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p53 Functions in Endothelial Cells to Prevent Radiation-Induced Myocardial Injury in Mice

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Abstract

p53 functions in the heart to promote myocardial injury after multiple types of stress. However, how p53 regulates radiation-induced myocardial injury, which develops after radiation therapy, is not well understood. Here, we utilize the Cre-loxP system to demonstrate that p53 functioned in endothelial cells to protect mice from myocardial injury after whole-heart irradiation. Mice with an endothelial cell-specific deletion of p53 succumbed to heart failure after whole-heart irradiation due to myocardial necrosis, systolic dysfunction and cardiac hypertrophy. Moreover, the onset of cardiac dysfunction was preceded by alterations in myocardial vascular permeability and density, which resulted in cardiac ischemia and myocardial hypoxia. Mechanistic studies using primary cardiac endothelial cells irradiated *in vitro* indicated that p53 signaling caused mitotic arrest and protected cardiac endothelial cells against radiation-induced mitotic catastrophe. Furthermore, mice lacking the cyclin-dependent kinase inhibitor p21, which is a transcriptional target of p53, were also sensitized to myocardial injury after wholeheart irradiation. Together, our results demonstrate that the p53/p21 axis functions to prevent radiation-induced myocardial injury in mice.

INTRODUCTION

The tumor suppressor protein 53 (p53) is a transcription factor that serves as a key executor of the DNA damage response to control cell survival and cell death (1, 2). In the heart, p53 functions to promote cardiac injury from pressure overload (3), ischemic injury (4), telomere attrition (5), and doxorubicin-induced oxidative stress (6–8). Therefore, blocking p53 with

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pharmacological inhibitors has been proposed as a promising approach to prevent cardiac injury from multiple stresses. However, the role of p53 in regulating radiation-induced myocardial injury is unknown.

Radiation-related heart disease is a well-described late effect of radiation therapy (9). In a meta-analysis from several randomized trials of women with breast cancer, mortality from heart disease was substantially increased for women who were randomized to receive adjuvant fractionated radiation therapy ranging from 35 to 65 Gy (10). Further support for the hypothesis that radiation causes heart disease in breast cancer patients comes from the observation that excess mortality from heart disease is observed in women receiving radiation therapy for left-sided breast cancer (11). A prospective study of left-sided breast cancer patients has been performed with cardiac single-photon emission computed tomography (SPECT) scans to measure blood flow to the myocardium. Patients receiving cardiac SPECT scans prior to and 6 months after radiation therapy had perfusion defects within the part of the left ventricle that received high dose irradiation (12). These perfusion defects persisted on follow-up cardiac SPECT scans 3 to 8 years after radiation therapy (13). Therefore, an important consequence of radiation therapy to the heart is decreased blood flow to the myocardium.

Damage to the microvasculature of the heart after irradiation occurs in animal models prior to pathological changes in the myocardium (14–18). For example, Fajardo and Stewart studied the pathogenesis of radiation-induced myocardial fibrosis in rabbits exposed to a single dose of 20 Gy (14, 15). In these elegant studies, focal areas of myocardial fibrosis were observed by two months after irradiation (15). From day 20 through 49 after irradiation, there was considerable damage to endothelial cells, including decreased microvessel density, within the myocardium (14). Lauk and co-workers observed similar histopathology in rats in which the heart received a single dose of 15 to 20 Gy. They found a substantial reduction in capillary density of the irradiated heart prior to any obvious histological damage to the cardiomyocytes (16). Follow-up studies comparing radiation-induced heart disease in Wistar and Sprague-Dawley rats showed that microvessel density was reduced by approximately 50% one month after a single dose of 17.5 to 20 Gy, whereas focal areas of myocardial necrosis were noted at two months (17). Seemann and colleagues reported alterations in the microvasculature in the myocardium of mice 40 weeks after a single dose of 16 Gy to the heart, with associated sudden death in one-third of the mice (18). Although it has been established that microvascular loss precedes myocardial necrosis in radiation-induced myocardial injury, the molecular mechanisms controlling the loss of the myocardial capillaries remain to be fully defined (19–21).

Radiation induces p53 in the heart *in vivo* (22) and in endothelial cells from various sources *in vitro* (23–25). In endothelial cells, whether p53 functions as a pro-survival or pro-death factor remains controversial. For example, lovastatin, a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor, protects human umbilical vein endothelial cells (HUVECs) from radiation-induced cell death by apparently blocking p53 (23). This finding is further supported by the observation that the small-molecule inhibitor p53 pifithrin increases the viability of HUVECs after irradiation (23). Moreover, blocking p53 has been suggested to suppress radiation-induced damage to human dermal microvascular endothelial cells and mouse endothelial progenitor cells (24, 25). In contrast, others have reported that when p53 is inhibited or deleted in tumor-associated endothelial cells, xenograft tumors are sensitized to radiation (26), suggesting that p53 functions to protect endothelial cells against radiation. Because endothelial cell dysfunction appears to play a crucial role in initiating radiation-induced myocardial injury, clarifying how p53 regulates the radiation response of cardiac endothelial cells may reveal the molecular mechanism of radiation induced cardiac toxicity. Moreover, understanding whether p53 plays a pro-death or pro-survival role in irradiated

cardiac endothelial cells will provide critical information to design clinical trials that combine radiation therapy with inhibitors of the DNA damage response pathway.

To investigate how p53 functions in cardiac endothelial cells to regulate radiation response of the heart *in vivo*, we deleted p53 in endothelial cells using Tie2Cre and VECadherin-Cre mice. Tie2 is a receptor tyrosine kinase present almost exclusively by endothelial cells (27–29) and VE-Cadherin is a transmembrane adhesion protein in endothelial cells (30). The promoters of both genes drive Cre expression in the endothelium of adult mice (31, 32). Deleting p53 in endothelial cells with either Tie2Cre or VE-Cadherin-Cre caused mice to be sensitized to myocardial injury after whole-heart irradiation. The onset of cardiac injury was preceded by increased endothelial cell death, increased vascular permeability and decreased microvessel density in the myocardium. In addition, cell-based studies using primary cardiac endothelial cells from these mice showed that p53-mediated cell cycle arrest protected cardiac endothelial cells from radiation-induced mitotic catastrophe. Moreover, mice lacking the cyclin-dependent kinase inhibitor p21, which is a transcriptional target of p53, also developed myocardial injury after whole-heart irradiation. Taken together, our results demonstrate that p53 functions in endothelial cells *in vivo* to prevent radiation-induced myocardial injury.

RESULTS

Deletion of p53 in endothelial cells by Tie2Cre sensitizes mice to radiation-induced cardiac injury

To investigate the role of p53 in endothelial cells in regulating the radiation response of the heart, we utilized Tie2Cre mice to delete p53 in endothelial cells *in vivo* (33). Because Tie2Cre has been reported to recombine genes in some cardiomyocytes in addition to endothelial cells (34), we examined the cell types that express Cre recombinase in the heart by crossing Tie2Cre mice to membrane-Tomato/membrane-GFP (mTmG) reporter mice (35). In *Tie2Cre; mTmG/+* mice, cells expressing Cre were labeled by enhanced green fluorescent protein (EGFP), whereas cells that do not express Cre were labeled by tdTomato protein. In *Tie2Cre; mTmG/+* mice, cells expressing EGFP in the myocardium are labeled by *Griffonia simplicifolia* isolectin B₄ (GS-IB₄) which marks endothelial cells (36) (Fig. S1). The pattern of Cre expression is similar in Tie2Cre mice regardless of the presence (*Tie2Cre; p53^{FL/+}; mTmG/+*) or absence (*Tie2Cre; p53^{FL/-}; mTmG/+*) of p53 in endothelial cells (Fig. S1). The results indicate that, in these Tie2Cre mice, Cre is expressed ubiquitously in endothelial cells and not in cardiomyocytes.

To study the radiation response of the heart, *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice were treated with whole-heart irradiation using either a single dose of 12 Gy or 10 daily fractions of 3 Gy to model a dose and schedule of radiation therapy used in the clinic. Focal irradiation to the heart was performed using a micro-computed tomography (CT)/micro-irradiator so that the radiation field included the heart and a margin of normal lung tissue (Fig. 1A). *Tie2Cre; p53^{FL/+}* mice did not show any signs of morbidity after 210 days following whole-heart irradiation, whereas all *Tie2Cre; p53^{FL/-}* mice succumbed to late effects of radiation between 39 to 69 days post-whole-heart irradiation, with the median survival of 55 days (Fig. 1B). In addition, *Tie2Cre; p53^{FL/-}* mice treated with 10 daily fractions of 3 Gy (3Gy × 10) whole-heart irradiation also succumbed to late effects of radiation, with the median survival of 33 days after the last treatment (Fig. 1C).

We performed histopathological examination of the lungs and heart in moribund *Tie2Cre; p53^{FL/-}* mice after 12 Gy or 3 Gy × 10 whole-heart irradiation. Although there was no detectable lesion in the lungs, hematoxylin and eosin-stained (H&E) sections of the heart showed multifocal areas of myocardial degeneration and necrosis throughout the

myocardium (Fig. 1, D and E). Myocardial degeneration and necrosis was characterized by loss of cross striations, decreased number of cardiomyocytes, and indistinct cytoplasmic borders and nuclei. In addition, the loss of sarcomeric structure in necrotic regions was further characterized by the absence of sarcomeric α -actin filaments (Fig. 1F). Infiltration of neutrophils and macrophages as well as hemorrhage was also present within necrotic foci (Fig. 1E). As shown in Masson's trichrome-stained sections, the necrotic cardiomyocytes were replaced by small amounts of fibrous connective tissue, which indicates that the lesion was an acute process (Fig. 1G). To investigate the damage of cardiomyocytes at the cellular level, uptake of Evans blue dye of the heart was assessed in *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice 5 weeks after 12 Gy whole-heart irradiation. Evans blue dye is a vital dye that penetrates into cardiomyocytes with a disrupted sarcolemma (37). Evans blue dye-positive cardiomyocytes were not present in irradiated *Tie2Cre; p53^{FL/+}* mice; in contrast, irradiated *Tie2Cre; p53^{FL/-}* mice showed increased Evans blue dye uptake by cardiomyocytes (Fig. 1, H and I). These results indicate that irradiated *Tie2Cre; p53^{FL/-}* mice showed cardiomyocyte sarcolemmal injury in the myocardium.

In addition to the pathological alterations in the myocardium, changes in the pericardium were also observed in some moribund *Tie2Cre; p53^{FL/-}* mice after 12 Gy whole-heart irradiation (Fig. S2). In these mice, certain areas of the pericardium were thickened by edema, proliferative fibroblasts, collagen fibrils and infiltration of lymphocytes. Collectively, our results suggest that deletion of p53 in endothelial cells by *Tie2Cre* sensitizes mice to radiation-induced cardiac injury, which is manifested as multifocal degeneration and necrosis in the myocardium as well as pericarditis.

***Tie2Cre; p53^{FL/-}* mice develop systolic dysfunction and cardiac hypertrophy following 12 Gy whole-heart irradiation**

To assess the effect of 12 Gy whole-heart irradiation on cardiac function in *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice, we performed serial echocardiography before and 3, 5, and 7 weeks after irradiation. Seven weeks after 12 Gy whole-heart irradiation, cardiac function of *Tie2Cre; p53^{FL/+}* mice remained normal, whereas *Tie2Cre; p53^{FL/-}* mice developed impaired systolic function manifested by significantly decreased fractional shortening, decreased heart rate corrected mean velocity of circumferential fiber shortening, and increased left ventricular end-systolic dimension (Fig. 2, A – D and Table S1). Echocardiography also showed significantly increased mass of the left ventricle in *Tie2Cre; p53^{FL/-}* mice (Fig. 2E). Cardiac hypertrophy in *Tie2Cre; p53^{FL/-}* mice was confirmed at necropsy by a markedly increased heart weight to body weight ratio (Fig. 2F). In addition, sections of the myocardium stained with wheat germ agglutinin demonstrated that average cardiomyocyte cross-sectional area was significantly increased in *Tie2Cre; p53^{FL/-}* mice 7 weeks after 12 Gy whole-heart irradiation (Fig. 2, G and H). The results from the echocardiography and histology studies indicate that myocardial systolic dysfunction is the primary cause of morbidity in *Tie2Cre; p53^{FL/-}* mice after 12 Gy whole-heart irradiation.

Radiation-induced systolic dysfunction is preceded by vascular damage and chronic ischemia in the myocardium of *Tie2Cre; p53^{FL/-}* mice

We hypothesized that cardiac dysfunction in *Tie2Cre; p53^{FL/-}* mice is preceded by the destruction of p53-deficient myocardial vessels. To test this hypothesis, we assessed the density of myocardial capillaries in *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice 3, 4 and 7 weeks after 12 Gy whole-heart irradiation. Although systolic function of *Tie2Cre; p53^{FL/-}* mice remained normal 4 weeks after 12 Gy whole-heart irradiation (Fig. 2), focal areas in the myocardium of these mice showed aberrant myocardial capillary morphology characterized by dilated and disorganized vessels (Fig. 3, A – C). In addition, microvessel density was significantly decreased within the necrotic foci (Fig. 3, D and E). By 7 weeks

after 12 Gy whole-heart irradiation in *Tie2Cre; p53^{FL/-}* mice, the necrotic region extended across the entire myocardium with a global decrease in microvessel density compared to unirradiated mice (Fig. 3E). Moreover, TUNEL stained myocardium showed a significant increase in dead endothelial cells in the necrotic regions of *Tie2Cre; p53^{FL/-}* mice 4 weeks after 12 Gy whole-heart irradiation (Fig. S3). To investigate functional alterations of myocardial capillaries *in vivo*, we assessed the permeability of the myocardial microvasculature with intravenous injection of low and high molecular weight dextrans (1×10^4 and 2×10^6 , respectively) into *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice 4 weeks after 12 Gy whole-heart irradiation. In *Tie2Cre; p53^{FL/+}* mice, dextrans of both molecular weights were restricted within myocardial capillaries. In contrast, in *Tie2Cre; p53^{FL/-}* mice, focal regions of the myocardium showed increased vascular permeability characterized by leakage of the low molecular weight dextran from myocardial capillaries (Fig. S4). Collectively, our results suggest that damage to the myocardial vasculature in *Tie2Cre; p53^{FL/-}* mice after 12 Gy whole-heart irradiation precedes myocardial necrosis and systolic dysfunction.

The effect of radiation on the myocardial microvasculature was further demonstrated in *Tie2Cre; p53^{FL/+}; mTmG/+* and *Tie2Cre; p53^{FL/-}; mTmG/+* mice, which contain EGFP⁺ microvessels and tdTomato⁺ cardiomyocytes. Histological evaluation in *Tie2Cre; p53^{FL/+}; mTmG/+* mice 7 weeks after 12 Gy whole-heart irradiation demonstrated tdTomato⁺ cardiomyocytes surrounded by EGFP⁺ microvessels in the myocardium, which is similar to the histology of the myocardium in mice without irradiation (Fig. 3F and Fig. S1). In contrast, *Tie2Cre; p53^{FL/-}; mTmG/+* mice had multiple regions of myocardial necrosis, which were composed of necrotic cardiomyocytes with a low tdTomato signal and disorganized EGFP⁺ microvessels. Adjacent to these necrotic areas, EGFP⁺ microvessels were dilated and disorganized although tdTomato⁺ cardiomyocytes remained histologically intact (Fig. 3F). These results further suggest that destruction of the microvasculature leads to myocardial injury in *Tie2Cre; p53^{FL/-}* mice after 12 Gy whole-heart irradiation.

To investigate if vascular injury in the myocardium leads to myocardial hypoxia, we used the 2-nitroimidazole agent EF5 as a surrogate to measure hypoxia (38) in the myocardium of *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice 4 and 5 weeks after 12 Gy whole-heart irradiation. Although EF5 did not label the myocardium of irradiated *Tie2Cre; p53^{FL/+}* mice, EF5 binding was observed in regions where myocardial vessels were damaged in *Tie2Cre; p53^{FL/-}* mice 4 weeks after 12 Gy whole-heart irradiation (Fig. 3G). Myocardial hypoxia was also present within regions of vascular injury in *Tie2Cre; p53^{FL/-}* mice 5 weeks after 12 Gy whole-heart irradiation (Fig. 3G). Therefore, our results indicate that damage to the myocardial capillaries in *Tie2Cre; p53^{FL/-}* mice after 12 Gy whole-heart irradiation leads to cardiac ischemia and myocardial hypoxia, which precedes systolic dysfunction and heart failure.

Deletion of p53 in endothelial cells by VE-Cadherin-Cre sensitizes mice to radiation-induced damage to the myocardial vasculature

Because recombination by Tie2Cre occurs in the majority of hematopoietic cells (31, 33, 39), it is possible that deletion of p53 in hematopoietic cells in Tie2Cre mice contributes to radiation-induced myocardial injury. To investigate if the radiation-induced myocardial injury in *Tie2Cre; p53^{FL/-}* mice is specifically due to loss of p53 in endothelial cells, we utilized VE-Cadherin-Cre (VECre) mice to delete p53 in endothelial cells. Similar to reports by others (32, 40), we observed that recombination in VECre mice occurs in a small subset (10 to 15%) of hematopoietic cells (Fig. S5), which is much lower than Tie2Cre mice (approximately 84%) (39). Tissue sections from *VECre; p53^{FL/+}; mTmG/+* and *VECre; p53^{FL/-}; mTmG/+* mice show that Cre is expressed specifically in endothelial cells in the myocardium (Fig. S6). In contrast to Tie2Cre mice, where Cre is expressed uniformly in

endothelial cells throughout the myocardium (Fig. S1), in VECre mice, Cre is predominantly expressed in endothelial cells in the epimyocardium and midmyocardium (Fig. S6).

We next investigated if deletion of p53 by VECre sensitized mice to myocardial injury after exposure to a single dose of 12 Gy whole-heart irradiation. We found that *VECre; p53^{FL/-}* mice succumbed to late effects of radiation after 12 Gy whole-heart irradiation, with the median survival of 67 days (Fig. 4A). Histopathological changes in the myocardium in *VECre; p53^{FL/-}* mice were similar to the radiation-induced myocardial necrosis observed in *Tie2Cre; p53^{FL/-}* mice (Fig. 4B). 9 weeks after 12 Gy whole-heart irradiation, cardiomyocytes in irradiated *VECre; p53^{FL/-}* mice showed increased Evans blue dye uptake compared to those in irradiated *VECre; p53^{FL/+}* mice (Fig. 4C). In addition, heart sections from *VECre; p53^{FL/-}; mTmG/+* mice 7 weeks after 12 Gy whole-heart irradiation showed focal areas of myocardial necrosis characterized by damaged EGFP⁺ microvessels and loss of tdTomato⁺ cardiomyocytes (Fig. 4D). Furthermore, a progressive increase in myocardial hypoxia was detected in the myocardium of *VECre; p53^{FL/-}* mice 5 and 7 weeks after 12 Gy whole-heart irradiation (Fig. 4E). Collectively, the phenotypes of myocardial injury observed from *VECre; p53^{FL/-}* mice are consistent with the phenotypes of irradiated *Tie2Cre; p53^{FL/-}* mice. In contrast to the *Tie2Cre; p53^{FL/-}* mice that developed myocardial injury after 12 Gy whole-heart irradiation throughout the myocardium, most necrotic lesions in *VECre; p53^{FL/-}* mice were located in the epimyocardium and midmyocardium where Cre is expressed in cardiac endothelial cells (Fig. 4B). Therefore, these results demonstrate that specific deletion of p53 in endothelial cells *in vivo* sensitizes mice to radiation-induced myocardial injury.

Impaired p53-mediated cell cycle arrest sensitizes cardiac endothelial cells to radiation-induced mitotic death

To determine if the susceptibility of radiation-induced endothelial cell damage in *Tie2Cre; p53^{FL/-}* mice is cell-autonomous, we assessed the viability of endothelial cells *in vitro* using primary cardiac endothelial cells isolated from the myocardium. Cardiac endothelial cells cultured *in vitro* displayed endothelial cell-like cobble stone morphology and surface markers of endothelial cells, CD31 and CD105, were present (Fig S7, A and B). p53 mRNA expression was lost in cardiac endothelial cells from *Tie2Cre; p53^{FL/-}* mice, whereas the abundance of p53 mRNA in cardiac endothelial cells from *Tie2Cre; p53^{FL/+}* mice was approximately 50% compared to wild type mice (Fig. S7C). To investigate the role of p53 in mediating radiation-induced cell death, we irradiated cardiac endothelial cells from *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice *in vitro*. Two major types of cell death have been reported in endothelial cells exposed to radiation *in vitro*: ceramide-mediated pre-mitotic apoptosis that occurs 4 to 6 hours post-irradiation, and post-mitotic cell death that occurs 24 to 96 hours post-irradiation (41, 42). We did not observe a significant increase in cell death 4 hours post-irradiation in cardiac endothelial cells with or without p53, but cell death was significantly increased in p53-deficient cardiac endothelial cells 96 hours post-irradiation in a dose-dependent manner (Fig. 5, A and B). In addition, a significantly higher percentage of p53-deficient cardiac endothelial cells underwent apoptosis, as shown by staining for annexin V and cleaved caspase 3, 24 to 72 hours after 12 Gy irradiation. However, no significant increase in apoptosis was detected in cardiac endothelial cells 4 hours after 12 Gy irradiation regardless of the presence or absence of p53 (Fig. 5, C – E). Collectively, these results show that p53 protects cardiac endothelial cells from post-mitotic cell death 24 to 96 hours after high dose irradiation *in vitro*.

Loss of p53-dependent cell cycle arrest in cells exposed to radiation can cause cells to proceed into mitosis with damaged DNA, resulting in mitotic catastrophe, a type of cell death following an aberrant mitosis (43, 44). Thus, we hypothesized that p53 protects cardiac endothelial cells from radiation by preventing mitotic catastrophe secondary to

premature entry into mitosis. We first examined if loss of p53 alters the mitotic ratio in cardiac endothelial cells after irradiation by quantifying cells with phosphorylated histone H3. *Tie2Cre; p53^{FL/-}* cardiac endothelial cells had a significantly higher mitotic ratio 8, 24, 48 and 72 hours after 12 Gy irradiation compared to *Tie2Cre; p53^{FL/+}* cardiac endothelial cells, indicating that p53-deficient cardiac endothelial cells have impaired cell cycle arrest after irradiation (Fig. 5F). To determine if premature entry into mitosis leads to mitotic catastrophe, we quantified the formation of micronuclei, which occurs as a result of incorrect chromosomal segregation during mitosis (43, 44). Cardiac endothelial cells from *Tie2Cre; p53^{FL/-}* mice contained a significantly higher percentage of cells with micronuclei compared to cardiac endothelial cells from *Tie2Cre; p53^{FL/+}* mice 24 and 48 hours after 12 Gy irradiation (Fig. 5, G and H). Most cells with micronuclei also showed intense staining of γ -H2AX foci (Fig. 5G), indicating that these cells contained unrepaired DNA damage during mitosis. Taken together, these results support a model in which p53-dependent mitotic arrest regulates radiation-induced mitotic catastrophe in cardiac endothelial cells.

p53 controls the G1/S and G2/M cell cycle checkpoints at least in part by inducing the cyclin-dependent kinase inhibitor p21 (45). Thus, we hypothesized that p53 controls survival of cardiac endothelial cells through a p21-dependent mechanism. We isolated mRNA from cardiac endothelial cells from wild type, *Tie2Cre; p53^{FL/+}*, *Tie2Cre; p53^{FL/-}* and *p53^{-/-}* mice and quantified mRNA expression of *p21* as well as *PUMA* and *Bax*, which are transcriptional targets of p53 that regulates premitotic apoptosis (46, 47). Our results show that mRNA expression of *p21*, but not that of *PUMA* or *Bax*, was significantly induced after 12 Gy in cardiac endothelial cells containing at least one allele of p53. (Fig. S8, A – C). However, *p21* mRNA was minimal at baseline in either *Tie2Cre; p53^{FL/-}* or *p53^{-/-}* cardiac endothelial cells and was not induced by radiation (Fig. S8A). These results suggest that radiation selectively induces p53-mediated pro-survival pathways in cardiac endothelial cells *in vitro*. Subsequently, we examined the susceptibility of primary cardiac endothelial cells from *p21^{+/-}* and *p21^{-/-}* mice to radiation 4 and 72 hours after 12 Gy irradiation *in vitro*. Although cell death was not significantly induced in both types of cardiac endothelial cells 4 hours after 12 Gy irradiation, p21-deficient cardiac endothelial cells were more susceptible to radiation-induced post-mitotic cell death 72 hours after 12 Gy irradiation (Fig. S8, D and E). Collectively, these results indicate that the mechanism by which p53 protects cardiac endothelial cells against radiation is at least partially through p21.

p21-deficient mice are susceptible to radiation-induced myocardial injury

To investigate if loss of p21 potentiates radiation-induced myocardial injury *in vivo*, *p21^{+/-}* and *p21^{-/-}* mice were exposed to 12 Gy whole-heart irradiation and followed for the development of cardiac injury. All *p21^{-/-}* mice were moribund 33 to 37 days after 12 Gy whole-heart irradiation (Fig. 6A). Histopathological examination indicated that *p21^{-/-}* mice developed acute to sub-acute myocardial degeneration and necrosis (Fig. 6B), which is similar to that observed in *Tie2Cre; p53^{FL/-}* mice after 12 Gy whole-heart irradiation. Echocardiography 4 weeks after 12 Gy whole-heart irradiation revealed that *p21^{-/-}* mice developed systolic dysfunction with significantly decreased fractional shortening and decreased mean velocity of circumferential fiber shortening (Fig. 6, C and D and Table S2). In addition, *p21^{-/-}* mice developed cardiac hypertrophy 4 weeks after 12 Gy whole-heart irradiation indicated by an increased left ventricular mass, heart to body weight ratio, and average cardiomyocyte cross-sectional area (Fig. 6, E – H).

Assessment of microvessel density in the myocardium 4 weeks after 12 Gy whole-heart irradiation showed decreased density of myocardial capillaries in *p21^{-/-}* mice (Fig. 7, A and B). This global decrease in microvessel density in the myocardium was preceded by focal increased vascular permeability (Fig. 7C). Furthermore, *p21^{-/-}* mice showed focal myocardial hypoxia 3 weeks after 12 Gy whole-heart irradiation, and the hypoxic areas

further increased in size and number at 4 weeks (Fig. 7D). Collectively, our results indicate that $p21^{-/-}$ mice phenocopy the sensitivity of $Tie2Cre; p53^{FL/-}$ mice to radiation-induced myocardial injury because after 12 Gy whole-heart irradiation, $p21^{-/-}$ mice developed (i) myocardial degeneration and necrosis, (ii) systolic dysfunction, (iii) a reduction in microvessel density, and (iv) increased vascular permeability and myocardial hypoxia prior to the onset of cardiac dysfunction. Taken together, our results demonstrate that p53 functions in endothelial cells to prevent radiation-induced myocardial injury through a mechanism at least partially regulated by p21.

DISCUSSION

In the heart, p53 promotes cardiac injury from pressure overload (3), ischemic injury (4), telomere attrition (5), and doxorubicin treatment (6–8). In contrast, in the present study, we demonstrate that p53 functions to protect mice from myocardial injury caused by radiation. Because radiation-related heart disease is a major cause of treatment-associated mortality for cancer patients after thoracic radiotherapy (9), our results suggest that combining radiation therapy with inhibitors of p53 (48) or other components of the DNA damage response that regulate mitotic arrest (49) may increase the risk of cardiac injury if the heart is in the field of radiation. However, it is important to note that complete deletion of p53 in cardiac endothelial cells is necessary to sensitize mice to radiation-induced myocardial injury. Therefore, the deleterious effects of reversibly blocking p53 by chemical inhibitors in combination with radiation therapy may be less than we have observed with complete deletion of p53.

Previous histological and ultrastructural studies of radiation-induced myocardial injury in the rabbit and rat suggest that loss of cardiac endothelial cells preceded damage to cardiomyocytes (14, 16). Despite these elegant experiments, the role of the vasculature in mediating late effects from radiation has been controversial (50) because it has been difficult to separate injury to the vasculature from injury to the parenchymal cells of an organ. By demonstrating that Tie2Cre mice or VECre mice with an endothelial cell-specific deletion of p53 are sensitized to radiation-induced cardiac injury *in vivo* (Fig. 1 and 4), we provide compelling genetic evidence that endothelial cells play a critical role in regulating radiation-induced injury to the heart. In addition, we demonstrate that damage in the myocardial vasculature results in cardiac ischemia and myocardial hypoxia (Fig. 3G and 4E), which leads to systolic dysfunction and heart failure. This system may also be useful for investigating the role of the vasculature in mediating other late effects of radiation.

Our experiments also clarify how p53 regulates the response of endothelial cells in the heart to radiation. Although HUVECs appear to undergo radiation-induced apoptosis that can be blocked by a p53 inhibitor (23), we find that cardiac endothelial cells do not undergo p53-dependent apoptosis 4 hours after radiation up to doses of 24 Gy. In contrast, cardiac endothelial cells undergo a delayed mitotic death following irradiation, which is potentiated by deletion of p53 (Fig. 5). This conclusion is consistent with a report that blocking p53 in tumor-associated endothelial cells can sensitize xenograft tumors to radiation (26). As additional evidence to support a model in which p53 functions in cardiac endothelial cells after irradiation to activate cell cycle checkpoints to prevent mitotic death and subsequent myocardial injury, we observed that mice lacking the p53 target gene $p21$ are also sensitized to radiation-induced cardiac injury (Fig. 6). Because p53 mediates increased abundance of the cyclin dependent kinase inhibitor p21 in cardiac endothelial cells (Fig. S8), our results indicate that the p53–p21 cell cycle arrest pathway is a mechanism by which cardiac endothelial cells survive radiation.

Our results do not exclude that cell types other than cardiac endothelial cells play a role in radiation-induced cardiac injury. After 12 Gy whole-heart irradiation, both *Tie2Cre; p53^{FL/-}* and *p21^{-/-}* mice showed destruction of the capillary network throughout the myocardium. Thus, loss of p53-dependent signaling may not only promote radiation-induced mitotic death in endothelial cells, but may also impair vessel regeneration in the myocardium. p21 plays an important role in regulating the turnover of mature endothelial cells and endothelial progenitor cells: *p21^{-/-}* mice show impaired neovascularization despite increased proliferation of mature endothelial cells and endothelial progenitor cells (51). In addition, p53 and p21 inhibit stromal-derived factor-1 (SDF-1) signaling through chemokine receptor 4 (CXCR4), which promotes inflammatory responses (52–54). After vascular wire injury, *p21^{-/-}* mice show impaired wound healing and increased migration and trafficking of inflammatory cells into areas of vascular damage as a consequence of increased abundance of vascular SDF-1 proteins (54). These results suggest that the p53–p21 axis may also regulate vessel regeneration after radiation. Indeed, the more rapid onset of myocardial necrosis in mice lacking p21 throughout the entire animal compared to mice lacking p53 specifically in endothelial cells (compare Fig. 1B and Fig. S6B with Fig. 6A) suggests that p21 may also function in cells other than endothelial cells to prevent radiation-induced myocardial injury. Therefore, how p53-dependent signaling functions in other stromal cells or in the cardiomyocytes to control radiation-induced heart disease warrants further investigation.

This study has several strengths. First, whole heart irradiation (whole-heart irradiation) was utilized with a micro-CT/micro-irradiator to deliver focal irradiation to the heart (Fig. 1A). Therefore, the potential contribution of radiation-induced damage from other tissues, such as the gastrointestinal tract or bone marrow, to the observed myocardial injury is limited. Second, we performed serial echocardiography on mice before and after whole-heart irradiation and documented systolic dysfunction and cardiac hypertrophy in mice lacking p53 in the endothelial cells or in *p21^{-/-}* mice (Fig. 2 and 6). These physiological measurements documented cardiac dysfunction independently of the histological endpoints thereby demonstrating that mice in this study succumbed to late effects of cardiac injury. Finally, we also studied the effect of deleting p53 in endothelial cells on radiation-induced cardiac injury in two independent Cre lines. By deleting p53 in endothelial cells with either *Tie2Cre* (Fig. 1) or *VECre* (Fig. 4), mice were sensitized to radiation-induced myocardial injury from whole-heart irradiation. Although multifocal myocardial necrosis was present throughout the myocardium of *Tie2Cre; p53^{FL/-}* mice (Fig. 1D), myocardial necrosis in *VECre; p53^{FL/-}* mice was prominent in the epimyocardium and midmyocardium (Fig. 4). Because the *VECre* mice did not have substantial necrosis in the endomyocardium after whole-heart irradiation in a region where the endothelial cells show poor expression of Cre recombinase (Fig. S6), the endomyocardium serves as an internal control to demonstrate that loss of p53 in endothelial cells, rather than other cell types, sensitizes mice to myocardial injury after whole-heart irradiation.

In addition to causing decreased blood supply to the myocardium, radiation to the heart also causes pericardial disease in humans and animal models (18, 55). In the pericardium of some mice lacking p53 in endothelial cells, we also observed increased edema, proliferative fibroblasts, collagen fibrils and infiltration of lymphocytes (Fig. S2). These findings suggest that, in addition to myocardial injury, damage to endothelial cells may also precede the pathological alterations in the pericardium. In the future, we plan to conduct studies to irradiate part of the pericardium of *Tie2Cre; p53^{FL/-}* or *VE-Cre; p53^{FL/-}* mice with tangent radiation fields, which may provide further insight into how damage to endothelial cells may regulate the progression of radiation-induced pericardial disease.

Accumulating data from mouse models reveal that activation of p53 by radiation *in vivo* can be either pro-survival or pro-death depending on the cell-type (1, 2). The diverse cell-type specific outcome of p53 activation after irradiation may be influenced by the ability of p53 to selectively activate its transcriptional targets through differential DNA binding cooperativity in a cell-type specific manner. Selectivity of p53 binding can be regulated by co-factors or by distinct post-translational modifications of p53 (56). For example, co-factors of p53 such as Hzf and Miz1 interact with the DNA binding domain of p53 to promote the expression of *p21*, but prevent the induction of the pro-apoptotic targets such as *PUMA* and *Bax* (57–59). In addition, whether p53 activation can induce premitotic apoptosis may depend on whether radiation causes p53 to tip the balance between pro-apoptotic and anti-apoptotic proteins. For example, in the hematopoietic system, radiation induces pro-apoptotic genes such as *PUMA* and *Bax* in both granulocyte and macrophage progenitors (GMPs) and hematopoietic stem cells (HSCs) (60). However, premitotic apoptosis is preferentially activated by radiation in GMPs compared to HSCs. This phenomenon can be at least partially explained because the expression of anti-apoptotic genes such as *Bcl-xl*, *Mcl-1* and *A1* (60) is higher in HSCs, which may inhibit the pro-apoptotic function of p53 target genes, such as *PUMA* and *Bax* or p53 itself acting at the mitochondria (61). In the present study, our results show that radiation does not induce premitotic apoptosis in cardiac endothelial cells *in vitro*. Consistent with this finding, mRNA expression analyses reveal that radiation selectively induces *p21* rather than pro-apoptotic genes *PUMA* and *Bax* (Fig. S8). Therefore, further studies are warranted to investigate whether cardiac endothelial cells harbor distinct co-factors or modifiers of p53 that regulate cell fate after activation in response to radiation.

In summary, we have demonstrated that p53 functions in endothelial cells to protect mice from radiation-induced myocardial injury. This finding may have important implications for cancer therapy. Several pharmacological inhibitors of proteins that regulate cell cycle checkpoints are now in clinical development including radiosensitizers, such as checkpoint kinases inhibitors (49), and radioprotectors, such as p53 inhibitors (48). Our findings raise the possibility that when these inhibitors are combined with radiation therapy, patients may experience increased late effects, such as radiation-related heart disease.

MATERIALS AND METHODS

Mouse strains

All animal procedures for this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Duke University. All of the mouse strains used in this study have been described previously including *p21^{-/-}*, *p53^{-/-}*, *p53^{FL/FL}*, Tie2Cre, VECre, mTmG and LSL-YFP mice (32, 33, 35, 62). The *p53^{FL/FL}* mice were kindly provided by Anton Berns and the *p21^{-/-}* and *p53^{-/-}* mice were kindly provided by Tyler Jacks. Tie2Cre, VECre, mTmG and LSL-YFP mice were obtained from Jackson laboratories. Experiments were carried out with mice that were between 8 to 10 weeks old and were on mixed genetic backgrounds. For every experiment, age-matched, littermate controls were utilized to minimize the effect of genetic background. Therefore, potential genetic modifiers of the response to radiation would be randomly distributed among the experimental and control groups. Because Cre expression can occur in the germ line of female Tie2Cre mice (63), *Tie2Cre; p53^{+/-}* males were crossed to *p53^{FL/FL}* females to avoid non-specific deletion of floxed alleles in the germ line.

Radiation treatment

Whole-heart irradiation was performed using a small-field biological irradiator, the X-RAD 225Cx (Precision X-Ray, Inc). The system was commissioned as previously described (64).

The heart was localized to isocenter (source to subject distance is approximately 30.76 cm) with fluoroscopy at 40 kVp, 2.5 mA with a 2 mm Al filter. Mice were irradiated with parallel-opposed anterior and posterior fields. Irradiation was performed with a collimating cone to produce a circular radiation field of 15 mm at treatment isocenter with an average dose rate of 300 cGy/min at target depth with a 225 kVp, 13 mA beam and a 0.3 mm Cu filter. Endothelial cells were irradiated with ionizing radiation *in vitro* using an X-RAD 320 Biological Irradiator (Precision X-ray, Inc). Irradiation was performed 50 cm from the radiation source with a dose rate of 200 cGy/min with 320 kVp X-rays, using 12.5 mA and a filter consisting of 2.5 mm Al and 0.1 mm Cu. The dose rate was measured with an ion chamber by members of the Radiation Safety Division at Duke University.

Echocardiography

Serial transthoracic echocardiography was performed on conscious mice for all groups with a Vevo 2100 high-resolution image system (VisualSonics) as previously described (65)

Histological analyses

To prepare paraffin-embedded tissues, tissues specimens were fixed in 10% neutralized formalin overnight, preserved in 70% ethanol and then embedded in paraffin. To prepare frozen tissues, tissue specimens were fixed in 4% paraformaldehyde in PBS for 2 hours at 4°C and transferred to 30% sucrose in PBS overnight at 4°C. Tissues were immersed in OCT compound (Sakura Finetek), snap frozen in dry ice/isopentane slurry, and stored at -80°C. H&E and Masson's trichrome-stained sections were analyzed by a veterinary pathologist (YK) blinded to genotype and treatment.

Evans blue dye (10 mg/ml in PBS; Sigma Aldrich) was administered to mice through intraperitoneal injection at the volume of 10 μ l/ g body weight and the hearts were collected 24 hours after injection. Frozen sections were prepared for histological analyses.

The 2-nitroimidazole agent EF5 was kindly provided by Cameron Koch. EF5 (10 mM in PBS) was administered to mice through intraperitoneal injection at the volume of 26.5 μ l/ g body weight and the hearts were collected 3 hours after injection. Frozen sections were prepared for histological analyses. EF5 was detected by staining with Cy3-conjugated anti-EF5 IgG (provided by Cameron Koch).

For quantification of the averaged cardiomyocyte cross-sectional area and microvessel density, sections cut from paraffin embedded tissues were deparaffinized with xylene and rehydrated with a graded series of ethanol and water washes. Antigens were retrieved by Antigen Unmasking Solution (Vector Laboratories) according to the manufacturer's instructions. Sections were stained with Alexa Fluor 488-conjugated WGA (5 μ g/mL) and Alexa Fluor 647-conjugated GS-IB₄ (10 μ g/mL) (Invitrogen) 2 hours at room temperature.

For TUNEL staining, sections from the same tissues were used. Antigens were retrieved by proteinase K (0.8 U/mL, Sigma-Aldrich) for 15 min at room temperature. TUNEL staining was performed using In Situ Cell Death Detection Kit, TMR red (Roche) according to the manufacturer's instructions. Sections were counterstained with Alexa Fluor 488-conjugated WGA (5 μ g/mL) and Alexa Fluor 647-conjugated GS-IB₄ (10 μ g/mL) (Invitrogen) overnight at 4°C. Six images (200 $\times$) per slide were randomly taken from the myocardium with similar cross sections in all mice except *Tie2Cre; p53^{FL}*^{-/-} mice 4 weeks after 12 Gy. In these mice, 6 fields (200 $\times$) were chosen from representative non-necrotic regions, and 3 to 6 fields (200 $\times$) were chosen from representative necrotic regions. Necrotic regions were identified by abnormal morphology of cardiomyocytes and microvessels. The microvessel density, the averaged cardiomyocyte cross-sectional area and the percentage of microvessels

containing TUNEL positive cells were quantified by MetaMorph (Molecular Devices). Data of all images from the same mouse were averaged.

Vascular permeability assay

Mice were injected intravenously with a mixture of TMR-conjugated high molecular weight (2×10^6 molecular weight, 1 mg/ml) and Alexa Fluor 647-conjugated low molecular weight (1×10^4 molecular weight, 0.4 mg/ml) dextrans (Invitrogen). Mice were euthanized 1.5 hours later. Ten μ m sections were cut from frozen tissues and stained with rat anti-mouse CD31 IgG (BD Pharmingen) diluted 1:50 in 5% rabbit serum overnight at 4°C. After washing with PBS, sections were incubated with Alexa Fluor 488-conjugated donkey anti-rat (Invitrogen) diluted 1:400 in 2% mouse serum for 30 min at room temperature.

Primary culture of cardiac endothelial cells

Isolation of cardiac endothelial cells was performed as described previously (66) with slight modifications. Primary cardiac endothelial cells were isolated from ventricles of 4 to 6-week-old mice. Ventricles were digested using Hank's Balanced Salt Solution (Invitrogen) with 200 U/ml of collagenase I (Worthington Biochemical Corporation) for 45 min at 37°C. Cardiac endothelial cells were captured using rat IgG magnetic beads (Invitrogen) coated with rat anti-mouse CD31 IgG (BD Pharmingen). After the initial purification, cardiac endothelial cells were cultured in DMEM supplemented with 25 mM HEPES, 20% FBS, penicillin/ streptomycin, 2 mM L-glutamine, 100 μ g/ml heparin, nonessential amino acid (1 $\times$), sodium pyruvate (1 $\times$) and 100 μ g/ml endothelial cell growth stimulant (Biomedical Technologies) on cell culture dishes coated with 0.1% of gelatin (Sigma). Cardiac endothelial cells were grown to 70% to 80% confluency followed by a second isolation using magnetic beads coated with rat anti-mouse CD102 IgG (BD Pharmingen) to further improve the purity. Cardiac endothelial cells were used for experiments from passage 1 to 4 after the secondary purification (total passage 3 to 6). The purity of cardiac endothelial cells was assessed by flow cytometry using FITC-conjugated anti-mouse CD31 and PE-conjugated anti-mouse CD105 antibodies (eBioscience).

Quantification of cell death by flow cytometry

Cardiac endothelial cells were irradiated at approximately 60% confluency and harvested at time points post-irradiation as indicated. Cells were washed with PBS and stained with either propidium iodide (5 μ g/ml, Sigma-Aldrich) alone or with FITC annexin V (BD Pharmingen) and propidium iodide according to the manufacturers' instructions. Data were collected by FACScan (BD Pharmingen) and analyzed by Flowjo (Tree Star, Inc).

Quantification of cleaved caspase-3 and pHH3 positive cells by flow cytometry

Cardiac endothelial cells were irradiated at approximately 60% confluency and harvested at time points post-irradiation as indicated. Cells were fixed with cold 70% ethanol and stored at -20°C. To quantify the percentage of cells positive for phosphorylated histone H3 (pHH3), cells were stained with rabbit anti-mouse pHH3 IgG (Abcam) diluted 1:500 in PBS/0.25% Triton-X 100/3% BSA for 1 hour at room temperature followed by staining with Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000) (Invitrogen) at room temperature for 1 hour. Cleaved caspase-3 staining was performed using PE Active Caspase-3 Apoptosis Kit (BD Pharmingen) according to the manufacturer's instructions. Data were collected by FACScan (BD Pharmingen) and analyzed by Flowjo (Tree Star, Inc).

Quantification of micronuclei

Cardiac endothelial cells were irradiated at approximately 60% confluency and harvested at time points post-irradiation as indicated. Cells were fixed with cold 70% ethanol and stored

at -20°C . Approximately 40,000 fixed cells were cytospun on poly-L-lysine coated slides (Azer scientific) at 500 rpm for 5 min. Slides were dried overnight at room temperature prior to staining. For γH2AX staining, cells were permeabilized with 0.2% Triton $\times$ -100 in PBS, blocked with 3% goat serum in PBS and stained with anti- γH2AX (Ser 139) antibody (Millipore) (1:500 dilution) for 1 hour at 37°C . Slides were then washed with PBS and stained with Alexa Fluor 488-conjugated goat anti-mouse IgG1 (Invitrogen) for 1 hour at 37°C . Hoechst 33342 (Sigma) was used for nuclear counterstaining. Cells with micronuclei were counted by examining the morphology of nuclei counterstained with Hoechst 33342. A hundred cells were counted per slide by a single observer (EJM).

Quantitative RT-PCR

Total RNA was extracted from the cardiac endothelial cells using the TRI reagent (Ambion) and RNeasy Mini kit (QIAGEN) following the manufacturer's protocol. RNA yield and quantity were determined by measuring absorbencies at 260 nm and 280 nm using NanoDrop (Thermo Scientific). Six hundred ng of total RNA were used for *in vitro* reverse transcription using iScript cDNA synthesis Kit (Bio-Rad). The expression of *p53*, *p21*, *PUMA*, *Bax* and *β 2-microglobulin (*B2m*)* mRNA was quantified by qRT-PCR with Taqman probes (Applied Biosystems, Mm01731290_g1 for *p53*, Mm00432448_m1 for *p21*, Mm00519268_m1 for *PUMA*, Mm00432050_m1 for *Bax* and Mm00437762_m1 for *B2m*). *B2m* was used as an internal control to correct for the concentration of cDNA in different samples.

Statistics

Results are presented as mean $\pm$ SEM. Student's *t*-test (two-tailed) was performed to compare the means of two groups, and one-way ANOVA was performed to compare the means of three or more groups. Two-way ANOVA followed by Bonferroni post-hoc test was performed to examine the interaction between genotypes and radiation treatment. If the normality test failed, data were log-transformed prior to applying statistical tests. For survival studies, Kaplan-Meier analysis was performed followed by the log-rank test. Significance was assumed at $P < 0.05$. GraphPad Prism 5 (GraphPad Software, Inc) was used for the calculation of statistics.

Microscopy

Pictures were taken using a Leica DFC 340FX fluorescent microscope (Leica Microsystems). Images were captured by Leica suite software (Leica Microsystems).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Deletion of p53 by Tie2Cre sensitizes mice to radiation-induced myocardial injury. (A) Representative fluoroscscopy image of a mouse treated with whole-heart irradiation. Whole-heart irradiation was performed with a 15 mm circular collimator (red circle) (B and C) Kaplan-Meier survival analysis of *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice after a single fraction of 12 Gy or 10 daily fractions of 3 Gy whole-heart irradiation (3 Gy × 10). *P* value was calculated by long-rank test. (D – G) Histological analysis of the myocardium of moribund *Tie2Cre; p53^{FL/-}* mice after 12 Gy whole-heart irradiation. Representative sections of the myocardium of a *Tie2Cre; p53^{FL/-}* mouse 62 days after 12 Gy whole-heart irradiation (D) were stained with hematoxylin and eosin (H&E) (E), anti-sarcomeric α-actin IgG (SαA) (F) or Masson's trichrome (Trichrome) (G). The dashed line in (E), (F) and (G) demarcates the relatively normal appearing myocardium and the region of necrosis (labeled as N). Yellow arrows indicate necrotic cardiomyocytes replaced by blue collagen fibers. (H and I) Evans blue dye uptake (red) in *Tie2Cre; p53^{FL/+}* (H) and *Tie2Cre; p53^{FL/-}* (I) mice 5 weeks after 12 Gy whole-heart irradiation. Cell membranes were counterstained with wheat germ agglutinin (white). Images represent four mice per group. Scale bar, 100 μm.

Fig. 2.

Deletion of p53 by Tie2Cre sensitizes mice to systolic dysfunction and cardiac hypertrophy. (A) Representative echocardiographic recordings from *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice prior to and 7 weeks after 12 Gy whole-heart irradiation. (B to E) Changes in fractional shortening (B), heart rate corrected mean velocity of circumferential fiber shortening (mVcf) (C), left ventricular end-systolic dimension (LVDs) (D) and the left ventricular mass (E) in *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice after 12 Gy whole-heart irradiation. Data are presented as mean $\pm$ SEM (n=6 mice per group). (F) Ratio of heart weight to body weight (HW/BW) of *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice sham irradiated or 7 weeks after 12 Gy whole-heart irradiation. Data are presented as mean $\pm$ SEM (n=3 mice per group). (G) Representative images of wheat germ agglutinin (WGA)-stained myocardium of *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice sham irradiated or 7 weeks after 12 Gy whole-heart irradiation. Scale bar, 100 μ m. (H) Quantification of cardiomyocyte cross-sectional area in WGA-stained myocardium. Data are presented as mean $\pm$ SEM (n=3 mice per group). Asterisk shows individual differences ($P < 0.05$) by two-way ANOVA with Bonferroni post-hoc test.

Fig. 3.

Deletion of p53 by Tie2Cre sensitizes mice to vascular injury and chronic ischemia in the myocardium. (A – C) Representative sections of WGA (white) and GS-IB₄ (red)-stained myocardium of *Tie2Cre; p53^{FL/+}* (A) and *Tie2Cre; p53^{FL/-}* mice (B and C) 4 weeks after 12 Gy whole-heart irradiation. (D and E) Quantification of GS-IB₄ positive myocardial capillaries per high power (200×) field. For *Tie2Cre; p53^{FL/-}* mice 4 weeks after 12 Gy whole-heart irradiation, vessel density in normal and necrotic regions are scored separately. By 7 weeks, no normal appearing myocardium was present in *Tie2Cre; p53^{FL/-}* mice. Asterisk shows individual differences ($P < 0.05$) by one-way ANOVA with Bonferroni post-hoc test compared to sham irradiated mice. Data are presented as mean $\pm$ SEM (n=3 mice per group). (F) Representative sections of the myocardium of *Tie2Cre; p53^{FL/+}; mTmG/+* and *Tie2Cre; p53^{FL/-}; mTmG/+* mice 7 weeks after 12 Gy whole-heart irradiation. Myocardial vessels were counterstained with GS-IB₄. N marks an area of necrosis. High power magnification image shows disorganized vessels in the area adjacent to a necrotic region. Images represent three mice per group. (G) The hypoxia marker EF5 (red) in *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice 4 and 5 weeks after 12 Gy whole-heart irradiation. EF5 positive regions correlated with loss of myocardial capillaries, shown by GS-IB₄ staining (white). Images represent three mice per group. Scale bar, 100 μ m.

Fig. 4. Deletion of p53 by VE-Cadherin-Cre sensitizes mice to radiation-induced myocardial injury. (A) Kaplan-Meier survival analysis of *VECre; p53^{FL/+}* and *VECre; p53^{FL/-}* mice after 12 Gy whole-heart irradiation. *P* value was calculated by long-rank test. (B) A representative hematoxylin and eosin-stained (H&E) section of the heart from *VECre; p53^{FL/-}* mice 52 days after 12 Gy whole-heart irradiation. Multifocal myocardial necrosis was mainly observed in the epicardium (E) and midmyocardium (M). (C) Evans blue dye (red) uptake in *VECre; p53^{FL/+}* and *VECre; p53^{FL/-}* mice 9 weeks after 12 Gy whole-heart irradiation. Cell membranes were counterstained with WGA (white). Images represent three mice per group. (D) Representative sections of the myocardium of *VECre; p53^{FL/+}; mTmG/+* and *VECre; p53^{FL/-}; mTmG/+* mice 7 weeks after 12 Gy whole-heart irradiation. Myocardial vessels were counterstained with GS-IB₄. An area of necrosis is marked by N. Images represent three mice per group. (E) The hypoxia marker EF5 (red) in *VECre; p53^{FL/+}* and *VECre; p53^{FL/-}* mice 5 and 7 weeks after 12 Gy whole-heart irradiation. EF5 positive regions correlated with loss of myocardial capillaries, shown by GS-IB₄ staining (white). Images represent two mice per group. Scale bar, 100 μ m.

Fig. 5. p53 protects cardiac endothelial cells from radiation-induced mitotic catastrophe. Experiments were performed using cardiac endothelial cells from *Tie2Cre; p53^{FL/+}* and *Tie2Cre; p53^{FL/-}* mice at passage 3 to 5. (A – B) Quantification of cell death 4 and 96 hours after various doses of irradiation. Cell survival was determined by propidium iodide exclusion. (C–D) Quantification of the percentage of annexin V positive cells 4 and 72 hours after 12 Gy irradiation. (E and F) Quantification of the percentage of cleaved caspase-3 (Casp3) positive (E) and mitotic ratio (F) of cardiac endothelial cells at various time points after 12 Gy irradiation. (G) Representative images of cells with micronuclei. Cells were stained with anti- γ -H2AX antibody and Hoechst 33324 to label γ -H2AX foci (green) and nuclei (blue), respectively. Arrows indicate cells with the presence of micronuclei. The majority of micronuclei contain unrepaired DNA damage indicated by positive staining for γ -H2AX foci. A representative image of a cell with micronuclei at higher magnification is shown in the inset. (H) Quantification of the percentage of cardiac endothelial cells with micronuclei at various time points after 12 Gy irradiation. Asterisk shows individual differences ($P < 0.05$) by two-way ANOVA with Bonferroni post-hoc test. Data are presented as mean $\pm$ SEM (n=3 independent experiments).

Fig. 6.

p21-deficient mice are susceptible to radiation-induced myocardial injury. (A) Kaplan-Meier survival analysis of *p21*^{+/+} and *p21*^{-/-} mice after 12 Gy whole-heart irradiation. (B) A representative hematoxylin and eosin-stained (H&E) section of the heart from *p21*^{-/-} mice 34 days after 12 Gy whole-heart irradiation. Scale bar, 100 μ m. (C to E) Changes in fractional shortening (C), heart rate corrected mean velocity of circumferential fiber shortening (mVcfc) (D), and left ventricular mass (E) in *p21*^{+/+} and *p21*^{-/-} mice after 12 Gy whole-heart irradiation. Data are presented as mean $\pm$ SEM. (F) Ratio of heart weight to body weight (HW/BW) of *p21*^{+/+} and *p21*^{-/-} mice 0, 3 and 4 weeks after 12 Gy whole-heart irradiation. (G) Representative images of WGA-stained myocardium in *p21*^{+/+} and *p21*^{-/-} mice sham irradiated or 7 weeks after 12 Gy whole-heart irradiation. Scale bar, 100 μ m. (H) Quantification of cardiomyocyte cross-sectional area in wheat germ agglutinin-stained myocardium. Data are presented as mean $\pm$ SEM (n=3 mice per group). Asterisk shows individual differences ($P < 0.05$) by two-way ANOVA with Bonferroni post-hoc test.

Fig. 7.

Loss of p21 sensitizes mice to microvascular injury and chronic ischemia in the myocardium. **(A)** Representative sections of the myocardium stained with Hoechst 33324 (blue) and GS-IB₄ (red). Scale bar, 100 μ m. **(B)** Quantification of GS-IB₄ positive capillaries per high power (200 $\times$) field in the myocardium of *p21*^{+/+} and *p21*^{-/-} mice. Asterisk shows individual differences ($P < 0.05$) by two-way ANOVA with Bonferroni post-hoc test. Data are presented as mean $\pm$ SEM (n=3 mice for each group). **(C)** Measurement of vascular permeability in the myocardium of *p21*^{+/+} and *p21*^{-/-} mice 3 weeks after 12 Gy whole-heart irradiation. Mice were injected with a mixture of high (2×10^6) and low (1×10^4) molecular weight (molecular weight) dextrans. Blood vessels were stained by anti-CD31 IgG. Images represent three mice per genotype. These results show that dextrans of both low (red) and high (green) MWs are restricted in blood vessels in irradiated *p21*^{+/+} mice. In contrast, in irradiated *p21*^{-/-} mice, the high MW dextran is retained in blood vessels, whereas the low MW dextran (red) leaks out of blood vessels in the damaged regions. Images represent three mice per group. **(D)** The hypoxia marker EF5 (red) in *p21*^{+/+} and *p21*^{-/-} mice 3 and 4 weeks after 12 Gy whole-heart irradiation. EF5 positive regions correlated with loss of myocardial capillaries, shown by GS-IB₄ staining (white). Images represent two mice per group. Scale bar, 100 μ m.