion of Mdm4 in the embryonic heart does not cause any developmental defects.<sup>22</sup> We maintained mice for >1 year to examine the possible role of Mdm4 in fully differentiated adult cardiomyocytes. Cohorts of mutant Mdm4<sup>−/−</sup>αMyHC-Cre and control Mdm4<sup>−/−</sup>αMyHC-Cre mice were monitored. To make possible examination of Cre-specific recombination, some mice contained the ROSA26-lacZ reporter, as Cre-recombination at the ROSA26 locus allows expression of β-galactosidase (these mice were not part of the cohort study). Robust and specific LacZ staining was observed in the adult heart with frozen sections from 5-month-old mice with both αMyHC-Cre and Rosa26-lacZ transgenes (Figure 1A). Obvious blue staining in the cardiomyocytes was present in both right and left ventricles, as well as in the septum between the 2 ventricles (Figure 1A; other data not shown), which was consistent with previous studies that showed tissue-specific expression of Cre from the αMyHC promoter.<sup>28</sup> Specific recombination at the Mdm4 locus was previously observed in the adult mice with PCR primers that distinguish conditional and recombined alleles.<sup>22</sup> Because mice inherit an Mdm4-conditional and a null allele, every recombination event leads to a cell that completely lacks Mdm4.

The earliest abnormality was observed in Mdm4<sup>Δ2/Δ2</sup>αMyHC-Cre mutant mice at 3 months of age. By 8 to 10 months of age, most of the mutant mice had swollen bodies, showed difficulty moving, and were out of breath. Mutant mice eventually died as a result of heart failure. The survival of Mdm4<sup>Δ2/Δ2</sup>αMyHC-Cre mice was significantly shorter than
Mdm4<sup>-/-FX</sup> αMyHC-Cre control mice, with median survival of 234 and 318 days, respectively (P<0.0001) (Figure 1B). αMyHC-Cre mice survived up to 1 year of age,<sup>30</sup> and Mdm4<sup>-/-FX</sup> mice survived even up to 2 years of age, although some Mdm4<sup>-/-FX</sup> αMyHC-Cre mice died before 1 year of age, perhaps as a result of toxicity of Cre expression plus the loss of 1 allele of Mdm4. Interestingly, the mutant male mice died significantly earlier than the female mice, with median survival at 208 and 243 days, respectively (P<0.007) (Figure 1C). To examine the cause of the death in more detail, Mdm4<sup>-/-FX</sup> αMyHC-Cre mutant mice were euthanized and dissected when they were moribund. The mutant mice had obvious edema in the lung and/or abdomen. The mutant hearts were enlarged, and all 4 chambers were dilated and paler than the hearts of control mice (Figure 2A). Cross-sections of the heart showed that the ventricular walls in mutant hearts were thinner and obviously hypertrophic (Figure 2B and 2C). These observations indicated severe dilated cardiomyopathy in the mutant mice. Mason trichrome staining was also performed to detect collagen deposition, a marker of fibrosis in the heart. Positive blue staining, which indicated fibrosis, was clearly evident in mutant hearts in comparison to hearts from control mice (Figure 2B and 2D). Atrial natriuretic peptide is a molecular marker widely used to characterize cardiomyocyte hypertrophy and heart failure.<sup>31</sup> Immunohistochemistry staining showed atrial natriuretic peptide was also prominent in mutant but not control hearts (Figure 2E). Together, these data indicated that loss of Mdm4 induced dilated cardiomyopathy, which led to heart fibrosis and eventually heart failure in the mutant mice.

Loss of Adult Cardiomyocytes in Mdm4 Mutant Mice

Hearts from Mdm4<sup>-/-FX</sup> αMyHC-Cre mice showed thinner walls in the both left and right ventricles and were positive for atrial natriuretic peptide and Mason trichrome staining as compared with controls, which suggests loss of cardiomyocytes in the mutant hearts. To test whether loss of Mdm4 in the adult heart caused loss of fully differentiated cardiomyocytes, the cell number from cross-sections of both mutant and control hearts at 3 and 8 months of age was determined.

Although the number of cardiomyocytes at 3 months of age was similar in the control and mutant hearts (P=0.14), strikingly, the hearts of Mdm4<sup>-/-FX</sup> αMyHC-Cre mutant mice had half the number of cardiomyocytes at 8 months of age as compared with Mdm4<sup>-/-FX</sup> αMyHC-Cre control mice (Figure 3A and 3B) (P=0.015). To investigate whether loss of cardiomyocytes in the heart was caused by the apoptosis, TUNEL assays were performed. TUNEL-positive cells were clearly evident only in mutant mice at 3 months of age when the cardiomyocytes are terminally differentiated (Figure 3B). In line with the differentiated and quiescent nature of adult cardiomyocytes, bromodeoxyuridine labeling indicated a lack of proliferation in both mutant and control mice (data not shown). These experiments demonstrated that loss of Mdm4...
in adult cardiomyocytes induced apoptosis, which indicates that Mdm4 is still required in these differentiated and quiescent cells.

**DCM Caused by Loss of Mdm4 Was Dependent on p53 Dose**

To understand whether the DCM phenotype caused by loss of Mdm4 is p53-dependent, the p53-null and p53-conditional alleles were introduced into Mdm4<sup>lox/lox</sup> αMyHC-Cre mutant mice. On loss of 1 p53 allele, Mdm4<sup>lox/lox</sup> αMyHC-Cre mice showed an extended median survival to 274 days compared with 234 days for Mdm4<sup>lox/lox</sup> αMyHC-Cre mice with 2 wild-type p53 alleles (P<0.0001). These data indicated that loss of a single p53 allele alleviated the severity of the phenotype. Because >90% of p53-null mice die by 6 months of age as a result of the development of lymphomas, we could not examine the survival advantage in Mdm4<sup>lox/lox</sup> αMyHC-Cre mice null for p53. We therefore used a p53-conditional allele<sup>26</sup> and combined it with the Mdm4 mutant alleles to generate Mdm4<sup>lox/lox</sup> p53<sup>-/-</sup> αMyHC-Cre mice to determine whether loss of both p53 alleles specifically in the heart could actually rescue the DCM phenotype. The median survival for Mdm4<sup>lox/lox</sup> p53<sup>lox/lox</sup> αMyHC-Cre mice lengthened to 403 days, which was significantly longer (P<0.0005) than Mdm4<sup>lox/lox</sup> p53<sup>+/−</sup> αMyHC-Cre mice (Figure 4A). Trichrome staining of the hearts of Mdm4<sup>lox/lox</sup> p53<sup>lox/lox</sup> αMyHC-Cre mice at about 6 months of age showed a significant reduction in staining as compared with the mutant mice with a single p53 allele (Figure 4B). Mdm4<sup>lox/lox</sup> p53<sup>lox/lox</sup> αMyHC-Cre mice were not edematous nor out of breath and died as a result of the development of various tumors (data not shown). These data demonstrated that the DCM phenotype caused by loss of Mdm4 was dependent on p53 dose.

**Discussion**

Loss of Mdm4 in terminally differentiated cardiomyocytes led to a p53-dependent lethal DCM. Mice that lacked Mdm4 in cardiomyocytes gradually lost these cells by apoptosis, and eventually died of heart failure. These data demonstrate that Mdm4-mediated inhibition of p53 in adult cardiomyocytes was essential to normal heart function. The rescue of the DCM phenotype by concomitant loss of p53 indicated that abnormal elevated p53 activity may induce DCM. It will be important to determine whether loss of Mdm4 and/or elevated p53 activity actually is a mechanism that leads to heart failure in humans. Another interesting observation in this mouse model is that the phenotype of Mdm4<sup>lox/lox</sup> αMyHC-Cre mice recapitulates the gender differences of human heart failure. Women with heart failure survive substantially longer than men.<sup>33,34</sup> This genetically defined mouse model with loss of Mdm4 in the adult heart may provide a relevant model to understand the gender differences of DCM-induced heart failure.

Recently, the concept of stem cell therapy has attracted many clinicians to test heart repair with a variety of stem cells. Although the cardiac transfer of stem and progenitor cells shows a favorable impact on tissue perfusion and contractile performance of the injured heart, the mechanism of stem cell therapy is still unclear, and it is essential to determine the right stem cell type in the right clinical setting.<sup>35</sup> This genetically defined mouse model with loss of Mdm4 in the adult heart may provide a good model to test stem cell therapies in DCM.

Activity of p53 is high in proliferating progenitor cells before and after birth (G.L., unpublished observations, 2006). Loss of Mdm2 and Mdm4 in these cells results in p53-mediated cell-cycle arrest and apoptosis.<sup>19,20</sup> Additionally, p53 activity is inhibited by Mdm2 in differentiated smooth muscle cells in the small intestine, and loss of Mdm2 in these cells results in p53-dependent apoptosis. The role of Mdm4 in the inhibition of p53 in smooth muscle cells seems unimportant, as deletion of Mdm4 does not cause any obvious defects.<sup>21</sup> The difference between our study and loss of Mdm4 in smooth muscle cells is either a result of tissue specificity of Mdm4 function, or that Mdm4 loss requires a longer period to develop a severe phenotype. The second possibility is consistent with the notion that Mdm4 loss causes less severe phenotypes in all tissues examined thus far in comparison to loss of Mdm2.<sup>19–22</sup>

The importance of understanding the role of the p53 pathway in differentiated tissues is underscored by a strategy to treat cancer patients with molecular drugs to disrupt the binding of p53 to Mdm2.<sup>36,37</sup> This strategy is widely accepted as a treatment option in cancer patients with wild-type p53. Recently, Nutlin-3 and Rita, 2 small molecules that disrupt binding of p53 to Mdm2, have been shown to be effective in cancer cell lines<sup>38–40</sup> and in a xenograft model.<sup>41</sup> Because Mdm4 is another p53-negative regulator, and Mdm4 is overexpressed in many tumor cell lines and primary tumors such as tumors with wild-type p53,<sup>42,43</sup> it is also an attractive choice for the design of drugs to target Mdm4 interaction with p53. Additionally, because Mdm2 and Mdm4 bind the same domain of p53,<sup>44</sup> it is also possible that future drugs will disrupt both p53–Mdm2 and p53–Mdm4 interaction. In consideration of all possibilities for this therapeutic strategy, it is very important to know whether these drugs will affect the normal functions of human tissues. Recent data demonstrate that p53 activity is inhibited by Mdm2 even in adult mouse tissues.<sup>45</sup> The present study implicates the importance of the Mdm4–p53 interaction in normal heart function, which sug-

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**Figure 4.** DCM phenotype in Mdm4<sup>lox/lox</sup> αMyHC-Cre mutant heart depends on p53 dose. A, The survival of Mdm4<sup>lox/lox</sup> αMyHC-Cre mutant mice with 0, 1, or 2 p53 alleles. B, Trichrome staining of Mdm4<sup>lox/lox</sup> αMyHC-Cre mutant mice with or without p53 at 6 months. Scale bar=100 μm.
gests that drugs that disrupt this interaction in patients may have unintended side effects.

Acknowledgments
We thank Yasmine Valentine-Vega, Sean Post, and Tamara Terzian for helpful discussions, as well as Sara Amra for careful preparation of sections.

Sources of Funding
This research was supported by National Institutes of Health grants CA47296 (to Dr Lozano) and CA16672 (to University of Texas M.D. Anderson Cancer Center).

Disclosures
None.

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CLINICAL PERSPECTIVE

Although several loci for familial dilated cardiomyopathy have been mapped, the underlying mechanism(s) of a large percentage of dilated cardiomyopathy remains unclear. Mdm4 is an inhibitor of the p53 tumor suppressor, and mice with deletion of Mdm4 in the adult heart developed dilated cardiomyopathy with significantly earlier onset in male than in female mice, which thus recapitulates the gender differences observed in humans. The cause of dilated cardiomyopathy in this mouse model is a gradual loss of cardiomyocytes by p53-dependent apoptosis. Thus, this genetically defined mouse model may provide a good model to test stem cell replacement therapies. The present study also has important implications for cancer treatment with specific drugs to disrupt the interaction between p53 and its negative regulators, Mdm2 and Mdm4. Although 2 small molecules, Nutlin-3 and Rita, have been shown to be effective in cancer cell lines and in a xenograft model, our study suggests the importance of the Mdm4–p53 interaction in normal heart function, which indicates that drugs that disrupt this interaction in patients may have unwanted side effects.
Loss of Mdm4 Results in p53-Dependent Dilated Cardiomyopathy
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Circulation. 2007;115:2925-2930; originally published online May 28, 2007;
doi: 10.1161/CIRCULATIONAHA.107.689901
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
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