Mechanisms by which p53 Regulates Radiation-induced Carcinogenesis and Myocardial Injury

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Radiation therapy can cause acute toxicity and long-term side effects in normal tissues. Because part of the acute toxicity of radiation is due to p53-mediated apoptosis, blocking p53 during irradiation can protect some normal tissues from acute radiation injury and might improve the therapeutic ratio of radiation therapy. However, the mechanisms by which p53 regulates late effects of radiation are not well understood. Here, I utilized genetically engineered mouse models to dissect the role of p53 in regulating two of the most clinically significant late effects of radiation: radiation-induced carcinogenesis and radiation-induced myocardial injury.

It has been well characterized that mice with one allele of p53 permanently deleted are sensitized to radiation-induced cancer. Therefore, temporary inhibition of blocking p53 during irradiation could promote malignant transformation. Experiments with mice lacking functional p53 in which p53 protein can be temporarily restored during total-body irradiation (TBI) suggest that the radiation-induced p53 response does not contribute to p53-mediated tumor suppression. Here, I performed reciprocal experiments and temporarily turned p53 off during TBI using transgenic mice with reversible RNA interference against p53. I found that temporary knockdown of p53 during TBI not only ameliorated acute hematopoietic toxicity, but in both Kras wild-type and tumor-prone Kras\textsuperscript{LAI} mice also prevented lymphoma development. Mechanistic studies show that p53 knockdown during TBI improves survival of hematopoietic stem and progenitor cells (HSPCs), which maintains HSPC quiescence and prevents
accelerated repopulation of surviving cells. Moreover, using an in vivo competition assay I found that temporary knockdown of p53 during TBI maintains the fitness of p53 wild-type HSPCs to prevent the expansion of irradiated mutant cells. Taken together, our data demonstrate that p53 functions during TBI to promote lymphoma formation by facilitating the expansion of irradiated HSPCs with adaptive mutations.

p53 functions in the heart to promote myocardial injury after multiple types of stress, including ischemic injury, pressure overload and doxorubicin-induced oxidative stress. However, how p53 regulates radiation-induced myocardial injury, which develops after radiation therapy, is not well understood. Here, I utilized the Cre-loxP system to demonstrate that p53 functions in endothelial cells to protect mice from myocardial injury after a single dose of 12 Gy or 10 daily fractions of 3 Gy whole-heart irradiation (WHI). Mice in which both alleles of p53 are deleted in endothelial cells succumbed to heart failure after WHI 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. Mechanistic studies using primary cardiac endothelial cells (CECs) irradiated in vitro indicate that p53 signals to cause a mitotic arrest and protects CECs against radiation-induced mitotic catastrophe. Furthermore, mice lacking the cyclin-dependent kinase inhibitor p21, which is a transcriptional target of p53, are also sensitized to myocardial injury after 12 Gy WHI. Together, our results demonstrate that the p53/p21 axis functions to prevent radiation-induced myocardial injury in mice. Our findings raise the possibility that when combining radiation therapy with inhibitors of p53 or other components of the
DNA damage response that regulate mitotic arrest, patients may experience increased radiation-related heart disease.

Taken together, our results demonstrate crucial but distinct roles of p53 in regulating late effects of radiation: p53-mediated apoptosis promotes radiation-induced lymphomagenesis, but p53-mediated cell cycle arrest prevents radiation-induced myocardial injury. These findings indicate that p53 may generally play a protective role from radiation, particularly at high doses, in cells where p53 activation is uncoupled from the induction of the intrinsic pathway of apoptosis. Therefore, selectively inhibiting p53-mediated apoptosis may be a promising approach to ameliorate acute radiation toxicity without exacerbating late effects of radiation.
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Chapter 1: Introduction

Radiation therapy plays an important role in the curative treatment of cancer patients. However, patients treated with radiation therapy can suffer acute toxicity and are at risk for developing late effects from radiation. Acute radiation toxicity becomes manifest during or within weeks after completing of a course of radiation therapy. Depending on the anatomic site irradiated, possible symptoms include mucositis, skin erythema, nausea, diarrhea or low blood cell counts (1). These symptoms are usually transient and may resolve a few weeks after irradiation. In contrast, late effects typically develop after latent periods of months to years and include radiation-induced fibrosis, secondary malignancies and myocardial injury (1). Late effects of radiation tend to be irreversible and may progress in severity.

Because the ability to cure cancer with radiation therapy is frequently limited by acute toxicity and late effects, decreasing the acute and late effects of radiation therapy should improve the quality of life of cancer patients and may increase the probability of a cure if this approach increases the amount of radiation that can safely be administered to cancer patients. Therefore, understanding mechanisms of acute radiation injury and late effects from radiation may enable the development of better strategies to ameliorate normal tissue toxicity and thereby increase the effectiveness of radiation therapy.

Studies over the last two decades demonstrate that some kinds of the acute radiation injury are regulated by transformation related protein 53 (p53) (2). Thus, pharmacological inhibition of p53 is a promising approach to ameliorate acute radiation
toxicity (3). However, inhibition of p53 during radiation therapy could exacerbate late
effects of radiation because p53-deficient mice are susceptible to radiation-induced
cancers and life shortening (4, 5). In this dissertation, I utilized genetically engineered
mouse models to dissect the mechanisms by which p53 regulates two clinically
significant late effects of radiation: radiation-induced carcinogenesis and myocardial
injury (6).

1.1 Characterization of the p53 response to radiation

p53 is a transcription factor that contains two transcriptional activation domains
(TAD), which can independently enhance transcription of p53 target genes, and a DNA
binding domain that is responsible for sequence-specific binding of the protein to p53
response elements (5). The activity of p53 in cells is elegantly fine-tuned through
post-translational modifications and protein stabilization. Under conditions of
homeostasis, when p53 activity is not required, p53 protein is negatively regulated
mainly by MDM2-family members, MDMX and MDM2 (7, 8). In response to genotoxic
and oxidative stress, stress signals inhibit the interaction of MDM2 and p53 through
multiple mechanisms, which subsequently activate p53 to execute cellular response to
various types of stress. In the context of radiation, p53 is activated almost immediately
following irradiation through the DNA damage response (DDR) to promote cell cycle
arrest, which contributes to cell survival, or to induce the intrinsic pathway of apoptosis,
which leads to cell death (9).
Following irradiation, how p53 determines cell fate (cell cycle vs. apoptosis) following irradiation may be dictated by the intensity of the stress and/or the cell type (9, 10). For example, in hematopoietic cells, p53-driven radiation response increases BH3-only proteins such as Noxa and PUMA to activate the intrinsic pathway of apoptosis (11). However, in mouse embryonic fibroblasts (MEFs), activation of p53 by radiation does not stimulate apoptosis but induces cell cycle regulators such as GADD45 and the cycline-dependent kinase inhibitor p21 to cause cell cycle arrest at G1 and G2 (12). The distinct response of p53 activation following total-body irradiation (TBI) was also observed in vivo and can be generally divided into 3 groups of cell types based on the induction of p53 protein and its downstream targets (13). The first group contains tissues including hematopoietic cells that show accumulation of p53 protein and increased pre-mitotic apoptosis. The second group comprises tissues that accumulate p53 but do not undergo significant pre-mitotic apoptosis; for example, the myocardium and the lung epithelium. While pro-apoptotic genes such as PUMA are not induced in these tissues after irradiation, transcriptional targets of p53 that regulate cell-cycle arrest, such as p21, are upregulated. The third group contains tissues in which p53 is not upregulated after irradiation; for example, the skeletal muscle. Most tissues in this group are considered to be terminally differentiated and are generally resistant to radiation. However, the small subset of stem cells and progenitors in these tissues may have a distinct response to radiation compared to their more differentiated progenies (12). Collectively, these results demonstrate that the response of p53 to radiation is heterogeneous. Because of the diverse outcome of p53 activation in vivo, the effect of
blocking p53 on acute radiation toxicity and late effects of radiation may be cell-type dependent.

1.2 p53 and acute radiation injury

1.2.1 Acute hematopoietic toxicity

The hematopoietic system is extremely sensitive to radiation-induced cell death. In mouse models, the hematopoietic syndrome arises after exposure to total-body irradiation with doses of approximately 1.5 to 7.5 Gy (14, 15). Death may occur within 30 days as a result of radiation-induced damage to the rapidly proliferating hematopoietic progenitor cells in the bone marrow, which subsequently prevents the regeneration of essential elements of the peripheral blood such as platelets and neutrophils (15, 16).

The survival of hematopoietic progenitors following irradiation is primarily determined by the abundance of p53 because p53-deficient hematopoietic progenitors are markedly resistant to radiation-induced apoptosis (17). Moreover, genetic deletion of p53 or PUMA markedly ameliorates the hematopoietic syndrome (18-20). Taken together, these results demonstrate a critical role of p53 in controlling radiation-induced cell death in hematopoietic cells and therefore suggest that inhibition of p53-dependent apoptosis is a promising approach to ameliorate acute hematopoietic toxicity.

1.2.2 Acute gastrointestinal toxicity

Damage to the gastrointestinal (GI) epithelial cells following irradiation is a primary cause of acute GI toxicity. In mouse models, pure GI syndrome occurs following
abdominal irradiation at doses of 12 Gy or higher, and causes death within 5 to 10 days after irradiation (21). Death from the GI syndrome results from destruction of the lining of the GI tract with collapse of the villi, diarrhea, and loss of fluid and electrolyte transport in the gut (16). Intestinal stem cells located in the crypt epithelium are essential to support crypt regeneration (15, 21). Although multiple lines of evidence indicate that death of crypt epithelial cells is a major cause of the radiation-induced GI syndrome, it remains to be clarified how the intestinal crypt cells die from radiation (22). Remarkably, p53-deficient mice are resistant to the hematopoietic syndrome, but show increased sensitivity to the radiation-induced GI syndrome (18). Because mice lacking p21 were also sensitized to the GI syndrome, p53 and p21-dependent cell cycle arrest has been suggested to play an important role in regulating the survival of intestinal stem cells (5, 18).

1.3 p53 and late effects of radiation

1.3.1 p53 and radiation-induced carcinogenesis

It has been well characterized that p53 is indispensable to suppress malignant transformation induced by irradiation because p53−/− mice, in which one allele of p53 is permanently deleted, are susceptible to radiation-induced cancer (4). Recent studies suggest that the role of p53 in suppressing tumor development can be dissected from p53-driven pathological response (23, 24). Christophorou and colleagues used mice lacking functional p53 in which p53 protein can be restored temporarily to investigate whether activation of p53 by the DDR following 2.5 Gy TBI contributes to tumor
suppression (23). They showed that the presence of functional p53 during TBI markedly induced apoptosis, but did not alter lymphomagenesis in p53-deficient mice. In contrast, they showed that temporary activation of p53 after TBI delayed lymphoma formation primarily by eliminating cells with oncogenic alternations at least partially via p19ARF. In addition, Hinkal and co-workers used an inducible Cre-loxP model to permanently delete p53 in somatic cells prior to, concurrently with or after 2.5 Gy TBI (24). However, these mice showed no difference in the latency of lymphoma formation regardless of when p53 was deleted, suggesting that p53 does not function during the DDR to regulate lymphomagenesis in mice where p53 is deleted after irradiation. Taken together, these studies suggest that activation of p53 by the DDR is dispensable for p53 to suppress radiation-induced lymphomagenesis (Figure 1.1).

The elegant studies described above demonstrate that activation of p53 by TBI does not alter lymphomagenesis in mice lacking functional p53 after irradiation. However, one limitation of these studies is that p53-deficient mice are predisposed to tumorigenesis and they possess a distinct susceptibility to radiation-induced cancers compared to mice with wild-type p53 (4, 25-27). For example, while a single dose of 4 Gy TBI significantly accelerates tumor formation in p53+/− mice, it rarely induces tumors in adult mice with wild-type p53 (4). In addition, for reasons that remain to be elucidated, adult p53+/− mice are resistant to radiation-induced carcinogenesis after 4 Gy TBI (4). In contrast, fractionated irradiation with 1.5 to 2 Gy per fraction, which is a schedule frequently employed in the clinic to treat patients, efficiently induces lymphomas after TBI in mice with wild-type p53 (28). Therefore, how the induction of p53 by the DDR
affects radiation-induced carcinogenesis in mice with physiological levels of p53 remains
to be defined.

1.3.2 p53 and radiation-induced myocardial injury

In the heart, p53 functions to promote cardiac injury from pressure overload (29),
ischemic injury (30), telomere attrition (31), and doxorubicin-induced oxidative stress
(32-34). Therefore, blocking p53 with 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.

Damage to the microvasculature of the heart after irradiation occurs in animal
models prior to pathological changes in the myocardium (35-38). For example, Fajardo
and Stewart studied the pathogenesis of radiation-induced myocardial fibrosis in rabbits
exposed to a single dose of 20 Gy (35, 36). In these elegant studies, focal areas of
myocardial fibrosis were observed by two months after irradiation (36). From day 20
through 49 after irradiation, significant damage to endothelial cells within the
myocardium was seen including decreased microvessel density (35). 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 (37).
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, while focal areas of myocardial
necrosis were noted at two months (38). 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 (39-41).

Radiation induces p53 in the heart in vivo (42) and in endothelial cells from various sources in vitro (43-45). 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, has been shown to protect human umbilical vein endothelial cells (HUVECs) from radiation-induced cell death by apparently blocking p53 (43). This finding is further supported by the observation that inhibition of p53 by pifithrin, a small-molecule inhibitor of p53, increases the viability of HUVECs after irradiation (43). Moreover, blocking p53 has been suggested to suppress radiation-induced damage to human dermal microvascular endothelial cells and mouse endothelial progenitor cells (44, 45). In contrast, others have reported that when p53 is inhibited or deleted in tumor-associated endothelial cells, xenograft tumors are sensitized to radiation (46), 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 (CECs) may reveal the molecular mechanism of radiation induced cardiac toxicity. Moreover, understanding whether p53 plays a pro-death or pro-survival role in irradiated CECs will provide critical
information to design clinical trials that combine radiation therapy with inhibitors of the DNA damage response pathway.

1.4 Rationale and specific aims

Pharmacological inhibition of p53 to block p53-dependent apoptosis is a promising approach to ameliorate acute radiation toxicity; however, the impact of blocking p53 during irradiation on late effects is not well defined. In this dissertation, I utilized genetically engineered mice to investigate the role of p53 in regulating two of the clinically significant late effects that develop in patients treated with radiation therapy: radiation-induced carcinogenesis and myocardial injury.

In the first part of my dissertation, I clarified the role of p53 induction by the DDR in radiation-induced carcinogenesis. To study how p53 functions during irradiation to regulate tumor development, I utilized mice in which a miR-30-based p53.1224 small hairpin RNA can be temporally and reversibly induced in vivo (47). Remarkably, my results show that temporary inhibition of p53 during fractionated TBI not only ameliorates acute hematopoietic toxicity, but also prevents radiation-induced lymphomagenesis. Therefore, my results demonstrate that p53 functions during TBI to promote radiation-induced lymphomagenesis.

In the second part of my dissertation, I investigated how p53 functions in endothelial cells to regulate the response of the heart to radiation in vivo using the Cre-loxP system. Remarkably, deleting p53 in endothelial cells with either Tie2Cre or VE-Cadherin-Cre causes mice to be sensitized to myocardial injury after whole-heart
irradiation. The onset of cardiac injury is preceded by increased endothelial cell death, increased vascular permeability and decreased microvessel density in the myocardium. Moreover, p21-deficient mice also developed myocardial injury after whole-heart irradiation. Taken together, my results demonstrate that p53 functions in endothelial cells \textit{in vivo} to prevent radiation-induced myocardial injury.
Figure 1.1: Distinct pathways that activate p53 dictate acute radiation toxicity and p53-mediated tumor suppression.
Chapter 2: p53 acts during total-body irradiation to promote lymphoma formation

2.1 Introduction

Radiation therapy plays an important role in the curative treatment of cancer patients. However, patients treated with radiation therapy can suffer acute toxicity and they are at risk for developing late effects including radiation-induced cancer (48). The primary cause of acute radiation toxicity in some tissues, such as the hematopoietic system, is due to activation of the intrinsic pathway of apoptosis through the DNA damage response (DDR) (2, 9). Because radiation activates the DDR to induce apoptosis at least partially through the transformation related protein 53 (p53), inhibiting p53 may reduce acute radiation toxicity in normal tissues in which p53 engages the intrinsic pathway of apoptosis (2, 9). For example, genetic deletion or pharmacological inhibition of p53 markedly ameliorates hematological toxicity in mice exposed to total body irradiation (TBI) (17, 18, 49). However, suppressing acute radiation toxicity by inhibiting p53 could exacerbate late effects of radiation, such as secondary malignancies, because p53−/− mice, in which one allele of p53 is permanently deleted, are susceptible to radiation-induced cancer (4).

Recent studies suggest that the mechanism by which p53 suppresses tumor formation following 2.5 Gy TBI is independent of activation of p53 by the DDR (23, 24). Christophorou and colleagues used mice lacking functional p53 in which p53 protein
can be restored temporarily to investigate whether activation of p53 by the DDR following TBI contributes to tumor suppression (23). They showed that the presence of functional p53 during TBI markedly induced apoptosis, but did not alter lymphomagenesis in p53-deficient mice. In contrast, they showed that temporary activation of p53 after TBI delayed lymphoma formation primarily by eliminating cells with oncogenic alterations via p19ARF. In addition, Hinkal and co-workers used an inducible Cre-loxP model to permanently delete p53 in somatic cells prior to, concurrently with or after 2.5 Gy TBI (24). However, these mice showed no difference in the latency of lymphoma formation regardless of when p53 was deleted, suggesting that p53 does not function during the DDR to regulate lymphomagenesis in mice where p53 is deleted after irradiation. Taken together, these studies suggest that activation of p53 by the DDR is dispensable for p53 to suppress radiation-induced lymphomagenesis.

The elegant studies described above demonstrate that activation of p53 by TBI does not alter lymphomagenesis in mice lacking functional p53 after irradiation. However, one limitation of these studies is that p53-deficient mice are predisposed to tumorigenesis and they may therefore possess a distinct susceptibility to radiation-induced cancers compared to mice with wild-type p53 (4, 25-27). For example, while a single dose of 4 Gy TBI significantly accelerates tumor formation in p53+/− mice, it rarely induces tumors in adult mice with wild-type p53 (4). In addition, for reasons that remain to be elucidated, adult p53+/− mice are resistant to radiation-induced carcinogenesis after 4 Gy TBI (4). In contrast, fractionated irradiation with 1.5 to 2 Gy per fraction, which is a schedule frequently employed in the clinic to treat patients,
efficiently induces lymphomas after TBI in mice with wild-type p53 (28). Therefore, how the induction of p53 by the DDR affects radiation-induced carcinogenesis in mice with physiological levels of p53 remains to be defined.

To clarify the role of p53 induction by the DDR in radiation-induced carcinogenesis, I utilized mice in which a miR-30-based p53.1224 shRNA (shp53) can be temporally and reversibly induced in vivo (47). Remarkably, my results show that temporary inhibition of p53 during fractionated TBI not only ameliorates acute hematopoietic toxicity, but also prevents radiation-induced lymphomagenesis. Therefore, my results demonstrate that p53 functions during TBI to promote radiation-induced lymphomagenesis.

2.2 Results

2.2.1 Temporary knockdown of p53 during total-body irradiation ameliorates acute hematopoietic toxicity

To temporally and reversibly regulate p53 expression in vivo, I utilized transgenic mice in which expression of a miR-30-based p53.1224 short hairpin RNA (shRNA) is controlled by a tetracycline-responsive element (TRE) (47). In compound transgenic mice harboring TRE-shp53 and a reverse tetracycline-transcriptional activator (rtTA), doxycycline (Dox) significantly decreases p53 expression, but Dox does not alter p53 levels in control mice containing only rtTA or TRE-shp53 (47). I crossed TRE-shp53 mice to either CMV-rtTA or Actin-rtTA mice in which rtTA is expressed from either a cytomegalovirus (CMV) or a β-actin (Actin) promoter, respectively, to generate compound transgenic mice (CMV-rtTA; TRE-shp53 or Actin-rtTA; TRE-shp53). Both
promoters drive rtTA expression in multiple cell types in vivo (47, 50). To investigate the kinetics of shp53 turnover in vivo, I induced shp53 in CMV-rtTA; TRE-shp53 mice by Dox treatment for 10 days and examined the expression of shp53 and p53 mRNA in the bone marrow at different time points after withdrawal of Dox. Significant induction of shp53 and a corresponding decrease in p53 mRNA were observed in CMV-rtTA; TRE-shp53 mice 10 days after Dox treatment. Withdrawal of Dox for 7 days decreased shp53 and significantly restored p53 mRNA (Figure 2.1, A and B). These results show that expression of p53 in compound transgenic mice can be temporally and reversibly regulated in bone marrow cells in vivo by Dox.

Radiation causes acute hematopoietic toxicity in mice through induction of the intrinsic pathway of apoptosis in hematopoietic cells, which is at least partially via p53 (17, 18). Thus, I investigated whether p53 knockdown in vivo during total-body irradiation (TBI) ameliorates acute hematopoietic toxicity in CMV-rtTA; TRE-shp53 and Actin-rtTA; TRE-shp53 mice (referred to hereafter as shp53 mice). Because Dox treatment may ameliorate radiation-induced hematopoietic toxicity (51), all comparison groups for this and subsequent experiments received the same Dox treatment for the same period of time and effects of p53 knockdown were compared between compound transgenic mice (shp53) and littermate controls (Control) containing either rtTA or TRE-shp53.

I first examined how p53 knockdown regulates apoptosis in thymocytes and bone marrow mononuclear cells (BM-MNCs) in vivo 4 hrs after 2.5 Gy TBI. The percentage of thymocytes and BM-MNCs expressing cleaved caspase 3 (Casp3+) was markedly increased by irradiation in control mice, whereas p53 knockdown significantly inhibited
radiation-induced apoptosis in both CMV-rtTA and Actin-rtTA strains (Figure 2.2, A – D). To examine if inhibition of apoptosis protects hematopoietic stem and progenitor cells (HSPCs) from radiation, I assessed the frequency of Lineage (Lin) Sca1+ c-kit+ (LSK) cells, a population enriched with HSPCs (52), in the bone marrow 24 hrs after 2.5 Gy TBI (Figure 2.3A). Although radiation markedly decreased the frequency of LSK cells in control mice, p53 knockdown significantly protected LSK cells from radiation (Figure 2.3, A – C).

Because depletion of HSPCs after a lethal dose of TBI results in the hematopoietic syndrome within 30 days, I next investigated if temporary p53 knockdown during 7.5 Gy TBI ameliorates the hematopoietic syndrome. Both shp53 and control mice were fed a Dox-containing diet for 10 days prior to irradiation, and were switched to a Dox-free diet immediately after irradiation. Exposure to 7.5 Gy TBI caused more than 80% of control mice to develop the hematopoietic syndrome within 30 days after irradiation. In contrast, temporary p53 knockdown during 7.5 Gy TBI significantly ameliorated the hematopoietic syndrome (Figure 2.3, D and E). Collectively, these results demonstrate that temporary p53 knockdown during TBI ameliorates acute hematopoietic toxicity by protecting HSPCs against radiation-induced cell death.

2.2.2 Temporary knockdown of p53 during total-body irradiation prevents radiation-induced lymphomagenesis

To investigate how temporary knockdown of p53 during TBI regulates radiation-induced carcinogenesis, I followed tumor formation in mice treated with TBI
delivered in daily fractions of 1.8 Gy because fractionated TBI causes radiation-induced lymphomas in wild-type mice (28). All CMV-rtTA; TRE-shp53 and Actin-rtTA; TRE-shp53 mice (shp53) and littermate controls (control) containing either rtTA or TRE-shp53 were fed a Dox diet for 10 days followed by 4 daily fractions of 1.8 Gy TBI (1.8 Gy x 4). After the last dose of radiation was delivered, mice were switched to a Dox-free diet (Figure 2.4A). As shown in Figure 2.4B and C, shp53 mice in which p53 was knocked down temporarily during 1.8 Gy x 4 TBI had a longer life span compared to littermate controls (By log-rank test, $P=0.057$ and $P=0.04$ for CMV-rtTA and Actin-rtTA, respectively). As expected, the most common tumor type identified in control mice after TBI was lymphoma. However, the incidence of lymphoma formation in shp53 mice (in both CMV-rtTA and Actin-rtTA strains) was markedly decreased. Indeed, Kaplan-Meier analysis revealed that temporary p53 knockdown during 1.8 Gy x 4 TBI significantly prevented radiation-induced lymphomagenesis (By log-rank test, $P=0.004$ and $P=0.03$ for CMV-rtTA and Actin-rtTA, respectively) (Figure 2.4, D and E). In mice that developed lymphomas, the tumors primarily developed in the thymus (96% in control mice and 100% in shp53 mice), but in some mice, the lymphoma also disseminated to the spleen and/or liver (Figure 2.4F). All lymphomas examined (n=8 for shp53 and n=10 for control) were T-cell lymphomas because they stained for CD3 (Figure 2.4G). Together, these results indicate that temporary p53 knockdown during 1.8 Gy x 4 TBI significantly reduces the incidence of radiation-induced lymphoma without altering the type of lymphoma that develops in irradiated mice.
Because these experiments were performed in mice with a mixed genetic background, to control for the possibility that an unanticipated mutation co-segregates in shp53 mice to prevent radiation-induced lymphomagenesis, I followed tumor formation in Actin-rtTA; TRE-shp53 (shp53) mice and littermate controls (Control) containing either Actin-rtTA or TRE-shp53 after 3 daily fractions of 1.8 Gy TBI (1.8 Gy x 3) without Dox treatment. As shown in Figure 2.5, shp53 and control mice had a similar life span after 1.8 Gy x 3 TBI. The vast majority of mice in both groups developed radiation-induced lymphomas with similar latency (Figure 2.5, A and B). These data indicate that the susceptibility to develop a radiation-induced lymphoma in the absence of Dox is the same in control mice with one (rtTA or TRE-shp53) transgenic allele and in shp53 mice. Taken together, my results demonstrate that temporary p53 knockdown during TBI prevents the formation of radiation-induced lymphoma, thereby suggesting that p53 functions during TBI to promote radiation-induced lymphomagenesis.

2.2.3 Temporary knockdown of p53 during total-body irradiation prevents lymphoma formation in cancer-prone KrasLA1 mice

To further test whether p53 functions during irradiation to promote lymphomagenesis, I utilized cancer-prone KrasLA1 mice. KrasLA1 mice carry a latent oncogenic allele of KrasG12D that is randomly activated in somatic cells by a spontaneous recombination event (53). While KrasLA1 mice develop lung adenomas and low-grade lung adenocarcinomas with 100% penetrance, approximately 20% of these mice develop spontaneous thymic lymphoma (53). Therefore, KrasLA1 mice provide a model to
investigate how temporary p53 knockdown during TBI regulates radiation-induced carcinogenesis in a cancer-prone system.

Compound transgenic (shp53) mice (Kras\textsuperscript{LA1}; CMV-rtTA; TRE-shp53 and Kras\textsuperscript{LA1}; Actin-rtTA; TRE-shp53), and Kras\textsuperscript{LA1} littermate (control) mice (Kras\textsuperscript{LA1}; rtTA, Kras\textsuperscript{LA1}; TRE-shp53 or Kras\textsuperscript{LA1} alone) were fed a Dox diet for 10 days followed by exposure to 4 daily fractions of 1.8 Gy TBI. After the last dose of radiation was delivered, mice were switched to a Dox-free diet (Figure 2.6A). Almost all of the mice in this experiment succumbed to either lung tumors or lymphomas (Figure 2.6, B and C). Kras\textsuperscript{LA1} mice appear to develop lymphomas after TBI with shorter latency than Kras wild-type mice. In the absence of p53 knockdown, following TBI the median times for developing lymphomas in Kras\textsuperscript{LA1} mice (control mice in Figure 2.6) and Kras wild-type mice (control mice in Figure 2.4) are 129 (n=32) and 186 (n=28) days, respectively (By log-rank test, P<0.0001). Consistent with the results from irradiated Kras wild-type mice (Figure 2.4, D and E), temporary p53 knockdown during 1.8 Gy x 4 TBI in either CMV-rtTA or Actin-rtTA strains significantly protected Kras\textsuperscript{LA1} mice from developing radiation-induced lymphomas (Figure 2.6, D and E). In the CMV-rtTA strain, a significant number of mice with (n=9) and without p53 knockdown (n=15) succumbed to lung tumors without developing lymphomas. Although the dose and schedule of radiation was not optimized in this experiment to cause radiation-induced lung cancer (54), I compared the aggressiveness of lung cancers in these mice by measuring the tumor area in the lungs and the percentage of lung tumors with >50% of the area staining positive for phospho-p44/42 MAPK (p-Erk) (Figure 2.7) (55). Results from both
assays suggest that temporary p53 knockdown during TBI does not significantly change the aggressiveness of lung tumors in Kras^{LA1} mice (Figure 2.6. F and G). Taken together, my results from four independent experiments (Figure 2.4, D and E; Figure 2.6, D and E) demonstrate that temporary knockdown of p53 during TBI prevents lymphoma formation in both Kras wild type and cancer-prone Kras^{LA1} mice.

### 2.2.4 Temporary knockdown of p53 during total-body irradiation suppresses accelerated repopulation of HSPCs

The formation of radiation-induced thymic lymphomas can be suppressed by shielding part of the bone marrow or by intravenously infusing non-irradiated bone marrow cells to irradiated recipients shortly after TBI (56, 57), suggesting that bone marrow-derived hematopoietic cells can inhibit radiation-induced lymphomagenesis. Because temporary inhibition of p53 during fractionated TBI is sufficient to prevent radiation-induced lymphomagenesis, I hypothesized that activation of p53 by the DDR in hematopoietic cells promotes lymphomagenesis. Total-body irradiation activates p53 through the DDR to simultaneously cause acute toxicity in the thymus and bone marrow. Therefore, I investigated how temporary p53 knockdown during 1.8 Gy x 4 TBI regulates cell death and repopulation of hematopoietic cells in the thymus and bone marrow. The cellularity of thymocytes in both shp53 and control mice was markedly decreased 7 days after 1.8 Gy x 4 TBI, but the cellularity of CD4^{-}CD8^{-}, CD4^{+}CD8^{-} and CD4^{+}CD8^{+} thymocytes in shp53 mice recovered significantly faster than control mice 14 and 28 days after 1.8 Gy x 4 TBI. (Figure 2.8, A – D). These data show that the ability of
control mice to regenerate thymocytes after TBI is limited, but shp53 mice with temporary p53 knockdown during TBI regenerate thymocytes within two weeks. Therefore, these results indicate that bone marrow-derived T-cell precursors were protected from radiation in shp53 mice. Indeed, while the cellularity of BM-MNCs and the frequency of multiple lineages of HSPCs in control mice were dramatically decreased 7 and 14 days after 1.8 Gy x 4 TBI, temporary p53 knockdown during TBI significantly inhibited cell death in BM-MNCs, oligopotent progenitors (Lin- c-kit+ Sca-1-) (52), LSK cells as well as LSK CD48- multipotent progenitors and hematopoietic stem cells (MPPs/HSCs) (58) (Figure 2.9, A – D). Collectively, these results indicate that p53 knockdown during 1.8 Gy x 4 TBI significantly protects HSPCs in the bone marrow against radiation-induced cell death.

Radiation-induced cell death in hematopoietic cells may stimulate proliferation of surviving HSPCs in the bone marrow to facilitate repopulation. To investigate the proliferation of HSPCs after TBI in this system, I examined how temporary Dox treatment during 1.8 Gy x 4 TBI or sham irradiation regulates MPPs/HSCs quiescence and proliferation in shp53 and control mice 12 days after withdrawal of Dox. As shown by Ki67 staining, in the absence of irradiation, MPPs/HSCs in shp53 and control mice were equally quiescent, but a significantly higher percentage of MPPs/HSCs in irradiated control mice stained positive for Ki67 compared to irradiated shp53 mice (Figure 2.9E). I also assessed proliferation of the MPPs/HSCs by pulsing with BrdU 4 hrs prior to collecting the cells. Consistent with the results from Ki67 staining, the percentage of MPPs/HSCs labeled with BrdU was significantly higher in irradiated
control mice compared to irradiated shp53 mice (Figure 2.9F). Together, these results indicate that temporary knockdown of p53 during 1.8 Gy x 4 TBI not only protects HSPCs in the bone marrow against radiation-induced cell death, but also maintains the surviving MPPs/HSCs in a non-proliferative quiescent state.

I also investigated whether p53 knockdown alone altered quiescence and proliferation of MPPs/HSCs because deletion of p53 can affect HSPC quiescence (59). I used Ki67 and Hoechst 33324 staining to investigate how p53 knockdown alone or in combination with radiation regulates the cell cycle in MPPs/HSCs prior to and 24 hrs after 1.8 Gy TBI in vivo (Figure 2.10A). After 10 days of Dox treatment, MPPs/HSCs in shp53 and control mice showed no significant difference in cell quiescence prior to irradiation even though p53 was knocked down in shp53 mice (Figure 2.10B). However, 24 hrs after 1.8 Gy TBI the surviving MPPs/HSCs in irradiated control mouse had a significantly lower percentage of cells in G0 and a higher percentage of cells in S-G2-M phases compared to irradiated shp53 mice (Figure 2.10B and C). I also assayed BrdU incorporation to assess proliferation of the MPPs/HSCs 24 hrs after 1.8 Gy TBI. Consistent with the results from staining for Ki67 and Hoechst 33324, staining for BrdU showed that p53 knockdown significantly suppresses proliferation of irradiated MPPs/HSCs (Figure 2.10D). These data indicate that p53 knockdown does not alter quiescence of MPPs/HSCs in the absence of irradiation. However, p53 knockdown during TBI significantly inhibits proliferation of irradiated MPPs/HSCs. Together, my results demonstrate that radiation activates p53 to promote cell death in hematopoietic cells, which subsequently stimulates accelerated proliferation of MPPs/HSCs.
2.2.5 Temporary knockdown of p53 during total-body irradiation suppresses clonal expansion of irradiated p53+/− HSPCs

Mathematical modeling of radiation-induced carcinogenesis in mouse models and humans suggests that repeated cycles of cell death and repopulation during fractionated irradiation plays a crucial role in promoting the expansion of irradiated stem/progenitor cells with adaptive mutations (60-63). Therefore, I hypothesized that temporary p53 knockdown during TBI prevents radiation-induced lymphomagenesis by suppressing clonal expansion of irradiated HSPCs with adaptive mutations. To test this hypothesis, I performed an in vivo competition assay to investigate how temporary p53 knockdown during TBI affects clonal expansion of p53+/− HSPCs, which mimics HSPCs that have acquired a mutation that increases fitness during repopulation (64, 65). I mixed LSK cells from GFP-tagged p53+/− (GFP) mice with LSK cells from either CMV-rtTA; TRE-shp53 (shp53) mice or littermate controls containing either CMV-rtTA or TRE-shp53 (Control) in a 5% to 95% ratio and transplanted them into lethally irradiated recipients to generate chimeric mice (Figure 2.11A). Eight weeks after bone marrow transplant, I measured the percentage of CD4+, CD8+, B220+, and CD11b+ peripheral blood mononuclear cells (PB-MNCs) and the percentage of GFP+ PB-MNCs to compare the engraftment of shp53/GFP and control/GFP LSK cells in chimeric mice. The percentage of GFP+ blood cells in chimeric mice transplanted with either shp53/GFP or control/GFP LSK cells ranged from approximately 2 – 35%, but the difference in GFP+ cells between these two groups of mice was not statistically significant prior to irradiation (Figure 2.11A). In addition, the two groups of mice also had similar levels of CD4+, CD8+, B220+, and
CD11b+ PB-MNCs (Figure 2.11, B – E). These results show that chimeric mice transplanted with either shp53/GFP or Control/GFP LSK cells had similar levels of stem cell engraftment.

Chimeric mice were subsequently fed a Dox diet for 10 days, then treated with 3 daily fractions of 1.8 Gy TBI (1.8 Gy x 3), and subsequently placed on a Dox-free diet (Figure 2.12A). The percentage of GFP+ cells in CD4+, CD8+ and B220+ lymphocytes as well as CD11b+ monocytes in peripheral blood was measured 10 weeks after 1.8 Gy x 3 TBI (Figure 2.12A). As a surrogate for the expansion of GFP-p53+/− HSPCs in the chimeric mice, I compared the fold change of GFP+ cells prior to and 10 weeks after 1.8 Gy x 3 TBI in the peripheral blood. In the majority of control/GFP chimeric mice 10 weeks after 1.8 Gy x 3, expansion of GFP-p53+/− cells was observed in CD4+, CD8+ and B220+ lymphocytes (Figure 2.12, B – D; Log10 ratio > 0), suggesting that p53+/− HSPCs outcompeted the p53 wild-type HSPCs to expand the GFP+ lymphoid cells. Remarkably, temporary p53 knockdown during TBI significantly suppressed clonal expansion of GFP-p53+/− cells in the same lymphocyte populations (Log10 ratio ≤ 0; Figure 2.12, B – D) suggesting that p53+/− HSPCs were outcompeted by the HSPCs with p53 knockdown to prevent expansion of the GFP+ lymphoid cells. Although temporary p53 knockdown in shp53/control mice during TBI also suppressed the growth of GFP-p53+/− cells in CD11b+ monocytes, the difference between shp53/GFP and control/shp53 mice is not statistically significant because of the variability of GFP+ cells in CD11b+ monocytes in control/GFP mice (Figure 2.11E). Collectively, these results support a model where temporary p53 knockdown during TBI prevents clonal expansion of mutated HSPCs after irradiation.
My findings indicate that one mechanism by which p53 functions during TBI to promote radiation-induced lymphomagenesis is to facilitate clonal expansion of irradiated HSPCs with adaptive mutations.

### 2.3 Discussion

Pharmacological inhibition of p53 to block p53-dependent apoptosis is a promising approach to ameliorate acute radiation toxicity (3). However, one concern of this approach is that temporarily inhibiting p53 during radiation therapy will increase the risk of developing a radiation-related cancer. In the present study, I used transgenic mice to temporarily inhibit p53 in vivo by RNA interference during fractionated TBI, which is known to cause radiation-induced lymphomas (28). I selected a treatment schedule of 1.8 Gy per daily fraction because it is frequently employed in the clinic to treat patients with cancer. I show that temporary knockdown of p53 during TBI not only ameliorates acute hematopoietic toxicity, but also prevents radiation-induced lymphomagenesis. Therefore, my results demonstrate that acute activation of the tumor suppressor p53 by the DDR during fractionated TBI promotes radiation-induced lymphomagenesis.

After p53 was identified, it was initially labeled as an oncogene because transfection of mutant p53 with an oncogenic Ras was sufficient to transform cells (66-68). However, genetic studies demonstrated that in cancers with p53 mutation, the other allele was frequently mutated (69). In addition, transfection of wild-type p53 into p53 mutant cells prevented cell growth (70). Therefore, these studies demonstrated that p53
functions as a tumor suppressor. Indeed, more recent studies have confirmed that loss of p53 function is not only required for tumor initiation, but is also required for tumor maintenance (71-73). My results establish that in wild-type mice exposed to TBI, p53 does not function during irradiation as a tumor suppressor, but instead acts to promote lymphoma development. To support this extraordinary conclusion, I performed all carcinogenesis experiments in two strains of rtTA mice, CMV-rtTA and Actin-rtTA, to control for potential promoter-specific or genetic background-specific effects. Indeed, these two strains of rtTA mice have slightly different sensitivity to acute hematopoietic toxicity (Figure 2.3) and radiation-induced lymphomas (Figure 2.4 and 2.6).

Nevertheless, my results from both rtTA strains consistently show that temporary knockdown of p53 during fractionated TBI prevents radiation-induced lymphomagenesis (Figure 2.4). Moreover, I tested the model that p53 functions to promote radiation-induced lymphomagenesis in cancer-prone KrasLAI mice, in which somatic cells randomly harbor a KrasG12D mutation. It has been well characterized that oncogenic Kras cooperates with p53 loss to promote malignant transformation in multiple tumor types including hematopoietic malignancies (74). Thus, it is possible that temporary knockdown of p53 during TBI may cause distinct effects on radiation-induced carcinogenesis in KrasLAI mice compared to wild-type mice. However, my results show that although radiation induces lymphoma with a higher penetrance and shorter latency in KrasLAI mice in the absence of p53 knockdown, temporary p53 knockdown during TBI still significantly suppresses lymphomagenesis in both CMV-rtTA; KrasLAI and Actin-rtTA; KrasLAI mice (Figure 2.6). Taken together, my data
from four independent experiments including wild type and *Kras<sup>LAI</sup>* mice demonstrate that temporary p53 knockdown prevents radiation-induced lymphomagenesis. Therefore, activation of p53 by the DDR during TBI promotes the formation of radiation-induced lymphomas. Future studies with this in vivo shRNA system using radiation doses and field sizes that are optimized for other types of radiation-induced malignancies will be needed to determine whether this observation extends to other types of cancers, such as sarcomas.

My findings demonstrate that inhibition of p53-mediated acute hematopoietic toxicity during fractionated TBI prevents radiation-induced lymphomagenesis, suggesting that p53-mediated cell death in the hematopoietic system during fractionated TBI promotes the expansion of lymphoma-initiating cells. Mathematical modeling suggests that during fractionated TBI repeated cycles of cell death and repopulation play an important role in promoting radiation-induced cancers in mouse models and in humans (60-63). In mice that are exposed to fractionated TBI, radiation activates p53 through the DDR to cause cell death in lineage committed hematopoietic progenitors and multipotent HSPCs (Figure 2.9, A – D). Following p53-dependent cell death, surviving HSPCs are stimulated to proliferate to repopulate the hematopoietic system (Figure 2.9, E and F; Figure 2.10, B – D). Therefore, repeated cycles of cell death and repopulation may select for cells with adaptive mutations, such as p53 mutations, which have increased fitness compared to their wild-type counterparts. Indeed, I used a bone marrow competition model to show that the ratio of p53<sup>−/−</sup> to wild-type hematopoietic cells in peripheral blood significantly increased 10 weeks after fractionated TBI,
indicating that radiation selects for clonal expansion of p53⁺⁻ HSPCs \textit{in vivo}. In contrast, temporary p53 knockdown during TBI significantly suppressed the expansion of p53⁺⁻ hematopoietic cells in peripheral blood, suggesting that surviving p53⁺⁻ hematopoietic cells had equal or less fitness than cells with temporary p53 knockdown (Figure 2.12, B – E). This interpretation is consistent with other recent studies of competition and selection for p53 mutant HSPCs after irradiation (64, 65). Therefore, these results indicate that p53 knockdown during TBI prevents the expansion of mutated HSPCs by protecting wild-type HSPCs against radiation. Moreover, my findings are consistent with previous observations that non-irradiated bone marrow-derived hematopoietic cells play a crucial role in suppressing radiation-induced lymphomagenesis (56, 57). Taken together, my results support a model where p53 functions during TBI to facilitate clonal expansion of irradiated HSPCs with adaptive mutations.

Accumulating evidence indicates that p53-mediated apoptosis induced by the DDR is dispensable for p53 to suppress radiation-induced tumor formation (23, 24, 75). Instead, p53 appears to function after the DDR has subsided and p53 suppresses radiation-induced lymphomagenesis when oncogeneic stress activates p53 via p19Arf (23). Therefore, several studies have concluded that p53 activation during TBI plays no role in radiation-induced lymphomagenesis. For example, Christophorou and colleagues utilized a mouse model lacking functional p53, in which p53 was temporarily restored prior to 2.5 Gy TBI and concluded that the pathological response to radiation does not contribute to p53-mediated suppression of lymphomagenesis (23). However, one potential limitation of this elegant study is that although p53 could be restored during
irradiation to cause apoptosis, after irradiation all of the cells that regenerated the hematopoietic system were p53-deficient. Therefore, these p53-deficient cells would likely have similar fitness and an equal opportunity to regenerate, so that radiation may not result in clonal selection in this system regardless of whether p53 is present during irradiation. Indeed, this phenomenon may also explain why p53−/− mice are not sensitive to radiation-induced cancers. My results do not conflict with a model where p53 functions after irradiation to suppress lymphomagenesis. However, my results demonstrate that in mice with wild-type p53, the pathological response to DNA damage activates p53 to promote radiation-induced lymphomagenesis.

Interestingly, Leonova and co-workers reported that treatment of p53−/+ mice with PFTβ, a chemical p53 inhibitor, prior to 4 Gy TBI does not change tumor latency (49). Although PFTβ significantly ameliorates acute hematopoietic injury in wild-type mice, it is not clear how this would impact HSPC fitness in p53−/+ mice and the clonal expansion of cells carrying mutations. In addition, these mice were irradiated with a single fraction of 4 Gy TBI, which induces lymphoma formation less efficiently than fractionated TBI in wild-type mice (4). Thus, whether p53 inhibitors administered to wild-type mice during fractionated TBI affects lymphomagenesis warrants further investigation.

Although the specificity of the miR30-based shp53 has been previously characterized (47), it is conceivable that a potential off-target effect of shp53 could affect radiation-induced lymphomagenesis. However, recent reports that mice lacking one allele of p53 upregulated modulator of apoptosis (PUMA) are resistant to radiation-induced apoptosis and radiation-induced lymphomagenesis after fractionated
TBI (76, 77), indicate that efficient apoptosis after TBI is critical for lymphoma development. Therefore, these results support my interpretation that the decreased rate of lymphoma formation in shp53 mice treated temporarily with Dox is due to specific knockdown of p53 in vivo during irradiation.

In summary, I provide compelling genetic evidence to show that p53 acts during total-body irradiation to promote radiation-induced lymphomagenesis. Although maintaining a physiological level of p53 after irradiation is critical to prevent tumor formation, during TBI p53 does not function as a tumor suppressor, but instead acts to promote lymphoma development. Thus, reversibly blocking p53 during radiation therapy may ameliorate acute radiation toxicity without exacerbating radiation-related secondary malignancies.

2.4 Materials and Methods

2.4.1 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 TRE-shp53 (TRE-p53.1224A) (47), CMV-rTA (47), Actin-rTA (50), UBC-GFP (78), KrasLA1 (53) and p53−/− (27) mice. The TRE-shp53 and CMV-rTA mice were provided by Scott Lowe, Actin-rTA mice were provided by Steve Artandi and KrasLA1 and p53−/− mice were provided by Tyler Jacks. UBC-GFP mice and C57BL/6 mice were obtained from Jackson laboratories. Experiments were carried out with 3 to 4-week-old mice for carcinogenesis and cell proliferation
studies and 8 to 10 week-old mice for hematopoietic syndrome studies. Both CMV-rtTA; TRE-shp53 and Actin-rtTA; TRE-shp53 mice 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.

2.4.2 Doxycycline treatment

All mice were fed a Dox-containing diet (6,000 mg/Kg Doxycycline, Cat. No. TD.04580, Harlan Teklad) for 10 days prior to irradiation. Immediately after irradiation, all mice were switched to a regular (Dox-free) diet.

2.4.3 Radiation treatment

Total-body irradiation was performed 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.

2.4.4 Histological analysis

Tissues specimens were fixed in 10% neutralized formalin overnight, preserved in 70% ethanol and then embedded in paraffin. Hematoxylin and eosin-stained sections were analyzed by a two veterinary pathologists (YK and LB) blinded to genotypes and
treatments. Sections cut from paraffin embedded tissues were stained with anti-mouse CD3 antibody (Thermo Scientific) with a standard immunoperoxidase technique described previously (79). For characterization of lung tumors, separated formalin-fixed lung lobes were processed and paraffin embedded adjacent to each other. Five sections were taken 300 µm into the lungs, and the fifth section was stained with hematoxylin and eosin. Lung and tumor areas for each mouse were determined without knowledge of genotype using ImageJ (NIH, Bethesda) for manual segmentation by a single observer (EJM). The fourth section was stained with anti-phospho-p44/42 MAPK antibody (Cell Signaling) with a standard immunoperoxidase technique described previously (79) and scored by a single observer.

2.4.5 FACS analysis of HSPCs

Total bone marrow cells were isolated from one femur and one tibia by grinding the bones with HSC buffer (HBSS w/o Ca2+ and Mg2+, 5% FBS, 2 mM EDTA). Red blood cells were lysed using ACK lysing buffer (Lonza). Total number of BM-MNCs was counted by Coulter counter (Beckman Coulter). Three million BM-MNCs were stained with PE-Cy5 conjugated Lineage (Lin) cocktail (ant-mouse CD3, CD4, CD8, B220, CD11b, Gr-1 and Ter-119 antibodies), PE conjugated anti-mouse Sca-1, APC conjugated anti-mouse c-kit, and biotin conjugated anti-CD48 antibodies followed by staining with APC-e780 conjugated Streptavidin (eBioscience). Dead cells were excluded by staining with 7-AAD (BD Pharmigen). Data were collected from 1 million cells by FACSCanto (BD Pharmingen) and analyzed by Flowjo (Tree Star, Inc).
2.4.6 FACS sorting and bone marrow transplant

BM-MNCs were isolated following the same procedure described above and followed by myeloid depletion using Dynabeads (Invitrogen) conjugated with anti-CD11b and Gr-1 antibodies (eBioscience). Myeloid depleted BM-MNCs were stained with PE-Cy5 conjugated Lin cocktail, PE-conjugated anti-mouse Sca-1, and APC conjugated anti-mouse c-kit antibodies (eBioscience). Dead cells were excluded by staining with 7-AAD (BD Pharmigen). Viable LSK or LSK GFP⁺ cells were sorted by FACS Vantage (BD Pharmingen). Total number of viable LSK cells after sorting was counted by hemocytometer with trypan blue (Sigma-Aldrich) exclusion. Mixed LSK cells (3800 cells from either shp53 or control mice and 200 cells from UBC-GFP; p53⁻/⁻ mice) were transplanted into 8-weel-old C57BL/6 mice irradiated with two fractions of 4.5 Gy TBI with interval of 6 hrs. After irradiation, recipient mice were treated with Septra water for 8 weeks.

2.4.7 FACS analysis of HSPC proliferation

Each mouse was given 50 mg/Kg of 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) through intraperitoneal injection and euthanized by CO₂ asphyxiation 4 hrs after BrdU injection. BM-MNCs were prepared as described above. Total number of BM-MNCs was counted by Coulter counter (Beckman Coulter). Three million BM-MNCs were stained with PE-Cy5 conjugated Lin cocktail, PE conjugated anti-mouse Sca-1, APC conjugated anti-mouse c-kit, and biotin conjugated anti-CD48 antibodies followed by staining with APC-e780 conjugated Streptavidin (eBioscience). BM-MNCs were then fixed in HSC
buffer containing 1% paraformaldehyde overnight at 4°C. Fixed BM-MNCs were permeabilized using Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Pharmigen) according to the manufacturer’s instructions. For cell cycle analyses, BM-MNCs were stained with FITC conjugated anti-human Ki67 antibody (BD Pharmigen) according to the manufacturer’s instructions followed by resuspension in HSC buffer containing 20µg/ml of Hoechst 33324 (Sigma-Aldrich). For detecting BrdU+ cells, BM-MNCs were incubated with 300 µg/ml of DNaseI (Sigma-Aldrich) for 1 hr at 37 °C followed by staining with FITC conjugated anti-BrdU antibody (eBioscience) according to the manufacturer’s instructions. Data were collected from 1 million cells by FACSCanto (BD Pharmingen) and analyzed by Flowjo (Tree Star, Inc).

2.4.8 FACS analysis of GFP+ mononuclear cells in peripheral blood

Eight to ten drops of blood were collected from anesthetized mice using submandibular bleeding and were mixed with PBS containing 5 mM EDTA. Red blood cells were separated from peripheral blood mononuclear cells (PB-MNCs) using Ficoll-Plaque (GE) according to the manufacturer’s instructions. PB-MNCs were stained with either PE-Cy5 conjugated anti-mouse CD4 and PE conjugated anti-mouse CD8 antibodies or PE-Cy5 conjugated anti-mouse B220 and PE conjugated anti-mouse CD11b antibodies (eBioscience). Data were collected from 30,000 cells by FACSCanto (BD Pharmingen) and analyzed by Flowjo (Tree Star, Inc).

2.4.9 Quantification of thymocytes
Total thymocytes were isolated from the thymus with HSC buffer. Red blood cells were lysed using ACK lysing buffer (Lonza). Total number of thymocytes was counted by Coulter counter (Beckman Coulter). One million thymocytes were stained with PE-Cy5 conjugated ant-mouse CD4, PE conjugated anti-mouse CD8, APC conjugated anti-mouse CD44, and FITC conjugated CD25 antibodies (eBioscience). Dead cells were excluded by staining with Calcein Blue AM (Life Technologies). Data were collected from 100,000 cells by FACSCanto (BD Pharmingen) and analyzed by Flowjo (Tree Star, Inc).

2.4.10 FACS analysis of cleaved caspase-3 positive cells

BM-MNCs and thymocytes were isolated following the same procedure described above. Cleaved caspase-3 staining was performed using PE Active Caspase-3 Apoptosis Kit (BD Pharmingen) according to the manufacturer’s instructions. Data were collected from 20,000 cells by FACSCanto (BD Pharmingen) and analyzed by Flowjo (Tree Star, Inc).

2.4.11 Quantitative RT-PCR

Total RNA was extracted from BM-MNCs using the TRIzol (Life Technologies) reagent (Ambion) following the manufacturer’s protocol. RNA yield and quantity were determined by measuring absorbencies at 260 nm and 280 nm using NanoDrop (Thermo Scientific). For detection of mRNA expression, 400 ng of total RNA was used for in vitro reverse transcription using iScript cDNA synthesis Kit (Bio-Rad). The expression level of p53 and Hprt1 mRNA was quantified by qRT-PCR with TaqMan probes (Applied Biosystems, Mm00441964_g1 for p53 and Mm00446968_m1 for Hprt1). Hprt1 was used
as an internal control to correct for the concentration of cDNA in different samples. Expression of miR-30-based p53.1224 shRNA was detected using a Custom TaqMan MicroRNA Assay (Applied Biosystems). A reverse transcription primer and a TaqMan probe were designed to specifically detect p53.1224 siRNA (80). *In vitro* reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. The expression level of p53.1224 siRNA and snoRNA 202 was quantified by qRT-PCR with TaqMan probes (Applied Biosystems). SnoRNA 202 was used as an internal control to correct for the concentration of cDNA in different samples

### 2.4.12 Statistics

Results are presented as mean ± SEM. Where variance was heterogeneous, data were log-transformed prior to applying statistical tests. Student’s *t*-test (two-tailed) was performed to compare statistical significance between 2 groups. For comparing the significance of the overall variance, two-way ANOVA or one-way ANOVA followed by Bonferroni post-test was used. For survival analysis, 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.
Figure 2.1: Expression of p53.1224 shRNA and p53 mRNA in the bone marrow in CMV-rTA; TRE-shp53 mice after Dox withdrawal.

Figure 2.1: CMV-rTA; TRE-shp53 mice were fed a Dox-containing diet for 10 days and then euthanized at different time points after being switched to a regular diet. Expression of (A) p53.1224 shRNA and (B) p53 mRNA in the bone marrow in mice without Dox treatment or at different time points after withdrawal of Dox. Data are presented as scatter plot with mean (n=2 mice per group). By one-way ANOVA, $P<0.001$ and $P=0.016$ for (A) and (B), respectively.
Figure 2.2: Knockdown of p53 suppresses radiation-induced apoptosis in the thymus and bone marrow.

Figure 2.2: The percentage of cleaved caspase 3 positive (Casp3*) thymocytes (A and B) and bone marrow mononuclear cells (BM-MNCs) (C and D) in Dox-treated CMV-rtTA; TRE-shp53 (CMV-rtTA), Actin-rtTA; TRE-shp53 (Actin-rtTA) mice (shp53) and littermate controls (Ctrl) containing rtTA or TRE-shp53 4 hrs after sham irradiation (sham) or 2.5 Gy TBI (2.5 Gy). *P<0.05; P<0.01; P<0.001 by two-way ANOVA. Data are presented as mean ± SEM (n = 3 mice for each group).
Figure 2.3: Temporary knockdown of p53 during TBI ameliorates acute hematopoietic toxicity.

Figure 2.3: (A) Representative FACS plots of Lineage (Lin) Sca1⁺ c-kit⁺ (LSK) cells in the bone marrow from Dox-treated CMV-rTA; TRE-shp53 (shp53) and littermate controls (Ctrl) containing either CMV-rTA or TRE-shp53 24 hrs after sham irradiation (sham) or 2.5 Gy TBI (2.5 Gy). FACS plots show cell population gated from Lin⁻ cells. (B and C) The frequency of LSK cells in the bone marrow of CMV-rTA; TRE-shp53 (CMV-rTA), Actin-rTA; TRE-shp53 (Actin-rTA) mice (shp53) and littermate controls (Ctrl) containing rTA or TRE-shp53 24 hrs after sham irradiation (sham) or 2.5 Gy TBI (2.5 Gy). *P<0.05 by two-way ANOVA. Data are presented as mean ± SEM (n = 5 mice). (D and E) Kaplan-Meier analysis of mice succumbed to the hematopoietic syndrome after 7.5 Gy TBI. P-value was calculated by log-rank test.
Figure 2.4: Temporary knockdown of p53 during fractionated TBI prevents lymphoma formation.

Figure 2.4: (A) Three to four-week-old CMV-rtTA; TRE-shp53 or Actin-rtTA; TRE-shp53 (shp53) mice and littermate controls containing rtTA or TRE-shp53 (Control) were fed a doxycycline (Dox)-containing diet for 10 days followed by exposure to 4 daily fractions...
of 1.8 Gy TBI. All mice were switched to a regular (Dox free) diet after the last dose of radiation. (B and C) Overall survival and (D and E) lymphoma-free survival were calculated by Kaplan-Meier analysis. $P$-value was calculated by log-rank test. (F) Summary of anatomical sites where lymphomas developed in irradiated shp53 and Control mice. (G) Representative images of lymphomas that developed in the thymus, spleen and liver stained with hematoxylin and eosin (H&E) or anti-mouse CD3 antibody (CD3). Scale bar, 100µm.
Figure 2.5: In the absence of Dox, Actin-rtTA; TRE-shp53 mice and littermate controls have similar latency of developing radiation-induced lymphoma.

Figure 2.5: (A) Three to four-week-old Actin-rtTA; TRE-shp53 (shp53) mice and littermate controls containing Actin-rtTA or TRE-shp53 (Control) were fed a regular diet for 10 days followed by exposure to 3 daily fractions of 1.8 Gy TBI. (B) Tumor-free survival and (C) lymphoma-free survival were calculated by Kaplan-Meier analysis. P-value was calculated by log-rank test.
Figure 2.6: Temporary knockdown of p53 during fractionated TBI prevents lymphoma formation in Kras<sup>LA1</sup> mice.

**Figure 2.6:** (A) Three to four-week-old Kras<sup>LA1</sup>; CMV-rtTA; TRE-shp53 or Kras<sup>LA1</sup>; Actin-rtTA; TRE-shp53 (shp53) mice and littermate controls harboring a Kras<sup>LA1</sup> allele alone or in combination with either rtTA or TRE-shp53 (control) were fed a doxycycline...
(Dox)-containing diet for 10 days followed by exposure to 4 daily fractions of 1.8 Gy TBI. All mice were switched to a regular (Dox free) diet after the last dose of radiation. (B and C) Overall survival and (D and E) lymphoma-free survival were calculated by Kaplan-Meier survival analysis. *P*-value was calculated by log-rank test. (F) The percentage of the lung (total lung area) that contains lung tumors (disease area) in *Kras<sup>LA1</sup>; CMV-rtTA; TRE-shp53* (shp53) mice and littermate controls harboring a *Kras<sup>LA1</sup>* allele (control) that were lymphoma-free. (G) The percentage of lung tumors that contain >50% of the area stained positive for phospho-p44/42 MAPK (p-Erk) in each mouse. Data are presented as scatter plot with mean (*n* = 7 and 12 for control and shp53 mice, respectively). *P*-value was calculated by Student’s *t*-test (two-tailed). *P* = 0.38 and 0.98 for results in (F) and (G), respectively.
Figure 2.7: Lung tumors in irradiated Kras^{LA1} mice stained with phospho-p44/42 MAPK

**Figure 2.7:** (A – D) Representative images of lung tumors contain 0% (no stain), <25%, 25% to 50%, or >50% of the area stained positive for phospho-p44/42 MAPK (p-Erk). Scale bar, 500 µm
Figure 2.8: Three to four-week-old CMV-rtTA; TRE-shp53 (shp53) mice and littermate controls containing CMV-rtTA or TRE-shp53 (control) were fed a doxycycline (Dox)-containing diet for 10 days followed by exposure to 4 daily fractions of 1.8 Gy TBI. All mice were switched to a regular (Dox free) diet after the last dose of radiation. Total number of (A) CD4+CD8−, (B) CD4+CD8+, (C) CD4 CD8+ and (D) CD4 CD8− thymocytes in shp53 and control at different time points after 1.8 x 4 TBI were analysed by FACS. *P<0.05; **P<0.01; ***P<0.001 by two-way ANOVA with Bonferroni correction. Data are presented as mean ± SEM (n=3 mice for each group)
Figure 2.9: Temporary knockdown of p53 during fractionated TBI suppresses cell death and accelerated repopulation of HSPCs.

Figure 2.9: Three to four-week-old CMV-rtTA; TRE-shp53 (shp53) mice and littermate controls containing CMV-rtTA or TRE-shp53 (control) were fed a doxycycline (Dox)-containing diet for 10 days followed by exposure to 1.8 Gy TBI. All mice were switched to a regular (Dox free) diet after the last dose of radiation. (A) Total number of bone marrow mononuclear cells (BM-MNCs) in one femur and one tibia. The frequencies of (B) oligopotent progenitors (OPPs, Lin⁻ Sca1⁺ cKit⁺), (C) LSK cells (Lin⁻ Sca1⁺ cKit⁺), and (D) LSK CD48⁻ multipotent progenitors and hematopoietic stem cells (MPPs/ HSCs) in the bone marrow. *P<0.05; **P<0.01; ***P<0.001 by two-way ANOVA
with Bonferroni correction. Data are presented as mean ± SEM (n=4 mice for each group). (E) The percentage of Ki67+ and (F) BrdU+ MPPs/HSCs in shp53 and control mice 12 days after 1.8 Gy x 4 TBI or sham irradiation. *P<0.05; **P<0.01; by two-way ANOVA with Bonferroni correction. Data are presented as mean ± SEM (n=5 mice for each group).
Figure 2.10: Knockdown of p53 maintains cell quiescence in irradiated MPPs/HSCs in vivo.

**Figure 2.10:** Cell cycle analyses of MPPs/HSCs in Dox treated CMV-rtTA; TRE-shp53 (shp53) and littermate controls containing CMV-rtTA or TRE-shp53 (Ctrl) 24 hrs after sham irradiation (sham) or 1.8 Gy TBI (1.8 Gy). (A) Representative FACS plot of the cell cycle of MPPs/HSCs (LSK CD48-) in Dox-treated shp53 and control mice 24 hrs after 1.8 Gy TBI. MPPs/HSCs at different stages of the cell cycle were distinguished by staining with Ki67 and Hoechst 33324. (B) The percentage of MPPs/HSCs in G0 (Ki67 negative) in shp53 and Control mice 24 hrs after sham irradiation (sham) or 1.8 Gy TBI (1.8 Gy) *$P<0.05$ by two-way ANOVA. Data are presented as mean ± SEM (n=5 mice for each group). (C) The percentage of MPPs/HSCs at each stage of the cell cycle in shp53 and control mice 24 hrs after 1.8 Gy TBI. *$P<0.05$; ***$P<0.001$ by Student’s t-test (two-tailed). Data are presented as mean ± SEM (n=5 mice for each group). (D) The percentage of BrdU+ MPPs/HSCs in shp53 and control mice 24 hrs after 1.8 Gy TBI. ***$P<0.001$ by Student’s t-test (two-tailed). Data are presented as mean ± SEM (n=5 mice for each group).
Figure 2.11: Bone marrow reconstitution in chimeric mice 8 weeks after bone marrow transplant.

Figure 2.11: Eight-week-old lethally irradiated C57BL/6 mice were transplanted with mixed LSK cells (95% from either CMV-rTA; TRE-shp53 or littermate control containing rtTA or TRE-shp53; 5% from UBC-GFP; p53+/- mice). (a – e) Eight weeks after bone marrow transplant, the percentage of GFP⁺, CD4⁺, CD8⁺, B220⁺, and CD11b⁺ peripheral blood mononuclear cells (PB-MNCs) were quantified by FACS. Data are presented as scatter plot with mean (n = 11 and 13 for shp53 and control mice, respectively). P>0.05 by Student’s t-test (two-tailed) between all comparison groups.
**Figure 2.12**: Temporary knockdown of p53 during fractionated irradiation suppresses expansion of p53+/- HSPCs.

**Figure 2.12**: (A) Eight-week-old lethally irradiated C57BL/6 mice were transplanted with mixed LSK cells (95% from either CMV-rTA; TRE-shp53 or littermate control containing rtTA or TRE-shp53; 5% from UBC-GFP; p53+/- mice). Eight weeks after bone marrow transplant, the percentage of GFP⁺ peripheral blood mononuclear cells (PB-MNCs) were quantified by FACS. All mice were subsequently fed a Dox-containing diet (Dox) for 10 days followed by exposure to 3 daily fractions of 1.8 Gy TBI and were switched to a regular (Dox free) diet after the last dose of radiation. The percentage of GFP⁺ PB-MNCs...
was assessed prior to and 10 weeks after 1.8 Gy x 3. (B-E) Repeated measurement of GFP+ cells in CD4+, CD8+, B220+, and CD11b+ PB-MNCs were performed prior to (Pre-IR) and 10 weeks after (10 wks) TBI. Results were presented as fold change in the percentage of GFP+ cells. *P<0.05 *P<0.01 by Student’s t-test (two-tailed). Data are presented as scatter plot with mean (n = 11 and 13 for shp53 and control mice, respectively).
Chapter 3: p53 functions in endothelial cells to prevent radiation-induced myocardial injury

3.1 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 (81, 82). In the heart, p53 functions to promote cardiac injury from pressure overload (29), ischemic injury (30), telomere attrition (31), and doxorubicin-induced oxidative stress (32-34). Therefore, blocking p53 with 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 (83). In a meta-analysis from several randomized trials of women with breast cancer, mortality from heart disease was increased significantly for women who were randomized to receive adjuvant radiation therapy (84). 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 (85). Interestingly, a prospective study of left-sided breast cancer patients was 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 were found to have perfusion defects within the part of the left ventricle that received
high dose irradiation (86). Remarkably, these perfusion defects persisted on follow-up cardiac SPECT scans 3 to 8 years after radiation therapy (87). 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 (35-38). For example, Fajardo and Stewart studied the pathogenesis of radiation-induced myocardial fibrosis in rabbits exposed to a single dose of 20 Gy (35, 36). In these elegant studies, focal areas of myocardial fibrosis were observed by two months after irradiation (36). From day 20 through 49 after irradiation, significant damage to endothelial cells within the myocardium was seen including decreased microvessel density (35). 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 (37).

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, while focal areas of myocardial necrosis were noted at two months (38). Although it has been established that microvascular loss precedes myocardial necrosis in radiation-induced heart disease, the molecular mechanisms controlling the loss of the myocardial capillaries remain to be fully defined (39-41).
Radiation induces p53 in the heart in vivo (42) and in endothelial cells from various sources in vitro (43-45). 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, has been shown to protect human umbilical vein endothelial cells (HUVECs) from radiation-induced cell death by apparently blocking p53 (43). This finding is further supported by the observation that inhibition of p53 by pifithrin, a small-molecule inhibitor of p53, increases the viability of HUVECs after irradiation (43). Moreover, blocking p53 has been suggested to suppress radiation-induced damage to human dermal microvascular endothelial cells and mouse endothelial progenitor cells (44, 45). In contrast, others have reported that when p53 is inhibited or deleted in tumor-associated endothelial cells, xenograft tumors are sensitized to radiation (46), 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 (CECs) may reveal the molecular mechanism of radiation induced cardiac toxicity. Moreover, understanding whether p53 plays a pro-death or pro-survival role in irradiated CECs 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 CECs to regulate radiation response of the heart in vivo, I utilized Tie2Cre and VE-Cadherin-Cre mice to delete p53 in endothelial cells. Remarkably, deleting p53 in endothelial cells with either Tie2Cre or
VE-Cadherin-Cre causes mice to be sensitized to myocardial injury after 12 Gy whole-heart irradiation (WHI). The onset of cardiac injury is preceded by increased endothelial cell death, increased vascular permeability and decreased microvessel density in the myocardium. In addition, cell-based studies using primary CECs from these mice show that p53-mediated cell cycle arrest protects CECs 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 12 Gy WHI. Taken together, my results demonstrate that p53 functions in endothelial cells \textit{in vivo} to prevent radiation-induced myocardial injury.

\section*{3.2 Results}

\subsection*{3.2.1 Deletion of p53 in endothelial cells by Tie2Cre sensitizes mice to radiation-induced myocardial necrosis.}

To investigate the role of p53 in endothelial cells in regulating radiation-induced injury to the heart, I utilized Tie2Cre mice to delete p53 in endothelial cells (5). Because Tie2Cre has been reported to recombine genes in some cardiomyocytes in addition to endothelial cells (88), I examined the cell types that express Cre recombinase in the heart by crossing Tie2Cre mice to membrane-Tomato/ membrane-GFP (mTmG) reporter mice (89). In \textit{Tie2Cre; mTmG/+} mice, cells expressing Cre are labeled by enhanced green fluorescent protein (EGFP), whereas cells that do not express Cre are labeled by tdTomato protein. Figure 3.1 shows that in \textit{Tie2Cre; mTmG/+} mice, cells expressing EGFP in the myocardium are labeled by \textit{Griffonia simplicifolia} isolectin B4 (GS-IB4) which marks
endothelial cells (90). The pattern of Cre expression is similar in Tie2Cre mice regardless of the presence (Tie2Cre; p53FL/; mTmG/+; mTmG/+ of p53 in endothelial cells (Figure 3.1). The results indicate that, in these Tie2Cre mice, Cre is expressed specifically in endothelial cells and not in cardiomyocytes. To study radiation-induced myocardial injury, Tie2Cre; p53FL/+ and Tie2Cre; p53FL/+ mice were treated with a single dose of 12 Gy whole-heart irradiation (WHI). Focal irradiation to the heart was performed using a micro-CT/ micro-iradiator so that the radiation field included the heart and a margin of normal lung tissue (Figure 3.2A). As shown in Figure 3.2B, Tie2Cre; p53FL/+ mice did not show any signs of morbidity after at least 210 days following WHI, whereas all Tie2Cre; p53FL/+ mice succumbed to late effects of radiation between 39 to 69 days post-WHI, with the median survival of 55 days. In addition, Figure 3.2C shows that Tie2Cre; p53FL/+ mice treated with 10 daily fractions of 3 Gy (3Gy x 10) WHI also succumbed to late effects of radiation, with the median survival of 34 days after the last treatment. Histopathological examination was performed for the lungs and heart in moribund Tie2Cre; p53FL/+ mice. While 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 (Figure 3.3C). Myocardial degeneration and necrosis was characterized by loss of cross striations, indistinct cytoplasmic borders and nuclei, hemorrhage, and decreased number of cardiomyocytes. Infiltration of neutrophils and macrophages was also present within necrotic foci. As shown in Masson’s trichrome-stained sections, small amounts of fibrous connective tissue replacing the necrotic cardiomyocytes indicates that the lesion was in
acute process (Figure 3.3C). Collectively, my results suggest that deletion of p53 in endothelial cells by Tie2Cre sensitizes mice to radiation-induced myocardial necrosis.

3.2.2 Tie2Cre; p53\(^{FL/+}\) mice develop systolic dysfunction and cardiac hypertrophy following 12 Gy WHI.

To assess the effect of 12 Gy WHI on cardiac function in Tie2Cre; p53\(^{FL/+}\) and Tie2Cre; p53\(^{FL/-}\) mice, I performed serial echocardiography prior to and 3, 5, and 7 weeks after irradiation. Seven weeks after 12 Gy WHI, cardiac function of Tie2Cre; p53\(^{FL/+}\) 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 (mVcfc), and increased left ventricular end-systolic dimension (LVDs) (Figure 3.3, A – D). Echocardiography also showed significantly increased mass of the left ventricle in Tie2Cre; p53\(^{FL/+}\) mice (Figure 3.4A). Cardiac hypertrophy in Tie2Cre; p53\(^{FL/-}\) mice was confirmed at necropsy by a markedly increased heart weight to body weight (HW/BW) ratio (Figure 3.4B). In addition, sections of the myocardium stained with Wheat Germ Agglutinin (WGA) demonstrated that the averaged cardiomyocyte cross-sectional area was significantly increased in Tie2Cre; p53\(^{FL/-}\) mice 7 weeks after 12 Gy WHI (Figure 3.4, C and D). The results from the echocardiography and histology studies indicate that radiation-induced myocardial injury is the primary cause of morbidity in Tie2Cre; p53\(^{FL/-}\) mice after 12 Gy WHI.
3.2.3 Radiation-induced myocardial injury is preceded by vascular damage in Tie2Cre; p53^{FL/-} mice.

Because deletion of p53 in endothelial cells sensitized mice to radiation-induced myocardial injury, I hypothesized that myocardial injury in Tie2Cre; p53^{FL/-} mice results from the destruction of p53-deficient myocardial capillaries. Consistent with this hypothesis, histological examination of the myocardium in Tie2Cre; p53^{FL/-} mice 7 weeks after 12 Gy WHI revealed aberrant myocardial capillary morphology characterized by dilated and disorganized vessels (Figure 3.5A) and significantly decreased microvessel density across the myocardium (Figure 3.5B). 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 WHI demonstrated tdTomato+ cardiomyocytes surrounded by EGFP+ microvessels in the myocardium, which is similar to the histology of the myocardium in mice without irradiation (Figure 3.5C; Figure 3.1). 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 markedly disorganized EGFP+ microvessels. Remarkably, adjacent to these necrotic areas, EGFP+ microvessels were dilated and disorganized even though tdTomato+ cardiomyocytes remained histologically intact (Figure 3.5C). These results suggest that destruction of the microvasculature leads to myocardial injury in Tie2Cre; p53^{FL/-} mice after 12 Gy WHI.
To further investigate if the onset of cardiac dysfunction is preceded by damage to blood vessels in the myocardium, I assessed the density and permeability of myocardial capillaries in Tie2Cre; p53FL/+ and Tie2Cre; p53FL−− mice 3, 4 and 5 weeks after 12 Gy WHI. During these time points, cardiac function of both mouse genotypes remained normal (Figure 3.3). However, focal areas of myocardial degeneration and necrosis were observed in the myocardium in Tie2Cre; p53FL−− mice as early as 4 weeks after 12 Gy WHI (Figure 3.6, A and B). Necrotic regions contained cardiomyocytes and endothelial cells with abnormal morphology as indicated by staining with WGA and GS-IB4 (Figure 3.6, C – E). In addition, microvessel density was significantly decreased within necrotic foci (Figure 3.6, F and G). Moreover, TUNEL stained myocardium had significantly increased dead endothelial cell in the necrotic regions of Tie2Cre; p53FL−− mice 4 weeks after 12 Gy WHI. Remarkably, TUNEL positive endothelial cells were also present in regions that appeared histologically normal (Figure 3.7). To investigate functional alterations of myocardial capillaries in vivo, I assessed the permeability of the myocardial microvasculature with intravenous injection of low and high molecular weight (MW) dextrans (1 x 10⁴ and 2 x 10⁶ MW, respectively) into Tie2Cre; p53FL−− and Tie2Cre; p53FL−− mice 4 weeks after 12 Gy WHI. In Tie2Cre; p53FL−− mice, dextrans of both MWs were found to be restricted within myocardial capillaries. However, in Tie2Cre; p53FL−− mice focal regions of the myocardium showed increased vascular permeability characterized by leakage of the low MW dextran from myocardial capillaries (Figure 3.8). Taken together, these results further suggest that damage to the myocardial capillaries
in Tie2Cre; p53FL/− mice after 12 Gy WHI precedes multifocal myocardial necrosis and cardiac dysfunction.

### 3.2.4 Deletion of p53 in endothelial cells by VE-Cadherin-Cre sensitizes mice to radiation-induced myocardial necrosis.

Since recombination by Tie2Cre occurs in the majority of hematopoietic cells (5, 91, 92), 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; p53FL/− mice is specifically due to loss of p53 in endothelial cells, I utilized VE-Cadherin-Cre (VECre) mice to delete p53 in endothelial cells. Similar to reports by others (93, 94), I observed that recombination in VECre mice occurs in a small subset (10 to 15%) of hematopoietic cells (Figure 3.9), which is much lower than Tie2Cre mice (approximately 84%) (92). I examined the types of cells expressing Cre in the myocardium in VECre; p53FL/−; mTmG/+ and VECre; p53FL/−; mTmG/+ mice. Tissue sections from these reporter mice show that Cre is expressed specifically in endothelial cells in the myocardium. Remarkably, in contrast to Tie2Cre mice, where Cre is expressed uniformly in endothelial cells throughout the myocardium, in VECre mice, Cre is predominantly expressed in endothelial cells in the epimyocardium and midmyocardium (Figure 3.10). Having characterized Cre expression in VECre mice, I next investigated if deletion of p53 by VECre sensitizes mice to myocardial injury after exposure to a single dose of 12 Gy WHI. As shown in Figure 3.11A, I found that VECre; p53FL/− mice succumbed to late effects of radiation after 12 Gy WHI, with the median
survival of 52 days. Histopathological changes in the myocardium in VECre; p53FL/ mice were similar to the radiation-induced myocardial necrosis observed in Tie2Cre; p53FL/ mice (Figure 3.11B). Furthermore, heart sections from VECre; p53FL/; mTmG/+ mice 7 weeks after 12 Gy WHI showed focal areas of myocardial necrosis characterized by markedly damaged EGFP+ microvessels and loss of tdTomato+ cardiomyocytes (Figure 3.11C). In contrast to the Tie2Cre; p53FL/ mice that developed myocardial injury after 12 Gy WHI throughout the myocardium, most necrotic lesions in VECre; p53FL/ mice were located in the epimyocardium and midmyocardium within the distribution of Cre expression in the CECs (Figure 3.10). Collectively, these results demonstrate that p53 functions in endothelial cells in vivo to prevent radiation-induced myocardial injury.

3.2.5 Impaired p53-mediated cell cycle arrest sensitizes CECs to radiation-induced mitotic death.

To determine if the susceptibility of radiation-induced endothelial cell damage in Tie2Cre; p53FL/ mice is cell-autonomous, I assessed the viability of endothelial cells in vitro using primary CECs isolated from the myocardium. CECs cultured in vitro displayed endothelial cell-like cobble stone morphology (95) and expressed surface markers of endothelial cells, CD31 and CD105 (Figure 3.12, A and B). Quantification of p53 mRNA levels in CECs demonstrated that p53 mRNA expression was lost in CECs from Tie2Cre; p53FL/ mice, whereas the p53 mRNA level in CECs from Tie2Cre; p53FL/+ mice was approximately 50% of the level of wild type mice (Figure 3.12C). To investigate the role of p53 in mediating CEC cell death from radiation, I irradiated CECs from
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 hrs post-irradiation, and post-mitotic cell death occurs 24-96 hrs post-irradiation (96, 97). I did not observe a significant increase in CEC cell death 4 hrs post-irradiation in CECs with or without p53, but cell death was significantly increased in p53-deficient CECs 96 hrs post-irradiation in a dose-dependent manner (Figure 3.13, A and B). In addition, a significantly higher percentage of p53-deficient CECs underwent apoptosis, as shown by Annexin V and cleaved caspase 3 staining, 48 and 72 hrs after 12 Gy, while no significant increase in apoptosis was detected in CECs 4 hrs after 12 Gy regardless of the presence or absence of p53 (Figure 3.13, C, D, and E). Collectively, these results show that p53 protects CECs from post-mitotic cell death 48 to 96 hrs after high dose irradiation in vitro.

It has been shown that 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 (98, 99). Thus, I hypothesized that p53 protects CECs from radiation by preventing mitotic catastrophe secondary to premature entry into mitosis. I first examined if loss of p53 alters mitotic arrest in CECs after irradiation by quantifying the percentage of cells expressing phospho-histone H3 (pHH3). Figure 3.13F shows that Tie2Cre; p53FL/CECs had a significantly higher mitotic index 8, 24, 48 and 72 hrs after 12 Gy compared to Tie2Cre; p53FL/CECs, indicating that p53-deficient CECs have impaired cell cycle arrest after irradiation. To determine if premature entry into mitosis leads to mitotic catastrophe, I
quantified the formation of micronuclei, which occurs as a result of incorrect chromosomal segregation during mitosis (98, 99). Quantitative results revealed that CECs from Tie2Cre; p53<sup>FL/−</sup> mice contained a significantly higher percentage of cells with micronuclei compared to CECs from Tie2Cre; p53<sup>FL/+</sup> mice 24 to 72 hrs after 12 Gy. Remarkably, most cells with micronuclei also showed intense staining of γ-H2AX foci, indicating that these cells contained unrepaired DNA damage during mitosis (Figure 3.13, G and H). Taken together, these results indicate that p53-deficient CECs show a higher mitotic index and corresponding induction of mitotic death 24 to 72 hrs after radiation, supporting 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 (100). Thus, I hypothesized that p53 controls survival of CECs via a p21-dependent mechanism. I first quantified mRNA expression of p21 and PUMA, a downstream target involved in p53-dependent apoptosis (101), in CECs isolated from wild type, Tie2Cre; p53<sup>FL/+</sup>, Tie2Cre; p53<sup>FL/−</sup> and p53<sup>−/−</sup> mice. My results show that p21, but not PUMA, mRNA was significantly induced after 12 Gy in CECs containing at least one allele of p53. However, p21 mRNA was minimal at baseline in either Tie2Cre; p53<sup>FL/−</sup> or p53<sup>−/−</sup> CECs and was not induced by radiation (Figure 3.14, A and B). These results suggest that radiation selectively induces p53-mediated pro-survival pathways in CECs in vitro. Subsequently, I examined the susceptibility of primary CECs from p21<sup>−/−</sup> and p21<sup>−/−</sup> mice to radiation 4 and 72 hrs after 12 Gy in vitro. While cell death was not significantly induced in both types of CECs 4 hrs after 12 Gy, p21-deficient
CECs were more susceptible to radiation-induced post-mitotic cell death 72 hrs after 12 Gy (Figure 3.14, C and D). Collectively, these results indicate that the mechanism by which p53 protects CECs against radiation is at least partially via p21.

### 3.2.6 p21-deficient mice are susceptible to radiation-induced myocardial injury.

To investigate if the loss of p21 potentiates radiation-induced myocardial injury *in vivo*, *p21*+/− and *p21*−/− mice were exposed to 12 Gy WHI and followed for the development of cardiac injury. As shown in Figure 3.15A, all *p21*−/− mice were found moribund 33 to 37 days after 12 Gy WHI. Histopathological examination indicated that *p21*−/− mice developed acute to sub-acute myocardial degeneration and necrosis (Figure 3.15B), which is similar to what was observed in *Tie2Cre; p53*FL/− mice after 12 Gy WHI.

Echocardiography 5 weeks after 12 Gy WHI revealed that *p21*−/− mice developed systolic dysfunction with significantly decreased fractional shortening and decreased mVcfc (Figure 3.15, C and D). In addition, *p21*−/− mice developed cardiac hypertrophy as early as 4 weeks after 12 Gy WHI indicated by an increased left ventricular mass, HW/BW ratio, and the averaged cardiomyocyte cross-sectional area (Figure 3.16, A – D). Furthermore, assessment of microvessel density in the myocardium 4 weeks after 12 Gy WHI showed that the density of myocardial capillaries was markedly decreased in *p21*−/− mice (Figure 3.17, A and B). Remarkably, this global decrease of microvessel density in the myocardium was preceded by focal increased vascular permeability (Figure 3.17C). Collectively, my results indicate that *p21*−/− mice phenocopy the sensitivity of *Tie2Cre;
p53fl/− mice to radiation-induced myocardial injury because after 12 Gy WHI, p21−/− mice developed (1) myocardial degeneration and necrosis, (2) systolic dysfunction and (3) a reduction in microvessel density and increased vascular permeability prior to the onset of cardiac dysfunction. Taken together, my results demonstrate that p53 functions in endothelial cells to prevent radiation-induced myocardial injury through a mechanism at least partially regulated by p21.

3.3 Discussion

In the heart, p53 promotes cardiac injury from pressure overload (29), ischemic injury (30), telomere attrition (31), and doxorubicin treatment (32-34). In contrast, in the present study, I 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 (83), my results suggest that combining radiation therapy with inhibitors of p53 (102) or other components of the DNA damage response that regulate mitotic arrest (103) may increase the risk of cardiac injury if the heart is in the field of radiation.

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 (35, 37). Despite these elegant experiments, the role of the vasculature in mediating late effects from radiation has been controversial (104) 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 p53 deleted
in endothelial cells are sensitized to radiation-induced cardiac injury \textit{in vivo} (Figures 3.2 and 3.11), I provide compelling genetic evidence that endothelial cells play a critical role in regulating radiation-induced injury to the heart. This system may also be useful for investigating the role of the vasculature in mediating other late effects of radiation.

My 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 an inhibitor to p53 (43), I find that CECs do not undergo p53-dependent apoptosis 4 hours after radiation up to doses of 24 Gy. In contrast, CECs undergo a delayed mitotic death following irradiation, which is potentiated by deletion of p53 (Figure 3.13). This conclusion is consistent with a report that blocking p53 in tumor-associated endothelial cells can sensitize xenograft tumors to radiation (46). Further supporting a model where p53 functions in CECs after irradiation to activate cell cycle checkpoints to prevent mitotic death and subsequent myocardial injury, I observed that mice lacking the p53 target gene p21 are also sensitized to radiation-induced cardiac injury (Figure 3.15). Because expression of the cyclin dependent kinase inhibitor p21 in CECs requires p53 (Figure 3.14), my results indicate that a mechanism by which CECs survive radiation is via the p53-p21 cell cycle arrest pathway.

My results do not exclude that cell types other than CECs play a role in radiation-induced cardiac injury. After 12 Gy WHI, both \textit{Tie2Cre; p53\textsuperscript{fl/fl} and p21\textsuperscript{−/−}} 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. Recent studies have shown that p21 plays an important role in regulating the turnover of mature endothelial cells and endothelial progenitor cells. Impaired neovascularization was observed in p21−/− mice despite increased proliferation of mature endothelial cells and endothelial progenitor cells (105). In addition, it has been shown that p53 and p21 negatively regulate SDF-1/CXCR4 signaling, which controls the inflammatory response (106-108). 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 vascular SDF-1 levels (108). 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 Figures 3.2B and 3.11A with Figure 3.15A) 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 (WHI) was utilized with a micro-CT/micro-irradiator to deliver focal irradiation to the heart (Figure 3.2A). 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, I performed serial echocardiography on mice before and after WHI and documented systolic dysfunction and cardiac hypertrophy in mice lacking p53 in the
endothelial cells or in p21−/− mice (Figures 3.3, 3.4, 3.15 and 3.16). 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, I 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 (Figure 3.2) or VECre (Figure 3.11), mice were sensitized to radiation-induced myocardial injury from WHI. Although multifocal myocardial necrosis was present throughout the myocardium of Tie2Cre; p53FL/− mice (Figure 3.2C), myocardial necrosis in VECre; p53FL/− mice was prominent in the epicardium and midmyocardium (Figure 3.11B). Because the VECre mice did not have significant necrosis in the endomyocardium after WHI in a region where the endothelial cells have poor expression of Cre recombinase (Figure 3.10), 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 WHI.

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 (81, 82). The diverse cell-type specific outcome of p53 activation after irradiation may be influenced by whether p53 engages the intrinsic pathway of apoptosis. For example, genetic or pharmacological inhibition of p53 significantly ameliorates the hematopoietic syndrome after total-body irradiation by protecting hematopoietic progenitors against apoptosis (18, 49). In contrast, I recently reported that p53 functions in the GI epithelium to protect
mice from the radiation-induced GI syndrome at least partially through p21, presumably by preventing intestinal stem cells from undergoing mitotic death (5, 109). Others have also found that p53 and p21 protect mice against the GI syndrome (18, 110). These findings together with the results in the present study indicate that p53 may generally play a protective role from radiation, particularly at high doses, in cells where p53 activation is uncoupled from the induction of the intrinsic pathway of apoptosis.

In summary, I 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 (103), and radioprotectors, such as p53 inhibitors (102). My 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.

3.4 Materials and Methods

3.4.1 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 and mTmG mice (5, 89, 93). 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 and mTmG
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 lines of female Tie2Cre mice (111), Tie2Cre; p53\(^{+/−}\) males were crossed to p53\(^{T/TL/FL}\) females to avoid non-specific deletion of floxed alleles in the germ line.

3.4.2 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 (112). The heart was localized to isocenter (source to subject distance is approximately 30.76 cm) via 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.

3.4.3 Echocardiography.

Serial transthoracic echocardiography was performed on conscious mice for all groups with a Vevo 2100 high-resolution image system (VisualSonics) as previously described (113).

3.4.4 Histological analysis.

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 hrs 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. 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 µg/mL) and Alexa fluor 647-conjugated GS-IB4 (10 µg/mL) (Invitrogen) 2 hrs 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 manufacturers’ instructions. Sections were counterstained with Alexa fluor 488-conjugated WGA (5 µg/mL) and Alexa fluor 647-conjugated GS-IB4 (10 µg/mL) (Invitrogen) overnight at 4°C. Six images (200x) per slide were randomly taken from the myocardium with similar cross sections in all mice except Tie2Cre; p53^{FL/FL} mice 4 weeks after 12 Gy. In these mice, 6 fields (200x) were chosen from representative non-necrotic regions, and 3 to 6 fields (200x) 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. Results are presented as mean ± SEM from 3 mice.

3.4.5 Vascular permeability assay.

Mice were injected intravenously with a mixture of TMR-conjugated high MW (2 x 10^6 MW, 1 mg/ml) and Alexa fluor 647-conjugated low MW (1 x 10^4 MW, 0.4 mg/ml) dextrans (Invitrogen). Mice were euthanized 1.5 hrs later. Ten µm sections were cut from frozen tissues and stained with rat anti-mouse CD31 IgG (BD Pharmigen) 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.

### 3.4.6 Primary culture of cardiac endothelial cells.

Isolation of CECs was performed as described previously (95) with slight modifications. Primary CECs 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. CECs were captured using rat IgG magnetic beads (Invitrogen) coated with rat anti-mouse CD31 IgG (BD Pharmigen). After the initial purification, CECs were cultured in DMEM supplemented with 25 mM HEPES, 20% FBS, penicillin/ streptomycin, 2 mM L-glutamine, 100 µg/ml heparin, nonessential amino acid (1X), sodium pyruvate (1X) and 100 µg/ml endothelial cell growth stimulant (Biomedical Technologies) on cell culture dishes coated with 0.1% of gelatin (Sigma). CECs were grown to 70% to 80% confluency followed by a second isolation using magnetic beads coated with rat anti-mouse CD102 IgG (BD Pharmigen) to further improve the purity. CECs were used for experiments from passage 1 to 4 after the secondary purification (total passage 3 to 6). The purity of CECs was assessed by flow cytometry using FITC-conjugated anti-mouse CD31 and PE-conjugated anti-mouse CD105 antibodies (eBioscience).
3.4.7 Quantification of cell death by flow cytometry.

CECs were irradiated with 12 Gy 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 µg/ml, Sigma-Aldrich) alone or with FITC Annexin V (BD Pharmingen) and propidium iodide according to manufacturer’s instructions. Data were collected by FACScan (BD Pharmingen) and analyzed by Flowjo (Tree Star, Inc).

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

CECs were irradiated with 12 Gy 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 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 hr at room temperature followed by staining with Alexa fluor 488-conjugated anti-rabbit IgG (1:1000) (Invitrogen) at room temperature for 1 hr. 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).

3.4.9 Quantification of micronuclei.

CECs were irradiated with 12 Gy 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 X-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 hr 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.

3.4.10 Quantitative RT-PCR.

Total RNA was extracted from the CECs 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 level of p53, p21 and β2-microglobulin (B2m) mRNA was quantified by qRT-PCR with Taqman probes (Applied Biosystems, Mm01731290_g1 for p53, Mm00432448_m1 for p21, and Mm00437762_m1 for B2m). B2m was used as an internal control to correct for the concentration of cDNA in different samples.
3.4.11 Statistics.

Results are presented as mean ± SEM. Student’s t-test was performed to compare statistical significance between 2 groups. For comparing the significance of the overall variance, two-way ANOVA followed by Bonferroni correction or one-way ANOVA followed by Tukey’s test was used. For survival analysis, 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.

3.4.12 Microscopy.

Pictures were taken using a Leica DFC 340FX fluorescent microscope (Leica Microsystems). Images were captured by Leica suite software (Leica Microsystems).
Figure 3.1: In Tie2Cre mice, Cre is expressed specifically in endothelial cells compared to cardiomyocytes.

**Figure 3.1:** In Tie2Cre mice, Cre is expressed specifically in endothelial cells compared to cardiomyocytes. Representative sections of the myocardium from (A) Tie2Cre; p53<sup>FL/+</sup>; mTmG/+ and (B) Tie2Cre; p53<sup>FL−/−</sup>; mTmG/+ mice without irradiation. Cells expressing Cre are labeled by enhanced EGFP (green), whereas cells that do not express Cre are labeled by tdTomato protein (red). Endothelial cells are labeled by Alexa-fluor 647-conjugated GS-IB<sub>4</sub> (blue). All EGFP expressing cells are stained by GS-IB<sub>4</sub>. Scale bar, 100 μm. Images represent three mice per genotype.
Figure 3.2: Deletion of p53 by Tie2Cre sensitizes mice to radiation-induced myocardial injury.

Figure 3.2: Deletion of p53 by Tie2Cre sensitizes mice to radiation-induced myocardial injury. (A) Representative fluoroscopy image of a mouse treated with whole-heart irradiation (WHI). A 15 mm circular collimator (red circle) was used for irradiation. Kaplan-Meier survival analysis of Tie2Cre; p53FL/FL and Tie2Cre; p53FL/− mice after (B) 12 Gy and (C) 3 Gy x 10 WHI. (D) Representative hematoxylin and eosin-stained (H&E) and Masson’s trichrome-stained (Trichrome) sections of the heart from Tie2Cre; p53FL/− mice 62 days after 12 Gy WHI. Yellow arrows indicate necrotic cardiomyocytes replaced by blue collagen fibers. Scale bar, 100 µm.
**Figure 3.3:** Radiation causes impaired systolic function in Tie2Cre; *p53*^FL/−^ mice.

**Figure 3.3:** (A) Representative echocardiographic recordings are shown from Tie2Cre; *p53*^FL/+^ and Tie2Cre; *p53*^FL/−^ mice prior to and 7 weeks after 12 Gy WHI. (B-D) Changes in fractional shortening, heart rate corrected mean velocity of circumferential fiber shortening (mVcfc) and left ventricular end-systolic dimension (LVDs) in Tie2Cre; *p53*^FL/+^ and Tie2Cre; *p53*^FL/−^ mice after 12 Gy WHI. Fractional shortening at 50% is defined as a threshold of normal cardiac function. **P<0.01; ***P<0.001 by two-way ANOVA with Bonferroni correction. Data are presented as mean ± SEM (n=6 mice per genotype).
Figure 3.4: *Tie2Cre; p53<sup>FL/+</sup>* mice develop cardiac hypertrophy 7 weeks after 12 Gy WHI.

**Figure 3.4:** (A) Changes in the mass of the left ventricle in *Tie2Cre; p53<sup>FL/+</sup>* and *Tie2Cre; p53<sup>FL/-</sup>* mice after 12 Gy WHI were assessed by echocardiography. ***P<0.001 by two-way ANOVA with Bonferroni correction. Data are presented as mean ± SEM (n=6 mice per genotype). (B) Ratio of heart weight to body weight (HW/BW) of *Tie2Cre; p53<sup>FL/+</sup>* and *Tie2Cre; p53<sup>FL/-</sup>* mice sham irradiated or 7 weeks after 12 Gy WHI. ***P<0.001 by two-way ANOVA. Data are presented as mean ± SEM (n=3 mice per group). (C) Representative images of WGA-stained (green) cell membrane in the myocardium in *Tie2Cre; p53<sup>FL/+</sup>* and *Tie2Cre; p53<sup>FL/-</sup>* mice sham irradiated or 7 weeks after 12 Gy WHI. Scale bar, 100 µm. (D) Quantification of cardiomyocyte cross-sectional area in WGA-stained myocardium. *P<0.05 by two-way ANOVA. Data are presented as mean ± SEM (n=3 mice per group).
Figure 3.5: Decreased microvessel density in Tie2Cre; p53FL/ mice 7 weeks after 12 Gy WHI.

Figure 3.5: (A) Representative sections of the myocardium stained with Hoechst 33324 (blue) and GS-IB4 (red). (B) Quantification of GS-IB4 positive capillaries per 200x field in the myocardium in Tie2Cre; p53FL/+ and Tie2Cre; p53FL/- mice. **P<0.01 by two-way ANOVA. Data are presented as mean ± SEM (n=3 mice per group). (C) Representative sections of the myocardium from Tie2Cre; p53FL/; mTmG/+ and Tie2Cre; p53FL/; mTmG/+ mice 7 weeks after 12 Gy WHI stained with GS-IB4 (blue). An area of necrosis is marked by the arrow. Scale bar, 100 µm. Images represent three mice per genotype.
Figure 3.6: Focal loss of myocardial capillaries in Tie2Cre; p53FL/− mice 4 weeks after 12 Gy WHI.

**Figure 3.6:** (A-B) Representative hematoxylin and eosin-stained sections (100x) of the heart from Tie2Cre; p53FL/+ and Tie2Cre; p53FL/- mice 4 weeks after 12 Gy WHI. The dashed line demarcates a focal region of myocardial degeneration and necrosis in the myocardium (labeled as N). Infiltration of neutrophils and macrophages is present in regions adjacent to the injured region. (C-E) Representative sections (200x) of the myocardium stained with WGA (green) and GS-IB4 (red). Necrotic regions show aberrant staining of WGA and GS-IB4. Scale bar, 100 µm. (F-G) Quantification of GS-IB4 positive capillaries per 200x field. For Tie2Cre; p53FL/- mice 4 weeks after 12 Gy WHI, normal and necrotic regions are scored separately. Data are presented as mean ± SEM (n=3 mice per group). *P<0.05 **P<0.01 by one-way ANOVA and Tukey’s test.
Figure 3.7: Increased TUNEL positive myocardial capillaries in Tie2Cre; p53^FL/^- mice 4 weeks after 12 Gy WHI.

Figure 3.7: (A) Representative sections (200x) of the myocardium stained with GS-IB4 (red) and TUNEL (green). Necrotic regions show aberrant staining of GS-IB4. Scale bar, 100 μm. (B-C) The percentage of GS-IB4 vessels containing TUNEL positive cells per 200x field. Data are scatter plot with mean (n=3 mice per group). *P<0.05 by one-way ANOVA and Tukey’s test.
Figure 3.8: *Tie2Cre; p53<sup>FL/-</sup>* mice show increased vascular permeability in the myocardium 4 weeks after 12 Gy WHI.

**Figure 3.8:** *Tie2Cre; p53<sup>FL/-</sup>* and *Tie2Cre; p53<sup>FL/+</sup>* mice 28 days after 12 Gy WHI were injected with a mixture of TMR-conjugated high MW (2 x 10<sup>6</sup> MW) and Alexa fluor 647-conjugated low MW (1 x 10<sup>4</sup> MW) dextrans to assess the permeability of myocardial capillaries. Blood vessels were stained by FITC-conjugated anti-mouse CD31 antibody. Representative images of blood vessels highlighted by squares show that while dextrans of both low and high MWs are restricted in blood vessels in the *Tie2Cre; p53<sup>FL/+</sup>* mouse, in the *Tie2Cre; p53<sup>FL/-</sup>* mouse only the high MW dextran is completely retained within the vessels, whereas the low MW dextran leaks out of the blood vessels in the damaged regions. Images represent three mice per genotype. Scale bar, 100 µm.
Figure 3.9: A small subset of hematopoietic cells expresses VE-Cadherin-Cre.

Figure 3.9: (A) Representative FACS plots of yellow fluorescent protein (YFP) expression in hematopoietic cells in peripheral blood in VECre; LSL-YFP/+ mice. (B) Quantification of the percentage of YFP positive cells in hematopoietic cells in peripheral blood. Data are presented as scatter plot with mean (n=2 mice).
Figure 3.10: In VE-Cadherin-Cre mice, Cre is specifically expressed in a subset of endothelial cells in the myocardium.

**Figure 3.10:** Representative sections of the myocardium from (A) VECre; p53\textsuperscript{FL/} \textsuperscript{FL/}; mTmG/+ and (B) VECre; p53\textsuperscript{FL/} \textsuperscript{FL/}; mTmG/+ mice without irradiation. Cells expressing Cre are labeled by EGFP (green), whereas cells that do not express Cre are labeled by tdTomato protein (red). Endothelial cells are labeled by Alexa-fluor 647-conjugated GS-IB\textsubscript{4} (blue). EGFP expression is mostly present in the epimyocardium (E) and midmyocardium (M) but is largely absent from the endomyocardium (N), where there is extensive GS-IB\textsubscript{4} staining. Scale bar, 100 \( \mu \text{m} \). Images represent three mice per genotype.
Figure 3.11: Deletion of p53 in endothelial cells sensitizes VE-Cadherin-Cre mice to radiation-induced myocardial injury.

Figure 3.11: (A) Kaplan-Meier survival analysis of VECre; p53FL+/mice and VECre; p53FL−/mice after 12 Gy whole-heart irradiation (WHI). Censored points represent mice that are still alive. By log-rank comparison, P<0.05. (B) A representative hematoxylin and eosin-stained (H&E) section of the heart from VECre; p53FL−/mice 52 days after 12 Gy WHI. Multifocal myocardial necrosis was mainly observed in the epicardium (E) and midmyocardium (M). Necrotic cardiomyocytes with eosinophilic cytoplasm and indistinct nuclei are present. Hemorrhage as well as infiltration of neutrophils and macrophages is also observed. Scale bar, 100 µm. (C) Representative sections of the myocardium from VECre; p53FL+/; mTmG/+ and VECre; p53FL−/; mTmG/+ mice 7 weeks after 12 Gy WHI stained with GS-IB4 (blue). An area of necrosis is marked by the arrow. Scale bar, 100 µm. Images represent three mice per genotype.
Figure 3.12: Characterization of cardiac endothelial cells cultured *in vitro*.

Figure 3.12: (A) Purified CECs grow as a monolayer in culture and exhibit cobblestone morphology. (B) The purity of CECs was determined by staining simultaneously with FITC-conjugated anti-mouse CD31 and PE-conjugated anti-mouse CD105 antibodies. More than 97% of CECs cultured *in vitro* were positive for both markers as determined by flow cytometry, indicating the endothelial cell origin of CECs cultured *in vitro*. (C) Expression of p53 mRNA in CECs isolated from mice of the indicated genotypes was quantified by qRT-PCR. B2m was used as an internal control for the concentration of cDNA in different samples. Data are presented as mean ± SEM (*n* = 3 independent experiments).
Figure 3.13: p53 protects cardiac endothelial cells from radiation-induced mitotic catastrophe.

Figure 3.13: All experiments were performed using cardiac endothelial cells (CECs) from Tie2Cre; p53FL/+ and Tie2Cre; p53FL− mice at passage 3 to 5. (A-B) Quantification of cell death 4 and 96 hrs 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 hrs after 12 Gy. (E) Quantification of the percentage of cleaved caspase-3 (Casp3) positive and (F) phospho-histone H3 (pHH3) positive CECs at various time points after 12 Gy. (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 CECs with micronuclei at various time points after 12 Gy. *P < 0.05; **P < 0.01; ***P < 0.001 by two-way ANOVA with Bonferroni correction. Data are presented as mean ± SEM (n=3 independent experiments).
Figure 3.14: p21 functions downstream of p53 to regulate radiation response in CECs.

(A-B) Quantification of p21 and PUMA mRNA in CECs of different genotypes sham irradiated or 5 hrs after 12 Gy by qRT-PCR. B2m was used as an internal control for the concentration of cDNA in different samples. Data are presented as mean ± SEM (n = 3 independent experiments). P value was calculated by Student’s t-test. **P < 0.01. (C-D) Quantification of cell death in p21+/− and p21−/− CECs 4 and 72 hrs after 12 Gy. Cell survival was determined by Annexin V – PI staining. *P < 0.05 by two-way ANOVA. Data are presented as mean ± SEM (n=3 independent experiments).
Figure 3.15: p21-deficient mice are susceptible to radiation-induced myocardial injury.

Figure 3.15: (A) Kaplan-Meier survival analysis of p21+/− and p21−/− mice after 12 Gy whole-heart irradiation (WHI). Censored points represent mice that are still alive. By log-rank comparison, \( P<0.0001 \). (B) A representative hematoxylin and eosin-stained (H&E) section of the heart from p21−/− mice 34 days after 12 Gy WHI. Necrotic cardiomyocytes with eosinophilic cytoplasm and indistinct nuclei are present. Hemorrhage and infiltration of neutrophils and macrophages are observed. Scale bar, 100 µm. (C and D) Changes in fractional shortening and heart rate corrected mean velocity of circumferential fiber shortening (mVfc) in p21+/− and p21−/− mice after 12 Gy WHI. Fractional shortening at 50% is defined as a threshold of normal cardiac function in mice. ***\( P<0.001 \) by two-way ANOVA with Bonferroni correction. Data are presented as mean ± SEM (n=3 mice per genotype).
Figure 3.16: *p21* mice develop cardiac hypertrophy 4 weeks after 12 Gy WHI.

Figure 3.16: (A) Changes in mass of the left ventricle in *p21*+− and *p21*−− mice after 12 Gy WHI were assessed by echocardiography. (B) Ratio of heart weight to body weight (HW/BW) of *p21*+− and *p21*−− mice 0, 3 and 4 weeks after 12 Gy WHI. (C) Representative images of WGA-stained (green) myocardium in *p21*+− and *p21*−− mice sham irradiated or 7 weeks after 12 Gy WHI. (D) Quantification of cardiomyocyte cross-sectional area in WGA-stained myocardium. *P<0.05; ***P<0.001 by two-way ANOVA with Bonferroni correction. Data are presented as mean ± SEM (n=3 mice for each group).
Figure 3.17: Decreased microvessel density and increased vascular permeability in $p21^{-/-}$ mice after 12 Gy WHI.

Figure 3.17: (A) Representative sections of the myocardium stained with Hoechst 33324 (blue) and GS-IB4 (red). Scale bar, 100 μm. (B) Quantification of GS-IB4 positive capillaries per 200x field in the myocardium in $p21^{+/+}$ and $p21^{-/-}$ mice. ***$P<0.001$ by two-way ANOVA with Bonferroni correction. Data are presented as mean ± SEM (n=3 mice for each group). (C) $p21^{+/+}$ and $p21^{-/-}$ mice 3 weeks after 12 Gy WHI were injected with a mixture of TMR-conjugated high MW (2 x 10^6 MW) and Alexa fluor 647-conjugated low MW (1 x 10^4 MW) dextrans to assess the permeability of myocardial capillaries. Blood vessels were stained by anti-mouse CD31 antibody. While dextrans of both low and high MWs are restricted to blood vessels in the $p21^{+/+}$ mouse, in the $p21^{-/-}$ mouse only the high MW dextran is completely retained within the vessels. In contrast, the low MW dextran leaks out of the blood vessels in the damaged regions. Images represent three mice per genotype. Scale bar, 100 μm.
Because activation of p53 regulates cell survival and death, the distinct outcome of p53 activation may ameliorate or exacerbate normal tissues injuries induced by various types of stress (2). In the context of radiation, p53 plays a cell-type dependent role in controlling the acute radiation syndrome: p53 functions in hematopoietic cells to promote the hematopoietic syndrome; however, it functions in gastrointestinal (GI) epithelial cells to prevent the GI syndrome (5, 18). In the present study, my results demonstrate crucial, but also distinct roles of p53 in regulating late effects of radiation: p53-dependent cell death promotes radiation-induced lymphomagenesis, but p53-mediated cell cycle arrest prevents radiation-induced myocardial injury.

4.1 p53 functions during total-body irradiation to promote lymphoma development

In the hematopoietic system, total-body irradiation (TBI) activates p53 to cause apoptosis in leukocytes and hematopoietic progenitors, which subsequently leads to acute hematopoietic toxicity. My results show that the p53-driven pathological response following TBI not only causes acute hematopoietic toxicity, but also promotes lymphoma development. These findings suggest that acute activation of p53 during TBI is sufficient to generate and/or promote lymphoma-initiating cells. Using an in vivo competition model, I show that TBI selects for p53+/− hematopoietic cells when surrounded by wild-type p53 hematopoietic cells. This selection process may occur during two different stages. First, p53+/− hematopoietic cells are relatively radioresistant
than p53 wild-type counterparts (65). Therefore, radiation increases the relative abundance of p53
clones in the surviving population. Second, radiation-induced cell death stimulates surviving cells to repopulate (114), which may further promote the clonal expansion of p53+/− hematopoietic cells. Remarkably, temporary knockdown of p53 in p53 wild-type hematopoietic cells during TBI significantly suppresses the expansion of p53+/− clones, suggesting that radiation-driven clonal selection of hematopoietic cells is dependent on the relative abundance of p53 between normal and premalignant cells.

These findings are consistent with a hypothesis that radiation-induced cancers are initiated through a selection process during radiation therapy that promotes the expansion of premalignant clones (60-63). My results support a model where the capability of mutated clones to grow out following TBI is determined by the relative abundance of p53 between wild-type and mutated hematopoietic cells because of the critical role of the p53-dependent DDR to promote cell death and attenuate the fitness of hematopoietic stem and progenitor cells (HSPCs) (64, 65). In contrast, temporary inhibition of p53 during TBI decreases cell death and promotes the fitness of p53 wild-type HSPCs, which prevents the outgrowth of mutated clones. However, whether inhibition of p53 during irradiation prevents clonal selection of premalignant cells in other tissues is not clear and may be dependent on how p53 regulates cell survival and fitness in tissue-specific stem/progenitor cells. Future studies with this in vivo shRNA system using radiation doses and field sizes that are optimized for other types of
radiation-induced malignancies will be needed to investigate whether this observation extends to other types of cancers, such as sarcomas and lung cancers.

**4.2 p53 functions in endothelial cells to prevent radiation-induced myocardial injury**

Damage to the myocardial vasculature has been proposed as an underlying mechanism of radiation-induced myocardial injury (39). Although radiation induces p53 in the heart (42), it remains unclear whether p53 functions in cardiac endothelial cells *in vivo* to protect cells from radiation or promote radiation-induced cell death. In the present study, I demonstrate that p53 functions in endothelial cells to protect mice from radiation-induced myocardial injury. My experiments clarify how p53 regulates the radiation response of cardiac endothelial cells (CECs). I found that CECs do not show p53-dependent apoptosis 4 hours after radiation up to doses of 24 Gy, but undergo a delayed mitotic death following irradiation, which is potentiated by deletion of p53. These results suggest a model where p53 functions in CECs after irradiation to activate cell cycle checkpoints to prevent mitotic death and subsequent myocardial injury. Further supporting this model, we observed that mice lacking the p53 target gene p21 are also sensitized to radiation-induced cardiac injury. Because expression of the cyclin dependent kinase inhibitor p21 in CECs requires p53, our results indicate that a mechanism by which CECs survive radiation is via the p53-p21 cell cycle arrest pathway.
Our lab recently reported that the p53/p21 axis also plays a pro-survival role in preventing the radiation-induced GI syndrome (5). Our results show that p53 functions in the GI epithelium to protect mice from the GI syndrome presumably by preventing intestinal stem cells from undergoing mitotic death (5, 109). These findings together with the results in the present study indicate that p53 may generally play a protective role from radiation, particularly at high doses, in cells where p53 activation is uncoupled from the induction of the intrinsic pathway of apoptosis.

4.3 Concluding remarks

Using genetically engineered mouse models, I demonstrated that complete deletion of p53 in cardiac endothelial cells is required to sensitize mice to radiation-induced myocardial injury. In contrast, mice with one p53 allele in cardiac endothelial cells were not sensitized to radiation-induced cardiac injury. Therefore, the deleterious effects of blocking p53 by p53 inhibitors in combination with radiation therapy may be less than I have shown in the present study where p53 is completely deleted. Indeed, mice treatment with p53 inhibitor pifithrin-alpha prior to total-body irradiation do not have increased sensitivity to the GI syndrome, suggesting that treatment with p53 inhibitors may not completely abrogate the transcriptional activity of p53 (18). In addition, during cancer treatment, chemical inhibitors of p53 or cell cycle checkpoints would only be given for a relatively short period of time compared to the permanent deletion of p53 or p21 in the mouse models used in the present study. Future studies with the inducible \textit{in vivo} RNA interference system to knock down p53 temporarily during whole-heart
irradiation would provide insight into whether specifically and reversibly blocking p53 during radiation therapy exacerbates radiation-induced myocardial injury.

In summary, my results show that the outcome of p53 activation following radiation is not only dependent on cell types, but also dependent on whether the full transcriptional activity of p53 is required to dictate downstream signaling pathways. In the hematopoietic system, temporary knockdown of p53, which does not completely abrogate p53 expression, is sufficient to suppress p53-dependent apoptosis and ameliorates acute hematopoietic toxicity. In contrast, in cardiac endothelial cells, loss one allele of p53 does not significantly alter p53-mediated p21 induction, whereas complete loss of p53 is required to impair p21 expression and to sensitize mice to radiation-induced myocardial injury. Taken together, these results suggest that fine-tuning the levels of p53 or selectively inhibiting p53-mediated apoptosis may provide a therapeutic window to ameliorate acute radiation toxicity without exacerbating late effects of radiation.
References


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