THERAPEUTIC EFFICACY OF P53 RESTORATION IN MDM2-OVEREXPRESSING TUMORS

Qin Li

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THERAPEUTIC EFFICACY OF P53 RESTORATION IN MDM2-OVEREXPRESSING TUMORS

by

Qin Li, M.S.

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THERAPEUTIC EFFICACY OF P53 RESTORATION IN MDM2-OVEREXPRESSING TUMORS

A
DISSETATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

BY

Qin Li, M.S.

Houston, Texas

December 2013
DEDICATION

To

My Father Yongpei Li
My Mother Zijun Tian
My Wife Fanlin Kong

For All The Understanding And Support

It Is Impossible For Me To Have Done This Without You
ACKNOWLEDGEMENTS

I sincerely express my gratitude to the many people who have helped me during my Ph.D. studies. This work would not have been accomplished without their support.

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ABSTRACT

THERAPEUTIC EFFICACY OF P53 RESTORATION IN MDM2-OVEREXPRESSING TUMORS

Qin Li

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The TP53 tumor suppressor is the most mutated gene in human cancers. Recent studies using genetically modified mouse models have shown that restoring the expression of wild-type p53 has led to tumor growth suppression in various types of tumors lacking p53. Other mechanisms, e.g. upregulation of Mdm2 levels, exist in tumors to inactivate the p53 pathway. Mdm2, an E3 ubiquitin-ligase that targets p53 for proteasomal degradation, is present at high levels in many tumors with wild-type p53. In this study, we probed the effects of restoring p53 activity in Mdm2-overexpressing tumors genetically using animal models. Here we demonstrated high levels of Mdm2 and low levels of p53 act additively to dampen p53 activity in DNA damage response and tumor development, suggesting a dose-dependent tumor-suppressive function of p53. Our data also indicate that restoration of wild-type p53 expression in spontaneous Mdm2-overexpressing angiosarcomas resulted in tumor stasis and regression in some cases. We further showed that the restored p53 suppressed
cell proliferation in the \textit{Mdm2}-overexpressing angiosarcomas but did not elicit apoptosis. Therefore, we conclude that restoration of wild-type \textit{p53} expression in tumors with high levels of Mdm2 represents a potential strategy to treat these tumors. This finding is of important clinic relevance since a large number of human tumors have high levels of Mdm2. Our present work suggests that \textit{p53} restoration confers therapeutic potentials even to tumors with high levels of Mdm2.
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CHAPTER 1. INTRODUCTION

The Basics of Tumor Suppressor p53

*p53* is the most frequently altered gene in human cancers (Cheok et al., 2011). In 1979, the p53 protein with a molecular weight of approximate 53 kDa was independently discovered in different studies via virologic and serologic approaches, respectively. In simian virus 40 (SV40) transformed cells, p53 was co-immunoprecipitated with SV40 large-T antigen, suggesting that p53 and large-T antigen exist in the same complex (Chang et al., 1979; Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero et al., 1979). Yet, p53 protein was also immunoprecipitated from the murine Meth A sarcoma cells or transformed mouse cells by mouse antisera against Meth A cells (DeLeo et al., 1979). Moreover, p53 expression was detected in all transformed mouse cells but not in non-transformed mouse cells (DeLeo et al., 1979). Although the nature and function of p53 was far from clear at that time, these early observations collectively suggested a correlation of p53 with cellular transformation.

It was therefore of interest to decipher the role of p53 in transforming events. Using a variety of approaches, several laboratories demonstrated that p53 expression positively correlated with induction of cell proliferation (Calabretta et al., 1986; Mercer et al., 1984; Mercer et al., 1982; Milner and McCormick, 1980; Reich and Levine, 1984). In addition, inhibition of p53 by a variety of means led to suppression of cell proliferation in both transformed and non-transformed cells (Mercer et al., 1984; Mercer et al., 1982; Shohat et al., 1987). Ectopic expression of *p53* not only immortalized normal cell lines (Jenkins et al., 1985; Jenkins et al.,
1984), but also cooperated with expression of other well-known oncogenes e.g. H-Ras to transform these cells (Eliyahu et al., 1984; Jenkins et al., 1984; Parada et al., 1984). All of these data suggested an oncogenic nature of p53 until it was finally revealed that the “wild-type” p53 used in these aforementioned transformation studies was indeed mutated.

In search for potential tumor suppressor genes on chromosome 17p, which were found deleted in more than 75% of colorectal carcinomas, a small region containing p53 gene was identified (Baker et al., 1989). Further analysis of gene status at the p53 locus revealed its compliance with the Knudson’s Two-hits theory of tumor suppressor genes during oncogenesis (Knudson, 1971): one copy of p53 gene was completely deleted while the other one was often altered by point mutations in these colorectal tumors (Baker et al., 1989). Later, similar p53 gene alterations were observed in a variety of tumor types (Nigro et al., 1989). These unexpected findings suggested p53’s role as a tumor suppressor and not as an oncogene, which was supported by additional observations that exogenous expression of wild-type p53 suppressed cell proliferation and oncogene-medicated transformation (Eliyahu et al., 1989; Finlay et al., 1989).

The tumor suppressor role of p53 was further confirmed by the studies of Li-Fraumeni syndrome. These patients carry germline mutations of p53 and are susceptible to an early onset of various types of tumors (Malkin et al., 1990; Srivastava et al., 1990). In order to examine the causal role of p53 loss during tumorigenesis, several research groups independently generated genetically engineered p53-null mice, in which both p53 alleles were deleted (Donehower et
al., 1992; Jacks et al., 1994; Lowe et al., 1993). These mice, although born normal, developed tumors at early ages with 100% penetrance, sealing the tumor suppressive role of the p53 protein.

The Structure of the p53 Protein

Due to its important role in tumorigenesis, extensive efforts have been dedicated to resolve the structure of the p53 protein. The analysis of p53 structure was possible with cloning of both human and mouse p53 cDNAs (Bienz et al., 1984; Zakut-Houri et al., 1985). Although the protein encoded by human cDNA with 393 amino acids is longer than that of mice by 2 amino acids, these two p53 proteins share a great deal of identity and homology at the primary sequence level (Zakut-Houri et al., 1985). To date, 5 functional domains have been identified within the p53 protein (Figure 1), which are from the N-terminus to the C-terminus, the transcription activation domain (TAD), the proline-rich domain (PRD), the central DNA binding domain (DBD), the tetramerization (TET) domain, and the C-terminus regulatory (CT) domain (Joerger and Fersht, 2008).
**Figure 1. Functional domains of the p53 protein.** The p53 protein contains five functional domains: a negatively charged transactivation domain (TAD), a proline-rich domain (PRD), a DNA binding domain (DBD) that binds a specific DNA sequence, a tetramerization domain (TET), and a C-terminus regulatory domain (CT). Numbers at the bottom indicate the beginning and ending amino acids of each domain.
The TAD, which remains unfolded, provides binding sites for various types of factors including the basic transcription machinery components, the transcriptional coactivators, and the negative regulators of p53 (Joerger and Fersht, 2008). One of the most important factors that binds the TAD of p53 is the transcriptional coactivator p300 (Teufel et al., 2007). Interaction with p300 via its TAD region is essential for the transcriptional functions of p53 (Avantaggiati et al., 1997; Gu et al., 1997; Scolnick et al., 1997). Interestingly, the sequence within the p53 TAD, which is responsible for interacting with two negative regulators, murine double minute 2 (Mdm2) and murine double minute 4 (Mdm4), overlaps the p300-binding site of p53 (Kussie et al., 1996; Popowicz et al., 2007; Teufel et al., 2007). In addition, Mdm proteins compete with p300 for binding to the TAD of p53 (Teufel et al., 2007). Therefore, the relative concentrations of these factors and their affinities of binding to the p53 TAD in certain cellular contexts dictate the activation or inhibition of p53 transcriptional functions (Joerger and Fersht, 2008).

The PRD of human p53 contains 5 PXXP motifs (where P stands for proline and X for any amino acid) and serves as a link between the TAD and DBD (Walker and Levine, 1996). In general, PXXP motifs mediate protein-protein interactions via binding to Src homology 3 (SH3) domains (Feller et al., 1994). Although it was demonstrated that p53 PXXPs impact on interaction with p300, peptidyl-prolyl isomerase Pin1, transcriptional corepressor mSin3A, and Mdm2 (Berger et al., 2001; Dornan et al., 2003; Zacchi et al., 2002; Zheng et al., 2002; Zilfou et al., 2001), the exact function of the PRD in p53 transactivation remains
largely unclear. Deletion of PRD significantly increases p53 sensitivity to Mdm2-mediated degradation and reduces transcriptional activities in mice (Toledo et al., 2006); however, disruption of the Pin1 binding sites or removal of PXXP motifs in the PRD did not significantly affect tumor suppression activities of p53 (Toledo et al., 2007). These data together suggest that the structure but not the primary sequence of PRD is essential for the functions of p53, which also explains for lack of conservation of PXXP motifs within the PRD among mammalian p53s (Toledo et al., 2007).

The p53 DBD, which mediates the interaction of p53 with specific DNA sequences, resides in the middle region of the p53 protein (Bargonetti et al., 1991; el-Deiry et al., 1992; Kern et al., 1991; Pavletich et al., 1993; Zambetti et al., 1992). The consensus sequence of the double-stranded DNAs that p53 binds contains two half-site palindromes 5’-PuPuPuC(A/T)(T/A)GPyPyPy-3’ (Pu represents A or G and Py for C or T) separated by a spacer sequence with 0-13 base pairs in length (el-Deiry et al., 1992). Crystallography has revealed that two p53 DBDs form a dimer to bind one of these two half sites (Kitayner et al., 2006). Due to the critical roles of p53 DBD in mediating p53-DNA interaction, mutations in p53 DBD disrupt its interaction with the target DNA sequence, and therefore compromise the transcriptional activity of p53. In human cancer cases, over 80% of p53 mutations are identified in the DBD domain (Olivier et al., 2002; Petitjean et al., 2007), suggesting the importance of DNA binding to p53 functions.

Early studies have clearly demonstrated that p53 binds to target DNA sequences as a tetramer (Friedman et al., 1993; Wang et al., 1994). The TET
domain at the C-terminus of p53, spanning residues 324-356, mediates the
tetramerization of p53 proteins (Sakaguchi et al., 1997; Veprintsev et al., 2006).
Although deletion of the TET domain does not completely abolish DNA-binding
ability and transcriptional activity of p53, p53 with TET domain deletion binds to
DNA with 10-100 times lower affinity (Chene, 2001), suggesting the importance
of tetramerization to p53 functions. In addition, the TET domain mediates protein-
protein interactions between other proteins, such as casein kinase 2 and Ca^{2+}-
dependent protein kinase C, which are also important for p53 regulation (Chene,
2001).

The CT domain of p53 represents an intrinsically unfolded structure at its C-
terminus, consisting of the last 30 amino acid residues of p53 (Joerger and
Fersht, 2008). Deletion of the CT domain leads to the constitutive activation of
p53 (Halazonetis and Kandil, 1993; Hupp et al., 1992), suggesting that it
negatively regulates p53 function. In response to stress, the p53 CT domain
undergoes extensive posttranslational modifications including phosphorylation,
acetylation, and ubiquitination (Jimenez et al., 1999). Although it is postulated
that these modifications are critical for p53 activation or stability, emerging \textit{in vivo}
evidence suggest that they may only contribute to the fine-tuning of p53 stress
responses but are dispensable for p53 stability and transactivation (Krummel et
al., 2005).
Biological Functions of p53

Under normal conditions, p53 remains dormant (Vogelstein et al., 2000). It is only activated in response to a number of intra- and extra-cellular insults including but not limited to DNA damage, oncogene activation and hypoxia (Vogelstein et al., 2000; Vousden and Lu, 2002). Cells exposed to such stresses are prone to becoming neoplastic, since they are more likely to accumulate mutations and/or undergo aberrant proliferation compared to unstressed cells (Vogelstein et al., 2000). In order to maintain genomic integrity at both single cell and organism levels, the p53 protein, activated in these cells, functions to induce apoptosis, cell cycle arrest and cellular senescence via transactivating a set of target genes involved in such cellular programs (such as Noxa and Puma for apoptosis, p21 and GADD45 for cell cycle arrest, and p21 and PML for cellular senescence) (Figure 2) (de Stanchina et al., 2004; Hofseth et al., 2004; Vogelstein et al., 2000; Vousden and Lu, 2002).
Figure 2. Function and Activation of p53. A variety of stresses including DNA damage, oncogene activation, and hypoxia activate p53 through upstream stress sensor proteins. Activated p53 induces expression of a set of target genes involved in apoptosis, cell cycle arrest, and senescence. Several p53 targets, such as Mdm2, COP1 and Pirh2, negatively regulate p53 function, which form negative feedback loops. Other proteins Mdm4, ARF-BP1, and Trim24 also negatively regulate p53 functions.
Cell cycle arrest

Cell cycle arrest is one of the first responses induced by p53 activation in nearly every type of mammalian cells (Vogelstein et al., 2000). Mechanically, p53 stimulates cell cycle arrest via transactivating its major cell cycle arrest target p21 (el-Deiry et al., 1993), which functions as a cycle-dependent kinase (CDK) inhibitor (Warfel and El-Deiry, 2013). As a member of the Cip/Kip family of CDK inhibitors, p21 interacts with and inhibits a broad spectrum of CDKs such as CDK2, CDK4, CDK6 and possibly CDK1 (Harper et al., 1995), and thus blocks cell cycle at the G1/S and G2/M transitions (Niculescu et al., 1998). Other p53 target genes such as GADD45 and 14-3-3σ have also been reported to induce cell cycle arrest (Chan et al., 1999; Kastan et al., 1992; Laronga et al., 2000; Wang et al., 1999). However, unlike p21, GADD45 and 14-3-3σ proteins can only inhibit the CDK1/cyclin B1 complex, and therefore induce a G2/M but not a G1/S arrest (Chan et al., 1999; Laronga et al., 2000; Wang et al., 1999). In human tumors, the identification of the p53 mutant p53R175P (arginine to proline at codon 175) that lacks apoptotic activity but retains cell cycle arrest activity (Ludwig et al., 1996; Rowan et al., 1996) allowed examination of the tumor-suppressive role of p53-induced cell cycle arrest. This question was clearly answered with the generation of knock-in mice carrying the p53<sup>515C</sup> allele that mimic the human p53R175P mutant. The p53<sup>515C/515C</sup> mice, although prone to tumor development, have a much later tumor onset compared with p53<sup>−/−</sup> mice (Liu et al., 2004), indicating the tumor-suppressive activity of p53-induced cell cycle arrest.
Cellular senescence

The role of p53 in inducing cellular senescence was clearly demonstrated in cells overexpressing oncogene $H\text{-}ras^{G12V}$ (Serrano et al., 1997). Ectopic expression of $H\text{-}ras^{G12V}$ in both rodent and human cells leads to cellular senescence accompanied by accumulation of p53 protein; deletion of p53 rescues the cells from senescence (Serrano et al., 1997), indicating a p53-dependent cellular senescence in response to oncogene activation. More recently, two independent studies showed that p53 restoration in liver carcinomas and sarcomas also resulted in cellular senescence in tumors, suggesting that p53 exerts tumor-suppressive activity by playing a causal role in induction of senescence in vivo (Ventura et al., 2007; Xue et al., 2007). Although the mechanisms by which p53 induces the senescence program remain largely unclear, both in vitro and in vivo studies suggest that p21 functions as the senescence effector (Bearss et al., 2002; Brown et al., 1997; el-Deiry et al., 1993).

The response to p53 activation varies in different contexts. For example, in response to DNA damage, p53 induces apoptosis in radiosensitive tissues such as spleen, thymus and hematopoietic system, and elicits cell cycle arrest and cellular senescence in other tissues such as fibroblasts and connective tissues (Di Leonardo et al., 1994; Kuerbitz et al., 1992; Lowe et al., 1993; Tsai et al., 2009). The molecular basis underlying context-dependent p53 responses remains largely unsolved. It has been speculated that p53 levels may serve as
one of the determinants dictating p53-induced responses. This notion is based on the finding that the promoters of cell cycle arrest/senescence genes show high affinity to p53, and low-affinity is observed for the promoters of apoptotic genes (Kaeser and Iggo, 2002; Weinberg et al., 2005). Therefore, cell cycle arrest/senescence but not apoptosis is likely to be induced in cells expressing low levels of p53, and the apoptosis pathway is activated when p53 is present at high levels. This is supported by a study in a hepatocellular carcinoma (HCC) cell line. Ectopically expressing p53 at high levels in HCC cells induces apoptosis, and low level p53 expression only evokes cell cycle arrest (Lai et al., 2007). Survival cytokines, e.g. IL-6, block p53-induced apoptosis (Liebermann et al., 2007), suggesting that distinct factors expressed by different cells may determine which pathways are activated by p53.

Apoptosis

Since first observed in the myeloid leukemia cells, overexpression of wild-type p53 has been reported to induce apoptosis in a wide range of cell types (Gottlieb and Oren, 1998; Yonish-Rouach et al., 1991). These observations provided the first set of evidence that high levels of p53 are capable of triggering apoptosis. In other studies, overexpression of adenovirus 5 oncogene E1A stabilizes endogenous wild-type p53 proteins in cells and elicits p53-dependent apoptosis (Debbas and White, 1993; Lowe and Ruley, 1993). Moreover, DNA damage caused by ionizing radiation or etoposide (a topoisomerase II inhibitor) also leads to induction of apoptosis in wild-type but not p53−/− thymocytes (Clarke
et al., 1993; Lowe et al., 1993). Together, these data convincingly documented a role for p53 in induction of apoptosis.

p53 induces apoptosis through both transcription-dependent and -independent pathways (Hofseth et al., 2004). Upon activation, p53 binds to the promoter regions of a set of proapoptotic genes such as *Puma*, *Noxa*, *Bax*, *APAF-1*, *Fas* and *PIDD*, and then induces the expression of such genes (Miyashita et al., 1994; Moroni et al., 2001; Nakano and Vousden, 2001; Oda et al., 2000). Upregulation of these gene products promotes activation of intrinsic or extrinsic apoptosis pathways (Haupt et al., 2003). As to the transcription-independent pathway, p53 directly interacts with anti-apoptotic proteins Bcl-2 and Bcl-XL, inhibits their activity, and promotes the release of cytochrome c, which activates the intrinsic pathway (Mihara et al., 2003). Thus, p53 is directly engaged in induction of an apoptosis program, which is essential for its tumor suppressive functions.

**Activation of p53**

As aforementioned, p53 can be activated by various stresses including DNA damage, oncogene activation and hypoxia (Vogelstein et al., 2000; Vousden and Lu, 2002). Although a limited number of studies suggest that p53 activation occurs at transcriptional or translational levels (Galban et al., 2003; Wang et al., 2004), stress signals mostly induce p53 activation at the protein level.

Intracellular and extracellular stress stimuli sensed by upstream factors activate different downstream pathways, which in turn stabilize and activate p53.
Among such pathways activating p53, responses to oncogenic insults and DNA damage have been best studied. Activation of oncogenes such as Ras and c-Myc results in upregulation of tumor suppressor ARF (Ozenne et al., 2010). In turn, ARF binds to two negative inhibitors of p53, Mdm2 and ARF-BP1, and releases p53 from their talons, which leads to elevated p53 levels and thus activation of p53 (Dai et al., 2006; Ozenne et al., 2010). In response to DNA damage caused by IR (ionizing radiation) or UV (ultraviolet light), ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia and Rad3 related) kinases are activated (Motoyama and Naka, 2004). ATM and ATR activate p53 by directly phosphorylating p53 at Ser15 and Ser20 or via two other kinases CHK1 and CHK2 (Motoyama and Naka, 2004). Phosphorylation at these sites disrupts the interaction between p53 and Mdm2 and thus blocks Mdm2-mediated p53 degradation, which leads to the stabilization and activation of p53 (Motoyama and Naka, 2004). Although HIF-1α (hypoxia-inducible factor 1α) and several other factors have been implicated in hypoxia-induced p53 activation (Sermeus and Michiels, 2011), no clear mechanism has yet been described for p53 activation under hypoxic conditions.

Clearly, p53 activation is a complicated but tightly controlled process involving a number of distinct pathways and numerous factors. Such fine regulation of p53 activation ensures that p53 remains inactive under non-stressed conditions and can be rapidly activated when cells suffer from a variety of insults.
Negative Regulators of p53

Deletion of Mdm2 or Mdm4, negative regulators of p53, leads to p53-dependent embryonic lethal phenotypes in mice due to elevated p53 activity (Jones et al., 1995; Migliorini et al., 2002; Montes de Oca Luna et al., 1995; Parant et al., 2001). In addition, expression of a p53 mutant with elevated activity in mice leads to a premature aging phenotype through p53-induced senescence (Tyner et al., 2002). These data collectively suggests that excessive activity of p53 in normal unstressed cells is detrimental to the organisms. Therefore, low levels of p53 activity are required for the growth of unstressed cells and normal development of whole organisms. Several mechanisms are in place to keep p53 activity checked, which mainly involve ubiquitination-mediated proteasomal degradation and block of transactivation. To date, a number of negative regulators of p53 engaged in such processes have been identified, which include Mdm2, Pirh2, COP1, TRIM24 and ARF-BP1 for p53 ubiquitination, and Mdm2, Mdm4 and Pirh2 for inhibiting p53 transcriptional activity (Figure 2). Among these factors, Mdm2 and Mdm4 have been the most studied.

Regulation of p53 Functions by Mdm2

Mdm2 was first identified as one of these genes amplified on double minute chromosomes in transformed mouse NIH-3T3 fibroblasts (Fakharzadeh et al., 1991). Biallelic deletion of Mdm2 results in an embryonic lethal phenotype in mice, which is completely rescued in a p53−/− background (Jones et al., 1995; Montes de Oca Luna et al., 1995). Deletion of Mdm2 in specific types of cells
such as neuronal progenitors and cardiomyocytes led to embryonic lethal phenotypes, which were completely rescued by deletion of p53 (Grier et al., 2006; Xiong et al., 2006). These compelling genetic observations undeniably indicate that Mdm2 functions as a critical negative regulator of p53 \textit{in vivo}.

Then, the question was asked how Mdm2 regulates p53. It has been well-documented that Mdm2 forms a tight complex with p53, masks its transactivation domain, inhibits p53 transcriptional activity, and thus suppresses p53-mediated transactivation (Momand et al., 1992; Oliner et al., 1993). These data collectively indicate Mdm2 negatively regulates p53 functions by blocking its transcriptional activity.

Cumulative evidence also establishes the role of Mdm2 in regulating p53 stability. Several studies have demonstrated that Mdm2 binds to p53, mediates ubiquitination of p53 as an E3 ubiquitin-ligase, which in turn targets p53 for proteasomal degradation (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). However, the p53 protein was not constitutively stabilized in an Mdm2\(^{-/-}\) background, although degradation was delayed (Ringshausen et al., 2006). This interesting observation indicates that Mdm2 is critical for p53 degradation but other proteins may also function to degrade p53.

As an important factor controlling p53 activities, Mdm2 expression is tightly regulated. Transcription of \textit{Mdm2} is controlled by two distinct promoters. The upstream promoter P1 of \textit{Mdm2} gene drives constitutive expression of it and is not evidently affected by p53 (Barak et al., 1994), which may function as a critical means to control p53 activity in normal cells under non-stressed conditions.
Activated p53 binds to the p53-responsive elements within intron 1 of *Mdm2* gene and drives transcription of Mdm2 from an internal *Mdm2* promoter (P2) (Barak et al., 1994). p53-mediated *Mdm2* expression together with Mdm2-mediated p53 degradation form a negative feedback loop, which is likely the key mechanism for rapid clearance of activated p53 after stress is withdrawn. A recent animal model has provided invaluable information on the role of both Mdm2 promoters *in vivo*. The *Mdm2*\textsuperscript{P2} allele carries mutations of the p53-binding sites in the P2 promoter and completely loses p53-induced expression but retains constitutive expression from the *P1* promoter (Pant et al., 2013). Surprisingly, *Mdm2*\textsuperscript{P2/P2} mice are viable but extremely sensitive to IR due to hematopoietic failure induced by high level of p53 (Pant et al., 2013). These observations clearly indicate constitutive expression of *Mdm2* from *P1* is sufficient for controlling p53 protein levels under physiological conditions, but induced expression from *P2* is required to dampen p53 activity under stressed conditions.

Transcripts from both *Mdm2* *P1* and *P2* promoters possess similar coding potentials (Barak et al., 1994), but translation efficiency of P2 transcript is 8-fold higher than that of *P1* (Landers et al., 1997). This enhanced translation of *Mdm2* *P2* mRNA may provide additional reinforcement to degrade p53 after its activation by stress.

Regulation of p53 by Mdm4
Mdm4 protein shares very high similarity with Mdm2 at the primary structure level (Shvarts et al., 1997). Two prominent conserved domains have been identified between Mdm4 and Mdm2, which are the RING-finger domain at the C-termini and the p53-binding domain at the N-termini (Shvarts et al., 1997; Shvarts et al., 1996). Biallelic deletion of Mdm4 leads to an embryonic lethal phenotype in mice, which is also rescued in a \( p53^{-/-} \) background (Finch et al., 2002; Migliorini et al., 2002; Parant et al., 2001). These observations clearly indicate that Mdm4 functions as a negative regulator of p53 \textit{in vivo}. Like Mdm2, Mdm4 binds to the transactivation domain of p53 and represses its transcriptional activity \textit{in vitro} (Shvarts et al., 1996). Upon deletion of \( Mdm4 \), expression of p53 target genes was elevated without concomitant increase in the p53 protein level in \( Mdm2^{-/-} \) MEFs (murine embryonic fibroblasts) (Francoz et al., 2006), which suggests that Mdm4 can suppress p53 function only by inhibiting transactivation but not by degradation. Consistently, overexpression of \( Mdm4 \) does not induce degradation of p53 (Jackson and Berberich, 2000; Stad et al., 2000). In addition, structural analysis indicates that Mdm4 cannot function as an active E3 ligase due to inability to recruit an E2 ubiquitin-conjugase (Linke et al., 2008). Collectively, these data indicate that Mdm4 regulates p53 activity by transcriptional inhibition but not degradation.

This difference in functionality between Mdm4 and Mdm2 provides a plausible explanation for the different phenotypes exhibited in mice with either \( Mdm4 \)- or \( Mdm2 \)-deficiency. Loss of \( Mdm4 \) in a variety of mouse tissues results in a set of phenotypes distinct from that of \( Mdm2 \) (Grier et al., 2006; Valentin-
Vega et al., 2009; Valentin-Vega et al., 2008; Xiong et al., 2007; Xiong et al., 2006), and concomitant deletion of \textit{Mdm2} and \textit{Mdm4} leads to a more severe phenotype compared to loss of either gene alone. Importantly, all of these phenotypes are rescued by deletion of \textit{p53}. Intriguingly, \textit{Mdm4}\textsuperscript{−/−} embryonic lethality can be rescued by overexpression of \textit{Mdm2} (Steinman et al., 2005), suggesting that Mdm2 at high levels may compensate Mdm4 loss at certain circumstances possibly via suppressing p53 functions. Collectively, these data suggest that Mdm2 and Mdm4 inhibit p53 functions in a non-overlapping fashion \textit{in vivo}.

Other Factors Mediating p53 Ubiquitination

Although it is widely accepted that Mdm2 is the major E3 ligase targeting p53 for degradation, recent studies suggest other ubiquitin-ligases also target p53 for degradation. In a study using inducible p53, loss of Mdm2 did not completely inhibit but only delayed degradation of p53, indicating that p53 is subjected to Mdm2-independent degradation (Ringshausen et al., 2006). To date, several ubiquitin E3 ligases have been shown to mediate p53 degradation. A RING finger domain E3 ligase Pirh2 (p53-induced RING-H2) binds to p53 at the DNA binding domain and mediates its degradation through ubiquitination (Leng et al., 2003). Deletion of Pirh2 RING finger domain, blocks Pirh2-mediated p53 ubiquitination, but does not remove repression of p53 transactivation by Pirh2 (Leng et al., 2003), which implies that binding of Pirh2 to p53 DBD compromises p53-DNA interaction. Another RING finger E3 ligase, COP1 (conconstutive
photomorphogenesis 1), also catalyzes Mdm2-independent ubiquitination of p53 (Dornan et al., 2004). Interestingly, both Pirh2 and COP1 are p53-inducible genes (Dornan et al., 2004; Leng et al., 2003), suggesting the other negative feedback loops other than the p53-Mdm2 feedback loop may exist to modulate p53 activity. A HECT domain containing E3 ubiquitin ligase ARF-BP1, and TRIM24 (Tripartite-motif protein 24) have been reported to target p53 for degradation by ubiquitination (Allton et al., 2009; Chen et al., 2005). Although these data collectively indicate that a number of E3 ligases mediate Mdm2-independent degradation of p53, the roles of these proteins as negative regulators of p53 remain to be verified in vivo using animal models.

Upregulation of Mdm2 Proteins in Tumors

Since Mdm2 functions as a major negative regulator of the tumor suppressor p53, a role for Mdm2 in tumor development is not surprising. High levels of Mdm2 are commonly found in various types of human cancers including sarcomas, gliomas, melanomas, carcinomas and hematological malignancies (Onel and Cordon-Cardo, 2004). Clinical data demonstrate that most human tumors that have elevated Mdm2 levels retain wild-type p53, which lead to a speculation that Mdm2 overexpression and p53 mutation are mutually exclusive since either mechanism inactivates the p53 pathway (Manfredi, 2010). Studies in human soft tissue sarcomas revealed that Mdm2 gene amplification results in overexpression of Mdm2 in tumors, but high levels of Mdm2 also exist in tumors lacking gene amplification of Mdm2 (Patterson et al., 1997), suggesting that other
mechanisms may contribute to Mdm2 upregulation in tumors. These mechanisms include increased transcription, prolonged mRNA half-life, enhanced translation potential and altered posttranslational modifications (Manfredi, 2010; Marine, 2011; Marine and Lozano, 2009; Riley and Lozano, 2012). Extensive studies also indicate that Mdm2 upregulation by such mechanisms is tightly associated with tumorigenesis and tumor progression (Manfredi, 2010; Marine, 2011; Marine and Lozano, 2009; Riley and Lozano, 2012).

A number of mouse models have been established to interrogate the role of Mdm2 overexpression in tumorigenesis. Mdm2 transgenic (Mdm2Tg) mice were established using a cosmid containing the entire Mdm2 gene including the promoter region, and therefore resembled Mdm2 gene amplification in human cancers (Jones et al., 1998). An average 4-fold increase of Mdm2 expression in a variety of tissues of the Mdm2Tg mice leads to development of multiple types of tumors that include a high percentage of lymphomas and sarcomas (Jones et al., 1998). Interestingly, Mdm2Tg/Tg mice have significantly earlier onset of tumors compared to Mdm2Tg mice (Jones et al., 1998), suggesting an Mdm2-dose-dependent effect during tumorigenesis possibly through inhibition of p53 activities. Overexpression of Mdm2 in mouse mammary glands and skin also results in development of spontaneous tumors (Alkhalaf et al., 1999; Ganguli et al., 2000; Lundgren et al., 1997). A human Mdm2 single nucleotide polymorphism SNP309 (T to G) is associated with earlier tumor onset in both sporadic and hereditary cancer patients (Bond et al., 2004). SNP309 results in enhanced transcription of
Mdm2 by generating an additional binding site for transcriptional activator Sp1, and therefore dampens p53 activity (Bond et al., 2004). Knock-in mice with a humanized \textit{Mdm2} intron 1 harboring SNP309 also show accelerated tumor development in both \textit{p53} wild-type and mutant backgrounds (Post et al., 2010). Collectively, these genetic mouse models reveal a causal role of Mdm2 overexpression in tumorigenesis.

\textbf{p53 Reactivation in Cancer Therapy}

Since the tumor suppressor gene \textit{p53} is mutated in over 50\% of human cancer cases and the p53 pathway is also muted by other means (e.g. overexpression of \textit{Mdm2}) in a large number of tumors, the question was asked whether reactivation of p53 in these tumors could suppress tumor growth or lead to tumor regression. To date, several approaches have been applied to reactivate p53 in tumors.

\textbf{Targeting Mdm2 to Reactivate p53}

Since the p53 inhibitor \textit{Mdm2} is overexpressed in multiple types of human tumors, inhibition of Mdm2 activity may constitute a means for reactivating p53 in cancer cells. The finding that a 20\% decrease in Mdm2 levels leads to tumor suppression without adverse side effects (Mendrysa et al., 2006) provides supportive evidence for targeting Mdm2 in cancer therapy. Extensive efforts have been advancing this approach rapidly.
A group of small molecules including nutlins, benzodiazepinediones and MI-63/MI-219 have been identified through structure-based design and high throughput screening (Ding et al., 2006; Grasberger et al., 2005; Shangary et al., 2008; Vassilev et al., 2004). Such molecules occupy the p53-binding pocket of Mdm2 and therefore disrupt Mdm2-p53 interaction (Ding et al., 2006; Grasberger et al., 2005; Shangary et al., 2008; Vassilev et al., 2004). Administration of these small molecules to a variety of tumors with wild-type p53 leads to accumulation of p53 protein and p53-induced cell cycle arrest and/or apoptosis (Ding et al., 2006; Grasberger et al., 2005; Shangary et al., 2008; Vassilev et al., 2004). More interestingly, treatment with nutlin-3 and MI-219 also results in tumor suppression in xenograft tumor models with no noticeable adverse side effects (Shangary et al., 2008; Vassilev et al., 2004). Another small molecule RITA (reactivation of p53 and induction of tumor cell apoptosis) binds to the p53 N-terminus and blocks the access of Mdm2 to p53 (Issaeva et al., 2004). RITA also induces accumulation of p53 and p53-mediated tumor suppressive activities in both p53 wild-type tumor cells and xenograft tumors (Issaeva et al., 2004). Two molecules HLI98 and JNJ-26854165 targeting the E3 ligase activity of Mdm2 promote stabilization of p53 and thus suppress the growth of tumor cells or mouse xenografts, respectively (Yang et al., 2005) (Arts et al., 2008). Currently, phase I clinical studies of JNJ-26854165 (NCT00676910) and a nutlin family member RG7112 (NCT01143740, NCT01164033, NCT00559533, NCT00623870 and NCT01605526) have shown acceptable safety of both drugs in patients with multiple types of tumors (Andreeff et al., 2010; Andreeff et al., 2011; Beryozkina
et al., 2011; Kurzrock et al., 2012; Ray-Coquard et al., 2011; Tabernero et al., 2009). In particular, RG7112 treatment of patients with leukemias, lymphomas, sarcomas and other solid tumors have shown favorable clinical outcomes including induction of apoptosis, reduced cell proliferation and complete remission (Andreeff et al., 2010; Andreeff et al., 2011; Beryozkina et al., 2011; Kurzrock et al., 2012; Ray-Coquard et al., 2011).

Although exciting, there are a number of caveats in the use of these drugs, which may limit their applications in clinic. First, the therapeutic effectiveness of these drugs is totally dependent on the presence of wild-type \( p53 \) in tumors, since no \( p53 \)-induced tumor suppressive activities have been observed in the tumor cells with mutant \( p53 \) (Ding et al., 2006; Grasberger et al., 2005; Issaeva et al., 2004; Shangary et al., 2008; Vassilev et al., 2004). However, over 50% human cancers have \( p53 \) mutations, and more importantly a very high percentage of tumors carrying \( p53 \) mutations undergo loss-of-heterozygosity (LOH) (Bellini et al., 2012; Coles et al., 1992; Fenoglio-Preiser et al., 2003; Venkatachalam et al., 2001). Therefore, wild-type \( p53 \) is not present in a large proportion of tumors, which will severely limit the administration of these drugs only to cancer patients carrying wild-type \( p53 \). Second, it is well-documented that Mdm2 also binds and degrades mutant \( p53 \) (Lukashchuk and Vousden, 2007; Midgley and Lane, 1997; Terzian et al., 2008). Disrupting the interaction of mutant \( p53 \) with Mdm2 will lead to the stabilization of mutant \( p53 \) (Lukashchuk and Vousden, 2007; Terzian et al., 2008) that possess gain-of-function oncogenic activities (Lang et al., 2004; Midgley and Lane, 1997; Olive et al.,
Therefore, administration of these Mdm2 inhibitors even to patients with both wild-type and mutant p53 alleles may lead to unfavorable responses. Lastly, in the preclinical studies, treatment with Mdm2-inhibitors resulted in accumulation of considerable amount of Mdm2 (Ding et al., 2006; Grasberger et al., 2005; Graves et al., 2012; Issaeva et al., 2004; Koblish et al., 2006; Tovar et al., 2006; Vassilev et al., 2004; Yang et al., 2005). Since p53-independent oncogenic activities of Mdm2 have been well documented (Li and Lozano, 2013), accumulation of Mdm2 by administration of Mdm2 inhibitors may also cause detrimental effects to the patients. Therefore, caution should be taken when administrating Mdm2 inhibitors to patients, and the safety, efficacy and effectiveness of these inhibitors should be monitored closely.

Gene delivery of wild-type p53

As aforementioned, in an early study, overexpression of wild-type but not mutant p53 in a murine myeloid leukemic cell line that lacks p53 resulted in induction of rapid apoptosis (Yonish-Rouach et al., 1991), suggesting that reactivation of p53 is an effective strategy to treat tumors with loss of p53 activities. Delivery of exogenous wild-type p53 into tumor cells with p53 loss using retroviral vectors leads to growth suppression in both human cancer cell lines and orthotopic mouse models (Cai et al., 1993; Fujiwara et al., 1994). These data together indicate that reactivation of p53 may serve as an effective therapeutic strategy in human cancers.
Recently, four research groups have independently established genetically engineered mice using distinct approaches, which allow p53 restoration in vivo. Utilizing these mice as tools, p53 restoration in established tumors has been modeled for multiple types of tumors (Martins et al., 2006; Ventura et al., 2007; Wang et al., 2011; Xue et al., 2007). Martins and colleagues evaluated the effects of p53 restoration in an Eµ-Myc mouse model to address the question whether p53 loss is required for tumor maintenance in lymphomas (Martins et al., 2006). To this end, they used a specific p53 knock-in allele (p53\textsuperscript{KI}), which encodes a wild-type p53 protein fused with a modified form of estrogen receptor (p53ER). This fusion protein can be only activated when 4-hydroxytamoxifen is administrated; otherwise, p53ER remains inactive in the cytoplasm. Activated p53ER enters the nucleus and functions as wild-type p53. A proportion of B-cell lymphomas developed in Eµ-Myc p53\textsuperscript{KI/+} mice underwent LOH and became tumors of an Eµ-Myc p53\textsuperscript{KI/0} genotype, in which p53 was functionally null. When transplanted into syngeneic mice, Eµ-Myc p53\textsuperscript{KI/0} lymphomas developed rapidly. However, 4-hydroxytamoxifen administration led to regression of these Eµ-Myc p53\textsuperscript{KI/0} lymphomas by p53-mediated apoptosis, which also extended the survival of tumor-carrying mice. Interestingly, tumors resistant to 4-hydroxytamoxifen showed deletion of Arf or p53\textsuperscript{KI} after short-term p53 restoration, implying a powerful selection against the p53 pathway.

Ventura and colleagues addressed the same question using a distinct mouse model (Ventura et al., 2007). In this study, they generated a p53 allele (p53\textsuperscript{LSL}) with a loxP-flanked STOP cassette inserted in the intron 1 of the p53
locus. p53 expression from p53$^{LSL}$ is normally blocked by the STOP cassette, but restored when Cre recombinase recognizes loxP sites and removes the STOP cassette. p53$^{LSL/LSL}$ and p53$^{LSL/LSL}$ Cre-ER$^{T2}$ mice develop spontaneous lymphomas and sarcomas rapidly. p53 restoration by tamoxifen-activated Cre-ER$^{T2}$ led to tumor regression in p53$^{LSL/LSL}$ Cre-ER$^{T2}$ tumors. However, tamoxifen injection did not suppress p53$^{LSL/LSL}$ tumor progress, since no p53 expression was restored. Intriguingly, tumor-specific responses to p53 restoration were observed in this study. Upon p53 restoration, lymphomas showed apoptosis predominantly, and sarcomas showed senescence.

In another study, Xue and colleagues evaluated therapeutic efficacy of p53 restoration in a transplant murine liver carcinoma model (Xue et al., 2007). H-Ras$^{V12}$ transformed embryonic liver cells with repressed p53 expression by shRNA formed liver carcinomas quickly when transplanted into immunocompromised mice. p53 restoration by turning off shRNA expression led to complete regression of these liver carcinomas.

Wang et al. (2011) modeled p53 restoration in the presence of mutant p53 allele p53$^{515A}$ (encoding the p53R172H mutant protein) in spontaneous lymphomas and sarcomas. They generated a hypomorphic p53 allele (p53$^{Ne0}$) with loxP-flanked PGKneo cassette inserted in the intron 4 of the p53 locus. p53 expression from p53$^{Ne0}$ is repressed by the PGKneo cassette under normal conditions, and restored to wild-type levels when active Cre recombinase removes the PGKneo Cassette. p53 restoration by tamoxifen-activated Cre-ER resulted in tumor regression in p53$^{Ne0/-}$ Cre-ER tumors, while p53 restoration in
p53\textsuperscript{Neo/S15A} Cre-ER tumors only led to tumor stasis, suggesting that mutant p53R172H partially inhibits the restored wild-type p53 in the tumors. These four studies together indicate that p53 restoration \textit{in vivo} renders tumor suppression and therefore supports the concept that p53 gene delivery is an effective approach for cancer treatment.

**p53 Restoration in an Mdm2-overexpressing Background**

Clinical data demonstrate that a large number of human tumors have elevated Mdm2 levels but lack p53 mutations (Momand et al., 1998; Oliner et al., 1992), which suggest Mdm2 overexpression and p53 mutations are mutually exclusive since either mechanism inactivates the p53 pathway (Manfredi, 2010; Momand et al., 1998). Accumulating evidence also indicates that a proportion of human tumors exist, which feature both Mdm2 overexpression and loss of wild-type p53 (Alazzouzi et al., 2007; Burton et al., 2002; Ganly et al., 2000; Gorgoulis et al., 2000; Gorgoulis et al., 1996; Gunther et al., 2000; Jablkowski et al., 2005; Jiao et al., 2002; Li et al., 2000; Marks et al., 1996; Ohnstad et al., 2013; Watanabe et al., 1994). More interestingly, patients with tumors carrying both alterations generally show more malignant disease and poorer prognosis, compared to the ones with tumors bearing either alteration alone (Gorgoulis et al., 2000; Gorgoulis et al., 1996; Li et al., 2000; Marks et al., 1996). These observations suggest that alterations in both Mdm2 and p53 may confer additional growth advantages to tumor cells.
Previously, Jones et al demonstrated that $Mdm2^{Tg}$ mice with an average 4-fold increase in $Mdm2$ levels compared to wild-type mice developed tumors with 100% penetrance and long latency (Jones et al., 1998). The tumor onset in $Mdm2^{Tg} \ p53^{-/-}$ mice, occurred much earlier than that in $Mdm2^{Tg}$ mice, but showed no difference from that in $p53^{-/-}$ mice (Jones et al., 1998). Currently, there is no direct evidence showing that elevated Mdm2 levels and dampened p53 expression additively contribute to tumorigenesis in vivo. Moreover, coexistence of Mdm2 overexpression and loss of wild-type $p53$ in tumors raises a question whether restoration of wild-type $p53$ expression in tumors with both alterations will alleviate the diseases. The $p53^{Neo}$ allele developed in our laboratory serves as a unique tool to examine these two questions in vivo. First, as a hypomorphic allele, $p53^{Neo}$ expresses wild-type $p53$ at a very low level, which provides the best opportunity to test the suppression imposed on already decreased p53 levels by high levels of Mdm2. Second, the $p53^{Neo}$ allele can be converted to a fully functional $p53$ allele at will by addition of active Cre recombinase, which well serves the purpose of restoring wild-type p53 expression in established tumors.

To this end, I established a mouse model with ectopic expression of $Mdm2$ and dampened expression of $p53$ to examine the combinational effects of these two alterations on tumorigenesis and subsequently to investigate therapeutic efficacy of p53 restoration in tumors with high Mdm2 levels. In this dissertation, I demonstrated that high levels of Mdm2 and low levels of p53 act additively to dampen p53 activity in DNA damage response and tumor
development. Our data indicate that restoration of wild-type p53 expression in Mdm2-overexpressing angiosarcomas resulted in tumor stasis and regression in some cases. I further showed that the restored p53 suppressed cell proliferation in the Mdm2-overexpressing angiosarcomas but did not elicit apoptosis. Therefore, I conclude that restoration of wild-type p53 expression represents a potential strategy to treat tumors with high levels of Mdm2.
CHAPTER 2. RESULTS

Additive Effects of Mdm2 Overexpression and Decreased p53 Expression

Mdm2\textsuperscript{Tg} mice carrying an integrated Mdm2 cosmid and expressing on average 4-fold higher levels of Mdm2 compared to that of wild-type mice, are tumor prone (Jones et al., 1998), while the p53\textsuperscript{Neo/Neo} mice inheriting two copies of a hypomorphic p53 allele have decreased expression of wild-type p53 and are also tumor prone (Wang et al., 2011). To test whether Mdm2 overexpression and decreased p53 levels will combinatively affect p53 function, I crossed Mdm2\textsuperscript{Tg} mice with p53\textsuperscript{Neo/Neo} mice to generate Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} mice. Since the Mdm2 transgene in the Mdm2\textsuperscript{Tg} mice is under control of its native promoter (Jones et al., 1998) and p53 drives the expression of Mdm2 from its P2 promoter (Barak et al., 1994), it was of interest to see whether the hypomorphic p53\textsuperscript{Neo} allele with dampened p53 expression will affect expression of Mdm2 in the Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} mice. Therefore, I measured the Mdm2 mRNA level by quantitative real-time PCR (qRT-PCR) in wild-type, p53\textsuperscript{Neo/Neo}, Mdm2\textsuperscript{Tg}, Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} mice in the spleen, a radiosensitive tissue. As shown in Figure 3, the Mdm2 transcript levels in the spleens of Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} mice were comparable with that of Mdm2\textsuperscript{Tg} mice, and approximately 4-fold higher than that of wild-type and p53\textsuperscript{Neo/Neo} mice, suggesting that the p53 status did not affect expression of the Mdm2 transgene under physiological conditions.
Figure 3. *Mdm2* overexpression in *Mdm2*Tg and *Mdm2*Tg *p53Neo/Neo* mouse spleens. qRT-PCR analysis of *Mdm2* mRNA levels in the spleens from 4-6 week old mice with wild-type (WT) (n=5), *p53Neo/Neo* (N/N) (n=4), *Mdm2*Tg (M2Tg) (n=3), and *Mdm2*Tg *p53Neo/Neo* (M2Tg N/N) (n=4) genotypes. Data were normalized to expression in a wild-type spleen and represent a mean± SEM.
To verify that high levels of Mdm2 further dampens the p53 protein levels in the $Mdm2^{Tg} p53^{Neo/Neo}$ mice with already decreased p53 levels, I compared the p53 protein levels in the wild-type, $Mdm2^{Tg}$, $p53^{Neo/Neo}$, and $Mdm2^{Tg} p53^{Neo/Neo}$ mice. Since p53 levels are undetectable under normal conditions, I treated these mice with 6 Gy IR to stabilize p53 in the animals. Upon IR treatment, less p53 was stabilized in the spleens of $Mdm2^{Tg}$ and $p53^{Neo/Neo}$ mice compared to that of wild-type spleens, and p53 levels in the IR-treated $Mdm2^{Tg} p53^{Neo/Neo}$ mouse spleens were even less than that in $Mdm2^{Tg}$ and $p53^{Neo/Neo}$ spleens (Figure 4).

To further test whether the reduced levels of p53 protein in the $Mdm2^{Tg} p53^{Neo/Neo}$ mice affect its transcriptional activity, I compared the mRNA levels of p53 target genes $p21$ and $Puma$ in the spleens of wild-type, $Mdm2^{Tg}$, $p53^{Neo/Neo}$, and $Mdm2^{Tg} p53^{Neo/Neo}$ mice after IR treatment. There was an over 40-fold increase in the $p21$ mRNA levels in the IR-treated wild-type mouse spleens compared to that of the untreated wild-type spleens. Induction of $p21$ by IR was significantly reduced in $Mdm2^{Tg}$ and $p53^{Neo/Neo}$ spleens and further dampened in $Mdm2^{Tg} p53^{Neo/Neo}$ mouse spleens compared to wild-type spleens (Figure 5). A similar pattern was also observed for the other p53 target gene $Puma$ (Figure 5). These data together indicate that $Mdm2$ overexpression and decreased $p53$ levels additively compromise p53 function during DNA damage response.
Figure 4. p53 protein levels in IR treated mouse spleens. Western blot analysis of p53 expression in the spleens of IR (6 Gy)-treated 4-6 week old mice with wild-type (WT), p53^Neo/Neo (N/N), Mdm2^{Tg} (M2Tg), and Mdm2^{Tg} p53^{Neo/Neo} (M2Tg N/N) genotypes. For each genotype, protein lysate samples from 3 mouse spleens were pooled together. Protein lysate from an Mdm2^{-/-} p53^{-/-} spleen served as negative control. The number under each lane indicates the relative p53 levels compared to wild-type (set as 100%).
Figure 5. Induction of p53 target genes by IR in mice with different genotypes. qRT-PCR analysis of mRNA levels of p53 target genes p21 and Puma in the spleens from 4-6 week old wild-type (WT) (n=5), p53<sup>Neo/Neo</sup> (N/N) (n=4), Mdm2<sup>Tg</sup> (M2Tg) (n=3), and Mdm2<sup>Tg</sup> p53<sup>Neo/Neo</sup> (M2Tg N/N) (n=4) mice with or without 6 Gy IR treatment. Data were normalized to expression in a wild-type spleen without IR treatment and represent a mean± SEM.
To examine the additive effects of *Mdm2* overexpression and *p53* loss in tumorigenesis, I established *Mdm2*\(^{\text{Tg}}\), *p53*\(^{\text{Neo/Neo}}\), and *Mdm2*\(^{\text{Tg}}\) *p53*\(^{\text{Neo/Neo}}\) mouse cohorts for tumor studies. Interestingly, *Mdm2*\(^{\text{Tg}}\) *p53*\(^{\text{Neo/Neo}}\) mice showed a significant shorter tumor latency (348 days) compared to that of *Mdm2*\(^{\text{Tg}}\) (666 days) and *p53*\(^{\text{Neo/Neo}}\) mice (484 days) (Figure 6 and Table 1), indicating that *Mdm2* overexpression and *p53* loss cooperate to accelerate tumor formation. The most common tumor types observed in these mice were lymphomas and sarcomas (Table 2).

In summary, I established a cohort of *Mdm2*\(^{\text{Tg}}\) *p53*\(^{\text{Neo/Neo}}\) mice, which has lower *p53* levels and activities compared with *Mdm2*\(^{\text{Tg}}\) and *p53*\(^{\text{Neo/Neo}}\) mice. Moreover, *Mdm2*\(^{\text{Tg}}\) *p53*\(^{\text{Neo/Neo}}\) mice have earlier tumor onset than *Mdm2*\(^{\text{Tg}}\) or *p53*\(^{\text{Neo/Neo}}\) mice.
Figure 6. Tumor-free survival curves of $p53^{Neo/Neo}$, $Mdm2^{Tg}$, and $Mdm2^{Tg}$ $p53^{Neo/Neo}$ mice. Numbers in the brackets indicate numbers of mice in the given cohorts. $p$-values indicate the difference between two survival curves. Difference between survival curves was determined via Log-rank (Mantel-Cox) test.
Table 1. Median Tumor-free survival of $p53^{\text{Neo/Neo}}$, $Mdm2^{Tg}$, and $Mdm2^{Tg}$ $p53^{\text{Neo/Neo}}$ mice.

<table>
<thead>
<tr>
<th>Median Tumor-free Survival</th>
<th>$p53^{\text{Neo/Neo}}$</th>
<th>$Mdm2^{Tg}$</th>
<th>$Mdm2^{Tg}p53^{\text{Neo/Neo}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>484</td>
<td>666</td>
<td>348</td>
</tr>
</tbody>
</table>
Table 2. Tumor spectra of $p53^{Neo/Neo}$, $Mdm2^{Tg}$ and $Mdm2^{Tg} p53^{Neo/Neo}$ mice.

<table>
<thead>
<tr>
<th>Tumor Types</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$p53^{Neo/Neo}$ (n=19)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>7 (28.0%)</td>
</tr>
<tr>
<td>Sarcoma</td>
<td></td>
</tr>
<tr>
<td>Angiosarcoma</td>
<td>15 (60.0%)</td>
</tr>
<tr>
<td>Spindle cell sarcoma</td>
<td>8</td>
</tr>
<tr>
<td>Other sarcomas</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
</tr>
</tbody>
</table>
p53 Restoration in Mdm2-overexpressing Cells

To examine the growth suppressing effects of p53 restoration in Mdm2-overexpressing cells, I restored p53 expression in Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER MEFs using 4-hydroxytamoxifen to induce the DNA recombinase activity of CreER. Upon 4-hydroxytamoxifen treatment, the mRNA levels of p53 increased by 8-fold in the Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER MEFs compared with that of the vehicle-treated control cells (Figure 7). In MTT assays, 4-hydroxytamoxifen treatment significantly suppressed the growth of Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER MEFs compared with the vehicle-treated control (Figure 8), suggesting a correlation between p53 restoration and suppression of cell growth. I then investigated whether the p53 restoration would lead to enhanced p53 function in response to DNA damage. Therefore, I treated the Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER MEFs with DNA damaging drug doxorubicin following 4-hydroxytamoxifen treatment. After treatment with doxorubicin, expression of p53 target genes p21 and Noxa in the 4-hydroxytamoxifen-treated Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER MEFs was significantly higher than that in the control cells (Figure 9). These data collectively indicate that p53 restoration in Mdm2-overexpressing and p53-insufficient cells can rescue p53 function and lead to growth suppression.
Figure 7. p53 restoration in 4-hydroxytamoxifen-treated Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER MEFs. qRT-PCR analysis of p53 expression in the Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER MEFs treated with 1 µM 4-hydroxytamoxifen or vehicle control. 4OH-Tam, 4-hydroxytamoxifen.
Figure 8. p53 restoration suppresses growth of $Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER$ MEFs. MTT assays of $Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER$ MEFs treated with 1 µM 4-hydroxytamoxifen or vehicle control. 4OH-Tam, 4-hydroxytamoxifen.
Figure 9. p53 restoration led to elevated induction of p53 target genes in $Mdm2^{Tg} p53^{Neo/Neo} CreER$ MEFs. qRT-PCR analysis of p53 target gene $p21$ and $Noxa$ expression in the $Mdm2^{Tg} p53^{Neo/Neo} CreER$ MEFs. $Mdm2^{Tg} p53^{Neo/Neo} CreER$ MEFs treated with 1 µM 4-hydroxytamoxifen or vehicle control for 48 hours, followed by doxorubicin (1 µg/mL) treatment for 4 hours or vehicle treatment. 4OH-Tam, 4-hydroxytamoxifen; Doxo, doxorubicin.
To further investigate the effects of restoring p53 under oncogene activation conditions, I infected Mdm2\(^\text{Tg}\) p53\(^{\text{Neo/Neo}}\) CreER MEFs with an H-Ras\(^{\text{G12V}}\) retrovirus. Ectopic expression of H-Ras\(^{\text{G12V}}\) led to stabilization of p53 in these MEFs, and p53 restoration by 4-hydroxytamoxifen treatment further resulted in higher p53 protein levels in H-Ras\(^{\text{G12V}}\)-infected Mdm2\(^\text{Tg}\) p53\(^{\text{Neo/Neo}}\) CreER MEFs compared with that of the control cells (Figure 10). To test whether increased p53 levels are associated with enhanced p53 function in the H-Ras\(^{\text{G12V}}\)-infected Mdm2\(^\text{Tg}\) p53\(^{\text{Neo/Neo}}\) CreER MEFs, I measured expression of p53 target genes in these cells. The expression of p53 target genes p21 and Noxa was induced upon p53 restoration by 4-hydroxytamoxifen in the Mdm2\(^\text{Tg}\) p53\(^{\text{Neo/Neo}}\) CreER regardless of H-Ras\(^{\text{G12V}}\) infection (Figure 11). Interestingly, upon 4-hydroxytamoxifen treatment, the expression levels of p21 and Noxa were significantly higher in the H-Ras\(^{\text{G12V}}\)-infected MEFs than that of control cells (Figure 11), suggesting that oncogene activation potentiates function of restored p53 in the Mdm2-overexpressing cells. As illustrated with MTT assays, H-Ras\(^{\text{G12V}}\)-infected Mdm2\(^\text{Tg}\) p53\(^{\text{Neo/Neo}}\) CreER MEFs grew significantly slower than the vehicle-treated cells upon 4-hydroxytamoxifen treatment (Figure 12). In addition, in the presence of 4-hydroxytamoxifen, the growth of H-Ras\(^{\text{G12V}}\)-infected MEFs was also significantly slower than that of the cells without H-Ras\(^{\text{G12V}}\) infection (Figure 12). These data together indicate that reintroducing p53 expression in oncogene-overexpressing cells may confer more potent growth suppressing activities even in an Mdm2-overexpressing background.
In summary, p53 restoration in $Mdm2^{Tg} \ p53^{Ne^o/Ne^o} \ CreER$ MEFs led to increased p53 levels, and thus elevated p53 activities in response to both DNA damage and oncogene activation. In addition, p53 restoration suppressed the growth of Mdm2-overexpressing MEFs.
Figure 10. p53 restoration in $H-Ras^{G12V}$-infected $Mdm2^{Tg}$ $p53^{Neo/Neo}$ CreER MEFs. Western blot analysis of p53 expression in $H-Ras^{G12V}$-infected $Mdm2^{Tg}$ $p53^{Neo/Neo}$ CreER MEFs upon 1 µM 4-hydroxytamoxifen treatment for 48 hours. H-Ras, H-Ras$^{G12V}$. 4OH-Tam, 4-hydroxytamoxifen.
Figure 11. Induction of p53 target genes upon p53 restoration in $H\text{-Ras}^{G12V}$-infected $Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER$ MEFs. qRT-PCR analysis of p53 target genes $p21$ and Noxa upon 1 µM 4-hydroxytamoxifen treatment for 48 hours. H-Ras, H-Ras$^{G12V}$. 4OH-Tam, 4-hydroxytamoxifen.
Figure 12. p53 restoration suppresses growth of $H-Ras^{G12V}$-infected $Mdm2^{Tg}$ $p53^{Neo/Neo}$ CreER MEFs. MTT assays of $H-Ras^{G12V}$-infected $Mdm2^{Tg}$ $p53^{Neo/Neo}$ CreER MEFs incubated with 1 µM 4-hydroxytamoxifen or vehicle control. H-Ras, H-Ras$^{G12V}$. 4OH-Tam, 4-hydroxytamoxifen.
p53 Restoration Leads to Tumor Suppression in vivo

In order to examine the therapeutic effects of p53 restoration on tumors in vivo, I established a mouse cohort with both $Mdm2^{Tg}$ p53$^{Neo/Neo}$ CreER and the $Mdm2^{Tg}$ p53$^{Neo/Neo}$ genotypes. Previous studies have shown that expression of Cre recombinase in transgenic animals leads to developmental defects, due to Cre functions at non-conventional recombination sites (Forni et al., 2006; Loonstra et al., 2001; Schmidt et al., 2000). To test whether CreER affects tumor development in our mouse cohort, I compared the tumor-free survival curves between $Mdm2^{Tg}$ p53$^{Neo/Neo}$ CreER and the $Mdm2^{Tg}$ p53$^{Neo/Neo}$ mice. The tumor-free survival curves of untreated mice from both genotypes overlap with each other (Figure 13), indicating that the presence of CreER transgene did not affect tumor development in the $Mdm2^{Tg}$ p53$^{Neo/Neo}$ CreER group. In order to screen spontaneous tumors at early stages, tumor formation in both genotypes was closely monitored with magnetic resonance imaging (MRI) bi-weekly. After tumors reached a size of $>50\text{mm}^3$, a proportion of tumor-bearing mice underwent weekly tamoxifen treatment for up to 4 weeks. Three major tumor types were captured in the tamoxifen-treated group, which were lymphomas, spindle cell sarcomas, and angiosarcomas. To examine the effects of p53 restoration in these tumors, I first analyzed the recombination rate of p53$^{Neo}$ allele in the $Mdm2^{Tg}$ p53$^{Neo/Neo}$ CreER tumors upon tamoxifen treatment. An average of 7% recombination rate was observed in the lymphomas after 4 doses of tamoxifen treatment (Figure 14). Three treated $Mdm2^{Tg}$ p53$^{Neo/Neo}$ CreER spindle cell
sarcomas showed highly variable recombination rates ranging from 9% to 61% (Figure 14). The $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}} \ CreER$ angiosarcomas had a uniform recombination rate with an average of 33% upon 4 doses of tamoxifen treatment (Figure 14). Therefore, I focused on these angiosarcomas and used them as a model system to study the effects of p53 restoration on Mdm2-overexpressing tumors (Table 3).

To examine the therapeutic potential of p53 restoration in mice with tumors overexpressing Mdm2, I compared the survival curves of tumor-carrying $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}} \ CreER$ and $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}}$ mice during tamoxifen treatment. Upon tamoxifen treatment, the angiosarcoma-bearing mice of the genotype $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}} \ CreER$ survived significantly longer than that of $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}}$ (Figure 15), suggesting that p53 restoration can prolong the survival of these tumor-carrying mice. To examine the effects of p53 restoration in tumor suppression, I then compared the changes in tumor volumes (post-treatment/pre-treatment ratio) between the $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}} \ CreER$ and $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}}$ groups. As shown in Figures 16 and 17, $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}} \ CreER$ angiosarcomas grew slower than the $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}}$ angiosarcomas upon tamoxifen treatment. To exclude the possibility that differences in post-treatment/pre-treatment ratios between these two mouse genotypes were due to the differences in initial tumor volumes, I compared the pre-treatment volumes (initial volumes) of angiosarcomas in both genotypes. There were no significant differences in tumor volumes between $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}} \ CreER$ and $\text{Mdm2}^{Tg} \ p53^{\text{Neo/Neo}}$ angiosarcomas at the time that tamoxifen treatment started (Figure
suggesting that p53 restoration by tamoxifen treatment led to growth suppression in the Mdm2-overexpressing angiosarcomas.

In summary, I have established a mouse model for restoring p53 in Mdm2-overexpressing tumors. Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER angiosarcomas were chosen as a p53 restoration model system due to their significant levels of recombined p53\textsuperscript{Neo} alleles after tamoxifen treatment. Upon p53 restoration in such tumors, growth suppression of tumors was observed, which is associated with prolonged survival of tumor-carrying mice.
Figure 13. Tumor-free survival curves of $Mdm2^{Tg} p53^{Neo/Neo}$ and $Mdm2^{Tg} p53^{Neo/Neo} CreER$ mice. Numbers in the brackets indicate mouse numbers in the given cohorts. Median tumor-free survival of $Mdm2^{Tg} p53^{Neo/Neo}$ mice is 348 days, and 357 days for $Mdm2^{Tg} p53^{Neo/Neo} CreER$. Difference between survival curves was determined via Log-rank (Mantel-Cox) test.
Figure 14. $p53^{\text{Neo}}$ recombination in tamoxifen-treated $\text{Mdm2}^{\text{Tg}}p53^{\text{Neo/Neo}} \text{CreER}$ tumors. a, PCR analysis of recombined $p53^{\text{Neo}}$ alleles in tumors treated with 4 doses of tamoxifen (A. angiosarcoma, L. lymphoma. S. spindle cell sarcoma) b, recombination rates of $p53^{\text{Neo}}$ alleles calculated from Figure 14a using ImageJ software.
Table 3. List of $Mdm2^{Tg\, p53^{Neo/Neo}}$ and $Mdm2^{Tg\, p53^{Neo/Neo\, CreER}}$ angiosarcomas monitored in the MRI study.

Tumor volumes are calculated from the stack of tumor MRI images; tumor volume changes are the fold changes of tumor volumes at the endpoint versus at the start point. Tumor growth rate is calculated based on the assumption that tumors undergo exponential growth.

<table>
<thead>
<tr>
<th>Tumor ID</th>
<th>Tumor Type</th>
<th>Genotype</th>
<th>Treatment Duration (Days)</th>
<th>Tumor Volumes (mm$^3$) Pre-Tx</th>
<th>Tumor Volume Changes (Post-Tx/Pre-Tx)</th>
<th>Tumor Growth Rate (%/Week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo}}$</td>
<td>22</td>
<td>263.81</td>
<td>587.25</td>
<td>2.23</td>
<td>29.00</td>
</tr>
<tr>
<td>2</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo}}$</td>
<td>22</td>
<td>142.5</td>
<td>1345.81</td>
<td>9.44</td>
<td>104.31</td>
</tr>
<tr>
<td>3</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo}}$</td>
<td>14</td>
<td>59.13</td>
<td>327</td>
<td>5.53</td>
<td>135.18</td>
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<tr>
<td>4</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo}}$</td>
<td>28</td>
<td>1065.31</td>
<td>1873.94</td>
<td>1.73</td>
<td>14.63</td>
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<td>5</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo}}$</td>
<td>28</td>
<td>65.25</td>
<td>170.19</td>
<td>2.61</td>
<td>27.08</td>
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<td>113.81</td>
<td>1310.31</td>
<td>11.51</td>
<td>64.20</td>
</tr>
<tr>
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<td>71.69</td>
<td>2.49</td>
<td>25.60</td>
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<tr>
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<td>236.07</td>
<td>269.47</td>
<td>1.14</td>
<td>3.36</td>
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<tr>
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<td>46.75</td>
<td>47.94</td>
<td>0.98</td>
<td>-0.40</td>
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<tr>
<td>10</td>
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<td>29</td>
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<td>400.12</td>
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<td>11</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo, CreER}}$</td>
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<td>37.56</td>
<td>58.63</td>
<td>1.56</td>
<td>11.76</td>
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<tr>
<td>12</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo, CreER}}$</td>
<td>28</td>
<td>18.36</td>
<td>12.30</td>
<td>0.64</td>
<td>-10.60</td>
</tr>
<tr>
<td>13</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo, CreER}}$</td>
<td>14</td>
<td>68</td>
<td>76.75</td>
<td>1.13</td>
<td>6.24</td>
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<tr>
<td>14</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo, CreER}}$</td>
<td>15</td>
<td>508.81</td>
<td>164.31</td>
<td>0.32</td>
<td>-40.99</td>
</tr>
<tr>
<td>15</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo, CreER}}$</td>
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<td>463.06</td>
<td>405.94</td>
<td>0.87</td>
<td>-3.50</td>
</tr>
<tr>
<td>16</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo, CreER}}$</td>
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<td>357.31</td>
<td>299.44</td>
<td>0.97</td>
<td>-9.52</td>
</tr>
<tr>
<td>17</td>
<td>Angiosarcoma $Mdm2^{Tg, p53^{Neo/Neo, CreER}}$</td>
<td>28</td>
<td>120.5</td>
<td>65.94</td>
<td>0.55</td>
<td>-13.99</td>
</tr>
</tbody>
</table>
Figure 15. Survival curves of angiosarcoma-carrying $Mdm2^{Tg}$ $p53^{Neo/Neo}$ CreER and $Mdm2^{Tg}$ $p53^{Neo/Neo}$ mice upon tamoxifen treatment. Numbers in the brackets indicate mouse numbers in the given cohorts. Difference between survival curves was determined via Log-rank (Mantel-Cox) test.
Figure 16. p53 restoration led to suppression of tumor growth in $Mdm^{2Tg}$ $p53^{Neo/Neo}$ CreER angiosarcomas. Tumor volume changes (post-treatment volume/pre-treatment volume ratio) of angiosarcomas in $Mdm^{2Tg}$ $p53^{Neo/Neo}$ CreER and $Mdm^{2Tg}$ $p53^{Neo/Neo}$ mice during tamoxifen treatment.
Figure 17. Representative MRI images of tamoxifen-treated $Mdm2^{Tg}$ $p53^{Neo/Neo}$ CreER angiosarcomas. Tumor MRI images and changes in tumor volumes of two representative angiosarcomas (Upper: $Mdm2^{Tg}$ $p53^{Neo/Neo}$ CreER; lower: $Mdm2^{Tg}$ $p53^{Neo/Neo}$). Day 0, the day treatment started; day 28, 28 days after initial tamoxifen treatment, etc.
Figure 18. Initial tumor volumes of Mdm2<sup>Tg</sup> p53<sup>Neo/Neo</sup> and Mdm2<sup>Tg</sup> p53<sup>Neo/Neo</sup> CreER angiosarcomas. Initial tumor volume, the tumor volume when the first tamoxifen treatment was initiated.
A Syngeneic Mouse Model for p53 Restoration

I then aimed to determine what types of responses occurred with restoring p53 in the Mdm2-overexpressing angiosarcomas. Although higher average levels of p53 target genes such as \( p21 \) and \( Mdm2 \) were observed in \( Mdm2^{Tg} p53^{Neo/Neo} \) CreER angiosarcomas compared to that of the \( Mdm2^{Tg} p53^{Neo/Neo} \) angiosarcomas in response to tamoxifen administration, the difference between two groups were not statistically significant (Figure 19). One plausible explanation for this unexpected observation is the heterogeneity of tumors, which might contribute to in-group variation and lack of differences between groups. To eliminate this in-group variation, I established a syngeneic tumor mouse model of angiosarcomas. Briefly, a spontaneous \( Mdm2^{Tg} p53^{Neo/Neo} \) CreER angiosarcoma was processed into single cell suspension, and injected into the subcutaneous flank regions of multiple syngeneic mice. In this transplant model, over 90% of \( p53^{Neo} \) alleles in the transplant tumors were recombined upon tamoxifen administration (Figure 20). In agreement with previous data, the tamoxifen-treated angiosarcomas grew significantly slower than the vehicle-treated controls (Figure 21). Since transplanted tumors reached the maximum allowable size faster in the vehicle-treated control group than that of tamoxifen-treated group, two groups of mice were under treatments for different durations. Therefore, the tumor volume changes during the treatment periods may not reflect the actual differences between these two groups of tumors. In order to illustrate the differences more accurately, I compared the tumor doubling times between the
tamoxifen-treated and control groups. Briefly, tumor doubling time was calculated based on the exponential curve fit of the tumor volume against time. The tumor doubling time of \( Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER \) angiosarcomas upon tamoxifen administration (10.9 days) was much longer than that of \( Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER \) angiosarcomas undergoing vehicle treatment (4.5 days) (Figure 22). These data collectively indicate a positive correlation between p53 restoration and tumor growth suppression in the transplant tumor model.

In order to examine upregulation of p53 activity after p53 restoration, I measured the protein levels of p53 and the p53 target p21 in both tamoxifen-treated and control \( Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER \) angiosarcomas. p53 restoration by tamoxifen injection in these transplant angiosarcomas led to accumulation of p53 and p21 proteins compared with control tumors (Figure 23). I then examined the induction of p53 target gene expression in the transplant tumors of \( Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER \) angiosarcomas upon tamoxifen injection. In comparison to the untreated tumors, transplant tumors treated with tamoxifen expressed significantly higher levels of p53 targets \( p21 \) and \( Puma \) (Figure 24). These data together indicate that tamoxifen administration in the transplant \( Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER \) angiosarcomas restored expression of functional p53 that led to elicitation of gene expression downstream to p53.

In summary, I have established a syngeneic mouse model for p53 restoration in \( Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER \) angiosarcomas. p53 restoration in such tumors led to upregulation of p53 and p53 target genes, which suppressed the growth of tumors.
Figure 19. Induction of p53 target genes in tamoxifen-treated $Mdm2^{Tg} p53^{Neo/Neo}$ and $Mdm2^{Tg} p53^{Neo/Neo} CreER$ angiosarcomas. qRT-PCR analysis of p53 target gene expression in tamoxifen-treated spontaneous $Mdm2^{Tg} p53^{ Neo/Neo}$ and $Mdm2^{Tg} p53^{ Neo/Neo} CreER$ angiosarcomas. 1X tamoxifen, 1 dose of tamoxifen treatment; 4X tamoxifen, 4 doses of tamoxifen treatment.
Figure 20. $p53^{\text{Neo}}$ Recombination in transplanted $\text{Mdm2}^{\text{Tg}}$ $p53^{\text{Neo}/\text{Neo}}$ $\text{CreER}$ angiosarcomas after tamoxifen treatment. PCR analysis of $p53^{\text{Neo}}$ allele recombination in two representative transplanted $\text{Mdm2}^{\text{Tg}}$ $p53^{\text{Neo}/\text{Neo}}$ $\text{CreER}$ angiosarcomas upon tamoxifen treatment.
Figure 21. p53 restoration led to growth suppression of transplant $Mdm2^{Tg}$ $p53^{Neo/Neo}$ CreER angiosarcomas. Transplant tumor sizes were measured with digital calipers twice a week and tumor volumes were calculated using the equation $[(\text{width}^2 \times \text{length})/2]$ as described by Bearss et al., 2000 (Bearss et al., 2000). a) Growth curves of transplanted angiosarcomas under tamoxifen and vehicle control treatments. b) Comparison of tumor volume changes between angiosarcomas undergoing tamoxifen and vehicle control treatments.
Figure 22. Tumor doubling times of tamoxifen-treated and control transplant *Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER* angiosarcomas. Tumor doubling time was calculated based on the exponential curve fit of the tumor volume against time.
Figure 23. p53 restoration resulted in increased protein levels of p53 and p21 in transplanted $Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER$ angiosarcomas. Western blot analysis of p53 and p21 levels in tamoxifen-treated and vehicle control $Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER$ transplanted angiosarcomas. Three representative tumors from both control and tamoxifen-treated groups were examined. $Mdm2^{-/-} \ p53^{-/-}$ spleen protein extract was used as a negative control.
Figure 24. Induction of p53 target genes upon tamoxifen treatment in transplanted $Mdm2^{Tg} p53^{Neo/Neo}$ CreER angiosarcomas. qRT-PCR analysis of expression of p53 target genes ($p21$ and $Puma$) in tamoxifen-treated and vehicle control transplant $Mdm2^{Tg} p53^{Neo/Neo}$ CreER angiosarcomas.
Suppression of Cell Proliferation Upon p53 Restoration

As aforementioned, activation of p53 can exert different biological functions including cell cycle arrest, senescence and apoptosis. To examine the biological and cellular effects of p53 restoration in the $Mdm2^{Tg} p53^{Neo/Neo}$ CreER angiosarcomas, I analyzed the proliferation index by immunohistochemistry in tumors with chronic (4 doses) tamoxifen treatment. Upon tamoxifen treatment, an average of 48% of cells were positive for proliferation marker Ki-67 in $Mdm2^{Tg} p53^{Neo/Neo}$ angiosarcomas, while approximately only 20% of cells were positively stained with Ki-67 antibody in the $Mdm2^{Tg} p53^{Neo/Neo}$ CreER angiosarcomas (Figure 25a, 25c, and 25d), indicating that chronic tamoxifen treatment-mediated p53 restoration leads to cell cycle arrest in $Mdm2$-overexpressing angiosarcomas. I also analyzed Ki-67 positivity in the $Mdm2^{Tg} p53^{Neo/Neo}$ CreER angiosarcomas at 3 days after 1 dose of tamoxifen treatment to examine the acute effects of p53 restoration. The percentage of Ki-67 positive cells between $Mdm2^{Tg} p53^{Neo/Neo}$ CreER angiosarcomas undergoing chronic and acute tamoxifen treatment was comparable to each other (Figure 25b, 25c and 25d), suggesting that p53 restoration may result in sustained cell cycle arrest in $Mdm2$-overexpressing angiosarcomas.
Figure 25. p53 restoration resulted in reduced cell proliferation in Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER angiosarcomas. Tamoxifen-treated Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} and Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER angiosarcomas were subjected to immunohistochemical staining for proliferation marker Ki-67. a), b) and c) Images of the representative angiosarcomas from the tamoxifen-treated Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} mice, and Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER mice with 1 dose or 4 doses of tamoxifen treatment, respectively. d) Percentages of cells positive for Ki-67 in tamoxifen-treated Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} and Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER angiosarcomas. Four random fields (X40 magnification) for each tumor were chosen to calculate the percentages. Differences in the percentages of Ki-67 positive cells between genotypes were analyzed with 2-tailed Student’s t test.
I also analyzed the expression of cellular senescence in the tamoxifen-treated spontaneous angiosarcomas. In $Mdm2^{Tg} p53^{Neo/Neo} CreER$ angiosarcomas, 3 out of 9 tumors treated with 4 doses of tamoxifen and 4 out of 5 treated with 1 dose of tamoxifen were positive for IHC staining of senescence marker PML; and no positive PML staining were observed in any tamoxifen-treated $Mdm2^{Tg} p53^{Neo/Neo}$ angiosarcomas (n=6) (Figure 26). Although no statistically significant difference ($p=0.11$, Chi-square analysis) was observed for the PML staining between tamoxifen-treated $Mdm2^{Tg} p53^{Neo/Neo}$ angiosarcomas and $Mdm2^{Tg} p53^{Neo/Neo} CreER$ angiosarcomas with 4 doses of tamoxifen, the PML staining of $Mdm2^{Tg} p53^{Neo/Neo} CreER$ angiosarcomas with 1 dose of tamoxifen was very significantly different from that of tamoxifen-treated $Mdm2^{Tg} p53^{Neo/Neo}$ angiosarcomas ($p<0.01$, Chi-square analysis). Therefore, the data here suggest that restoration of functional p53 expression induces a senescence response at least in a proportion of the $Mdm2$-overexpressing angiosarcomas.

In order to analyze the apoptotic effects of p53 restoration in the $Mdm2^{Tg} p53^{Neo/Neo} CreER$ tumors, I compared the levels of cleaved caspase 3, an apoptosis marker, by immunohistochemistry between the tamoxifen-treated $Mdm2^{Tg} p53^{Neo/Neo} CreER$ and $Mdm2^{Tg} p53^{Neo/Neo}$ angiosarcomas. Interestingly, no apoptosis was induced in the $Mdm2^{Tg} p53^{Neo/Neo} CreER$ angiosarcomas with either chronic or acute tamoxifen treatment compared to $Mdm2^{Tg} p53^{Neo/Neo}$ angiosarcomas (Figure 27), indicating that reintroduction of p53 in these tumors does not induce an apoptotic response mediated by cleaved caspase 3.
In summary, p53 restoration in $Mdm^{2Tg} \ p53^{Neo/Neo}$ angiosarcomas only induced suppression of cell proliferation and senescence but not apoptosis, which is consistent with observations reported by previous studies that p53 restoration in angiosarcomas lacking $p53$ did not result in an apoptotic phenotype (Ventura et al., 2007; Wang et al., 2011). These data collectively suggest that intrinsic characteristics of angiosarcomas dictate their responses to p53 restoration.
Figure 26. p53 restoration resulted in induction of senescence in $Mdm2^{Tg} p53^{Neo/Neo}$ CreER angiosarcomas. Tamoxifen-treated $Mdm2^{Tg} p53^{Neo/Neo}$ and $Mdm2^{Tg} p53^{Neo/Neo}$ CreER angiosarcomas were subjected to immunohistochemical staining for senescence marker PML. a), b) and c) Images of the representative angiosarcomas from the tamoxifen-treated $Mdm2^{Tg} p53^{Neo/Neo}$ mice, and $Mdm2^{Tg} p53^{Neo/Neo}$ CreER mice with 1 dose or 4 doses of tamoxifen treatment.
Figure 27. p53 restoration did not result in apoptosis in Mdm2\textsuperscript{Tg} \textit{p53\textsuperscript{Neo/Neo}}\textit{ CreER} angiosarcomas. Tamoxifen-treated Mdm2\textsuperscript{Tg} \textit{p53\textsuperscript{Neo/Neo}} and Mdm2\textsuperscript{Tg} \textit{p53\textsuperscript{Neo/Neo}} \textit{CreER} angiosarcomas were subjected to immunohistochemical staining for apoptosis marker cleaved caspase 3. a), b) and c) Images of the representative angiosarcomas from the tamoxifen-treated Mdm2\textsuperscript{Tg} \textit{p53\textsuperscript{Neo/Neo}} mice, and Mdm2\textsuperscript{Tg} \textit{p53\textsuperscript{Neo/Neo}} \textit{CreER} mice with 1 dose or 4 doses of tamoxifen treatment. d) Image of an IR-treated mouse spleen as a positive control. CC3, cleaved caspase 3.
CHAPTER 3. DISCUSSION

Mdm2 negatively regulates p53 functions by suppressing its transcription activity and targeting it for degradation. Thus, it is reasonable to postulate that restoring p53 expression in tumors with high levels of Mdm2 may not render therapeutic effectiveness. However, patients with tumors featuring both Mdm2 overexpression and p53 mutation have more malignant diseases and poorer prognosis than patients with tumors carrying either alteration alone. In addition, Mdm2\textsuperscript{Tg} mice in p53\textsuperscript{−/−} background develop tumors significantly earlier than Mdm2\textsuperscript{Tg} mice with wild-type p53. These data suggest an additive effect of Mdm2 overexpression with loss of p53. Therefore, p53 restoration in Mdm2-overexpressing tumors may lead to alleviated diseases. To test whether p53 restoration in Mdm2-overexpressing tumors would have therapeutic efficacy, we established a mouse model with high levels of Mdm2 and decreased p53. Upon restoring wild-type p53 expression, Mdm2-overexpressing angiosarcomas underwent growth suppression and regression, suggesting that increasing p53 expression levels can render therapeutic benefits to tumors with high levels of Mdm2. This finding provides in vivo experimental evidence for treating Mdm2-overexpressing tumors by p53 gene delivery.

p53 Reactivation in Mdm2-overexpressing Tumors

Growth suppression was observed in Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} CreER angiosarcomas after p53 restoration, similar to the effects of p53 restoration in
previous studies, which showed that p53 restoration in p53-deficient tumors results in tumor suppression (Wang et al., 2011; Xue et al., 2007). However, the findings from the present study are also distinct from previous reports. Both tumor stasis and regression were observed when two copies of wild-type p53 were restored in Mdm2<sup>Tg</sup> p53<sup>Neo/Neo</sup> CreER angiosarcomas, whereas restoration of a single copy of wild-type p53 led to regression in all angiosarcomas not carrying the Mdm2 transgene (Wang et al., 2011). Such attenuated response to p53 restoration in the present study may be ascribed to the higher levels of Mdm2 in Mdm2<sup>Tg</sup> p53<sup>Neo/Neo</sup> CreER angiosarcomas.

Our present study indicates that elevating p53 expression levels confers tumor suppression in Mdm2-overexpressing angiosarcomas, it is therefore of interest to ask why high levels of Mdm2 in these tumors does not totally mask the activities of restored p53 in these tumors. Previous studies have shown that components of Myc and Ras oncogenic pathways are overexpressed in majority of angiosarcoma cases (Fernandez-Medarde and Santos, 2011; Guo et al., 2011; Kurisetty and Bryan, 2013; Przygodzki et al., 1997; Shon et al., 2013; Zietz et al., 1998). Sustained activation of such oncogene pathways is indispensable for tumor progression (Weinstein, 2002), since blocking the signaling of these oncogenic pathways shows tumor-suppressive effects (Downward, 2003; LaMontagne et al., 2000; Singh et al., 2009; Soucek et al., 2008; Soucek et al., 2013). Activation of the Ras and Myc oncogenic pathways results in upregulation of tumor suppressor Arf that binds Mdm2 and releases p53 from its talons (Ozenne et al., 2010), and subsequently leads to accumulation and activation of
p53 (Dai et al., 2006; Ozenne et al., 2010). Interestingly, activation of Myc and Ras oncogenic pathways in cells also cause genomic instability by elevated production of reactive oxygen species (ROS) and suppression of DNA damage repair (Felsher and Bishop, 1999; Fest et al., 2002; Li et al., 2012; Saavedra et al., 1999; Saavedra et al., 2000; Soucek and Evan, 2002; Wade and Wahl, 2006). DNA damage sensor kinases such as ATM and ATR in response to such genomic stresses catalyze phosphorylation of p53, which disrupts Mdm2-p53 interaction and thus activates p53. Hence, in our Mdm2-overexpressing angiosarcomas, it is likely that constitutive activation of oncogenic pathways by overexpression of Myc and Ras pathway components results in activation of restored p53 in the presence of high levels of Mdm2. This notion is in agreement with our data in MEFs, which clearly showed that ectopically expressing oncogene $H-Ras^{G12V}$ led to accumulation of p53 and potentiated p53 transcriptional activity upon restoration even in an Mdm2-overexpressing setting.

It is well documented that tumors in general impose selective pressure on the p53 pathway in order to maintain aberrant growth (Sherr, 1998). And yet, in the present study, reactivated p53 induces expression of downstream target genes such as $p21$ and $Puma$, and therefore leads to growth suppression of tumors, suggesting that the p53 pathway remains intact except for the introduced alterations on $p53$ and $Mdm2$. One plausible explanation for such an observation is that due to the excessive amount of Mdm2 and the extremely low level of p53 in $Mdm2^{Tg} p53^{Neo/Neo} CreER$ angiosarcomas, it is unlikely that selection pressure is imposed on the p53 pathway, and thus other components of the p53 pathway
remain intact in these tumors, which warrant the therapeutic efficacy of p53 restoration. However, further investigation is needed to understand the mechanisms of activating restored p53 in the Mdm2-overexpressing angiosarcomas, which may lead to better therapies of tumors with high levels of Mdm2.

**Effects of p53 Gene Dosage**

In the present study, I have showed that Mdm2\(^{Tg}\) p53\(^{Neo/Neo}\) mice have dampened levels and activities of p53 compared to Mdm2\(^{Tg}\) or p53\(^{Neo/Neo}\) mice, which leads to earlier tumor onset in the Mdm2\(^{Tg}\) p53\(^{Neo/Neo}\) mice. This observation is consistent with earlier studies showing that p53\(^{-/-}\) mice develop tumor significantly earlier than p53\(^{+/−}\) mice (Donehower et al., 1992; Jacks et al., 1994). These data collectively indicate p53 exerts tumor-suppressive functions in a dose-dependent manner.

As to the dose-dependent function of p53 in Mdm2-overexpressing tumors, clear discrepancy exists in clinical data. The fact that majority of Mdm2-overexpressing tumors lack p53 mutations (Momand et al., 1998; Oliner et al., 1992) leads to the speculation that Mdm2 overexpression and loss of wild-type p53 are mutually exclusive events during tumor development. But the observation that concomitant existence of Mdm2 overexpression and p53 mutations in human cancers results in more malignant diseases and poorer prognosis than either alteration alone (Gorgoulis et al., 2000; Gorgoulis et al., 1996; Li et al., 2000; Marks et al., 1996) suggests that loss of p53 provides
additional growth advantage to tumor cells even in the present of high levels of Mdm2. However, there was no direct evidence for dose-dependent tumor-suppressive activity of p53 in Mdm2-overexpressing tumors. In the present study, the observation that $Mdm2^{Tg}$ $p53^{Neo/Neo}$ mice have earlier tumor onset than $Mdm2^{Tg}$ mice also suggests that loss of p53 confers growth advantage even in the presence of high levels of Mdm2, providing support for dose-dependent effects of p53 in Mdm2-overexpressing settings.

In a previous study, $Mdm2^{Tg/Tg}$ mice had significantly shortened tumor-free survival compared to $Mdm2^{Tg/+}$ mice (Jones et al., 1998). In Eu-myc transgenic mice, deletion of one copy of Mdm2 results in augmented p53 activities and thus delays onset of lymphomas driven by the Eu-myc transgene compared to transgenics with intact Mdm2 alleles (Alt et al., 2003). These observations together suggest that the Mdm2:p53 ratio is critical for tumorigenesis. In the present study, p53 restoration in $Mdm2^{Tg} p53^{Neo/Neo}$ CreER angiosarcomas leads to tumor growth suppression implies that the Mdm2:p53 ratio may also be important for the maintenance of established tumors.

**Clinical Implications**

Pharmaceutically restoring p53 function in Mdm2-overexpressing tumors includes identifying Mdm2 inhibitors. However, the nature of Mdm2 inhibitors determines the limitations of these pharmaceuticals in tumor treatments. Since the therapeutic efficacy of Mdm2 inhibitors is dependent on the presence of wild-type p53 in the tumors (Li and Lozano, 2013), treatments with the Mdm2
inhibitors are unlikely to induce anti-tumor effects in tumors with concurrent
Mdm2 overexpression and p53 loss. Moreover, a number of studies have
revealed that administration of Mdm2 inhibitors including nutlin-3 and Mi-219 to
tumor cells with wild-type p53 potently selects for p53 mutations and thus confers
resistance to such drugs (Aziz et al., 2011; Jones et al., 2012; Long et al., 2010;
Michaelis et al., 2011). Hence, alternative approaches, e.g. p53 gene delivery,
which overcomes such limitations of Mdm2 inhibitors, may benefit patients
carrying tumors with high levels of Mdm2. Previous studies have demonstrated
that ectopically expressing wild-type p53 via gene transfer confers anti-tumor
effects in both pre-clinical and clinical studies (Cai et al., 1993; Fujiwara et al.,
1994; Roth et al., 1996). However, there is no information available for p53 gene
delivery in Mdm2-overexpressing tumors in human. It is natural to speculate that
this approach may not be effective for treatment of Mdm2-overexpressing tumors,
since high levels of Mdm2 can readily degrade p53 and suppress its
transcriptional activity. Using a genetically engineered mice to model p53
restoration in Mdm2-overexpressing tumors, our present study indicates that
restoring expression of p53 in tumors with high levels of Mdm2 is able to
suppress tumor growth.

However, it should be noted that our present model does not accurately
mimic high levels of Mdm2 in human cancers. Many human tumors that retain
wild-type p53 have 10-50 fold increase in Mdm2 levels (Alazzouzi et al., 2007;
Forslund et al., 2008; Onel and Cordon-Cardo, 2004), which cannot be achieved
in the Mdm2Tg mouse model due to embryonic lethality caused by very high
levels of Mdm2 (Jones et al., 1998). And yet, our Mdm2\textsuperscript{Tg} p53\textsuperscript{Neo/Neo} mouse tumor model with ~4-fold increase in Mdm2 levels and ~5-fold decrease in p53 levels better mimics the Mdm2:p53 ratio in human Mdm2-overexpressing tumors with 20-fold increase in Mdm2 levels and wild-type p53. As to Mdm2:p53 ratio, p53 restoration in our Mdm2-overexpressing mouse tumors is similar to overexpression of p53 by 5 folds in human tumors with high levels of Mdm2. Therefore, growth suppression of Mdm2-overexpressing tumors by p53 restoration manifested in our current study suggests potential effectiveness of p53 gene therapy of human tumors featuring high Mdm2 levels.

A study recently published by our group demonstrated that treatment of mouse MMTV-Wnt1 mammary tumors with the DNA-damaging drug doxorubicin resulted in inferior antitumor responses such as less extent of regression and earlier relapse in tumors with wild-type p53 compared to tumors with mutant p53 (Jackson et al., 2012). Upon doxorubicin treatment, mammary tumor cells with mutant p53 failed to arrest, divided with extensive DNA damage, and underwent mitotic catastrophe, while tumor cells with wild-type p53 circumvented apoptosis via p53-mediated proliferative arrest (Jackson et al., 2012). Such wild-type p53-associated inferior responses to chemotherapies were also observed in other preclinical and clinical studies (Kim et al., 2009; Wong et al., 2013). These observations together suggest that the presence of wild-type p53 in tumors may undermine the effectiveness of therapeutic regimens at least in particular conditions. Therefore, it is of important clinical relevance to ask whether p53 reactivation will cooperate with or antagonize conventional DNA damage p53-
activating chemotherapies and radiotherapy in tumor treatment. Intriguingly, combining p53 restoration with IR treatment in *Eu-myc* lymphomas prolongs the survival of tumor-carrying mice compared to treatment of either regimen alone (Martins et al., 2006). Reactivation of p53 by inhibitors of Mdm2 and Mdm4 also cooperates with DNA-damaging pharmaceuticals to induce growth inhibition and apoptosis in various human cell lines of different tumor types including breast cancer, pancreatic cancer, colon cancer, liver cancer, neuroblastoma, retinoblastoma, and sarcomas (Azmi et al., 2011; Barbieri et al., 2006; Costa et al., 2013; Ohnstad et al., 2011; Zheng et al., 2010). These data collectively suggest p53 reactivation can sensitize tumors to treatments with DNA-damaging agents. Since it is out of our current focus, DNA-damaging drug treatment combined with p53 restoration to *Mdm2*-overexpressing tumors was not performed in the present study. And yet, our *in vitro* indicate that p53 restoration cooperates with doxorubicin treatment in induction of p53 target genes in *Mdm2*-overexpressing MEFs, suggesting that p53 restoration potentiates p53 activity induced by DNA damaging drugs in an *Mdm2*-overexpressing background. Future studies with such combinatorial treatments may help us to understand whether and how restored wild-type p53 affect tumor responses to DNA-damaging agents in *Mdm2*-overexpressing tumors.

**Future Directions**

Identification of cellular determinant(s) of p53-induced apoptosis and cell cycle arrest
Although p53 exerts its growth-suppressive activities by inducing apoptosis, cell cycle arrest and senescence programs (Jackson et al., 2011), different tissues respond differently to a variety of stimuli due to understudied mechanisms (Jackson et al., 2011). In an early study, p53 expression in colorectal cancer cell lines yielded arrest phenotypes in some lines and apoptotic response in others (Polyak et al., 1996). Interestingly, deletion of p21 (an effector of p53-mediated cell cycle arrest) in arrested cell lines led to p53-induced apoptosis (Polyak et al., 1996). Moreover, hybrid cells resulted from fusing an arrested line to an apoptotic line underwent apoptosis upon p53 expression (Polyak et al., 1996). In a recent report, a transcription factor AATF (apoptosis-antagonizing transcription factor) bound to the promoters of Puma, Bax and Bak, repressed the p53-induced expression of these apoptotic genes, and therefore inhibited p53-mediated apoptotic response (Hopker et al., 2012). These observations together indicate that specific factors exist in cells, which dictate cellular responses to p53 activation.

In the present study, cell cycle arrest but no apoptosis has been observed in the angiosarcomas after p53 restoration. This observation is consistent with previous studies showing that restoration of p53 in sarcomas, including angiosarcomas, results in cell cycle arrest but no apoptosis (Ventura et al., 2007; Wang et al., 2011). These data suggest that cell cycle arrest is determined as the default program induced by p53 restoration in angiosarcomas. It is therefore of interest to ask what factors dictate such an arrest response to p53 restoration in angiosarcomas. To this end, I propose to identify such factors via a genome-wide
shRNA screening strategy. Briefly, a barcoded lentiviral shRNA library will be used to transduce $Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER$ angiosarcoma cell lines established in the present study. Then, I will restore $p53$ expression by 4-hydroxytamoxifen treatment in pooled cells with stable shRNA expression. Subsequently, barcode sequencing will be performed to identify shRNAs which are underrepresented in the 4-hydroxytamoxifen-treated cells in comparison to that of control cells. Theoretically, genes repressed by such shRNAs may function to reinforce $p53$-mediated cell cycle arrest/senescence and/or repress $p53$-induced apoptosis. I will then perform functional studies, e.g. overexpression of identified factors in a set of selected cell lines to confirm their roles in determining $p53$-induced responses. Identification of such cellular determinants of $p53$-induced response will not only further our knowledge of the $p53$ pathway, but also provide therapeutic targets for treatment of tumors resistant to $p53$-induced apoptosis.

Paracrine Effects of Tumor Cells

One of the interesting findings from the present study is that the growth of $Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER$ angiosarcomas was completely stopped and some tumors even underwent regression upon $p53$ restoration; however, only 30-40% recombination was observed in the tamoxifen-treated $Mdm2^{Tg} \ p53^{Neo/Neo} \ CreER$ angiosarcomas, indicating that a large proportion of tumor cells did not have restored $p53$ expression. This inconsistency between $p53$ restoration and tumor response suggests that tumor cells without $p53$ restoration may sense the change from $p53$-restored cells. It has been reported that $p53$ induces Mdm2-
mediated HIF-1α (hypoxia-inducible factor 1-alpha) degradation and suppresses HIF-1α induced VEGF (vascular endothelial growth factor) expression (Ravi et al., 2000). In addition, expression of VEGF promotes proliferation of angiosarcoma cells in autocrine and paracrine manners (Tokuyama et al., 2010). These data suggest that p53 expression in angiosarcoma tumor cells may affect neighboring cells via secretory factors. Therefore, I hypothesize that p53 restoration in the \(Mdm2^{Tg}\ p53^{Neo/Neo}\ CreER\) cells leads to altered secretory profiles, which affect the growth of neighboring cells. To test this hypothesis, RNA-seq analyses can be performed to identify differences in expression profiles especially for secretory factors between tamoxifen-treated \(Mdm2^{Tg}\ p53^{Neo/Neo}\) and \(Mdm2^{Tg}\ p53^{Neo/Neo}\ CreER\) angiosarcomas. In particular, upon tamoxifen treatment secretory factors upregulated in \(Mdm2^{Tg}\ p53^{Neo/Neo}\ CreER\) angiosarcomas may contribute to growth inhibition of tumors, whereas secretory factors downregulated may promote tumor growth. Subsequently, the functions of such factors in inhibiting tumor growth will be tested in both in vitro and in vivo systems. Identification of such factors may advance our understanding of cell-cell communication in tumors, and help to develop better therapeutic approaches.

In summary, mouse models such as the present study have shed light on tumor development in the context of the whole-body system, intact immune system, and unstressed conditions. In addition, modeling tumor responses to alterations in specific genes in genetically modified mice provide unique tools to identify druggable candidates for targeted cancer therapies in human patients.
Hopefully, the invaluable findings generated from these studies will be translated to clinic and benefit cancer patients.
CHAPTER 4. MATERIALS AND METHODS

Mice

The $p53^{\text{Neo/Neo}}$ mice (Wang et al., 2011) were crossed with $\text{Mdm2}^{\text{Tg}}$ mice (Jones et al., 1998) to generate $\text{Mdm2}^{\text{Tg}}p53^{\text{Neo/+}}$ mice. The $\text{Mdm2}^{\text{Tg}}p53^{\text{Neo/+}}$ mice were backcrossed with $p53^{\text{Neo/Neo}}$ mice to generate $\text{Mdm2}^{\text{Tg}}p53^{\text{Neo/Neo}}$ mice. The $p53^{\text{Neo/Neo}}$ mice were crossed with $\text{CreER}$ transgenic mice to generate $p53^{\text{Neo/+}}\text{CreER}$ mice, and the $p53^{\text{Neo/+}}\text{CreER}$ mice were backcrossed with $p53^{\text{Neo/Neo}}$ mice to generate $p53^{\text{Neo/Neo}}\text{CreER}$ mice. $\text{Mdm2}^{\text{Tg}}p53^{\text{Neo/Neo}}$ mice were crossed with $p53^{\text{Neo/Neo}}\text{CreER}$ mice to generate $\text{Mdm2}^{\text{Tg}}p53^{\text{Neo/Neo}}$ and $\text{Mdm2}^{\text{Tg}}p53^{\text{Neo/Neo}}\text{CreER}$ mice. All $\text{Mdm2}^{\text{Tg}}, p53^{\text{Neo/Neo}}, \text{Mdm2}^{\text{Tg}}p53^{\text{Neo/Neo}},$ and $\text{Mdm2}^{\text{Tg}}p53^{\text{Neo/Neo}}\text{CreER}$ mice were maintained in a genetic background of over 95% C57BL/6J. The mice were bred and maintained in the mouse facility at the University of Texas M.D. Anderson Cancer Center in compliance with the Institutional Animal Care and Use Committee guidelines.

Magnetic Resonance Imaging (MRI)

All MRI studies were performed in the Small Animal Imaging Facility at the University of Texas M.D. Anderson Cancer Center. Mice were subjected to anesthesia during the MRI. Five percent isoflurane in oxygen was used to initiate anesthesia and then 2-3% isoflurane in oxygen was used to maintain anesthesia during MRI. Respiratory bellows in conjunction with a monitoring and gating system (Small Animal Instruments, Inc) were used to monitor physiological conditions of anesthetized mice. The magnetic resonance (MR) images were...
acquired using a 7 Tesla small animal MR system BioSpec USR70/30 (Bruker Biospin MRI) with a 6-cm imaging gradient. A RF coil with 3.5-cm internal diameter was used for signal excitation and detection. The MRI procedure was conducted as previously described (Wang et al., 2011). T$_2$-weighted coronal and axial MR image stacks were used to calculate tumor volumes using ParaVision 4.0 software (Bruker Biospin MRI).

**Tamoxifen Treatment**

Tamoxifen (Sigma-Aldrich) was prepared as a 30 mg/mL stock solution with corn oil:ethanol mixture (95.5:4.5, v/v). Tumor-carrying mice received weekly intraperitoneal (i.p.) injection of tamoxifen at a dose of 3 mg/40 g bodyweight. Tamoxifen treated mice were closely monitored for up to 4 weeks or till morbidity and then sacrificed.

**Transplantation**

The primary $Mdm2^{Tg}$ $p53^{Neo/neo}$ CreER angiosarcoma was excised from a euthanized mouse, extensively diced with a scalpel blade, and trypsinized at 37 °C for 10 minutes. Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum was used to inactivate trypsin and then the mixture was passed through a 40 µM filter to remove cell debris. Tumor cells were resuspended in PBS:Matrigel mixture (2:1, v/v) to a final density of $2 \times 10^7$/mL at 4 °C, following PBS washing. Two million tumor cells in PBS:Matrigel solution (100 µL) were injected subcutaneously into the flanks of C57BL/6J mice. Tumor formation was
monitored by palpation. The sizes of these tumors were monitored with digital calipers and tumor volumes were calculated using the equation 

\[ \text{Volume} = \frac{\text{width}^2 \times \text{Length}}{2} \]

as described previously (Bearss et al., 2000).

**Histopathology and Immunohistochemistry**

Mouse tissues were processed for histopathological analysis as previously described (Lang et al., 2004). Briefly, fresh tissue samples were excised from euthanized mice, washed with cold phosphate buffered saline, and then fixed in 10% formalin for 48 hours. Fixed tissue samples were embedded in paraffin and then sectioned into slices with 4 µM thickness. Histological preparation of mouse tissue samples and hematoxylin and eosin (H&E) staining were conducted in the Department of Veterinary Medicine at the University of Texas M.D. Anderson Cancer Center. Immunohistochemical staining was performed on paraffin-embedded tissue sections as previously described (Evans et al., 2001), using antibodies for Ki-67 (MM1, Leica Biosystems; 1:200), cleaved caspase-3 (5A1E, Cell Signal Technology; 1:200), and PML (PG-M3, Santa Cruz Biotechnology; 1:100). Signals were detected using the Vectastain Elite ABC Kit (Vector Labs), and counterstaining was performed using Nuclear Fast Red (Vector Labs).

**Murine Embryonic Fibroblast (MEF) Preparation, Cell Culture and Methyl Thiazolyl Tetrazolium (MTT) Assay**

MEFs were established from 13.5 days post coitum embryos as previously described (Terzian et al., 2007). The culture conditions for maintaining MEFs
were the same as previously described (Terzian et al., 2007). pBabe-puro based retroviral vectors were used to transduce Phoenix-eco cells to produce retroviral particles; and then retroviral particles collected from the Phoenix-eco cell medium were used to infect MEFs. MEFs infected with these retroviral particles were subjected to screening for puromycin-resistance in culture medium with 2 µg/mL puromycin for 4 days. 4-hydroxytamoxifen treatment to MEFs was performed using culture medium containing 1 µM 4-hydroxytamoxifen. In order to elicit DNA-induced p53 activation in MEFs, cells were incubated in culture medium with 1 µg/mL doxorubicin for 4 hours. For MTT assays, cells were incubated in culture medium with 125 µg/ml MTT for 3 hours followed by DMSO incubation for 20 minutes, and the optical absorbance was measured at the wavelength of 550 nm.

**Real-time Reverse Transcription-PCR (qRT-PCR)**

Total RNAs were isolated from MEFs or mouse tissues using TRIzol reagent (Life Technologies) following manufacturer’s instructions. Reverse transcription reactions for first-strand cDNA synthesis were performed using the First-Strand cDNA Synthesis Kit with random hexamers as primers (GE Healthcare). Real-time PCR was performed on a 7900HT Fast Real-time PCR system (Applied Biosystems) using iTaq SYBR Green Supermix with ROX or SsoAdvanced SYBR Green Supermix (Bio-Rad). Primer sets used in the real-time PCR probing for p21, Mdm2, Puma, Noxa, and Rplp0 are listed below: p21, 5’-CAGGCACCATGTCCAATCCT-3’ and 5’-GAGACAACGGCACACTTTGCT-3’; Mdm2, 5’-AGTCCCATGAAGAGATTGTACATGAC-3’ and 5’-AACATAGGCAACC
ACCAGGAA-3' Puma, 5'-GCGGCAGACAAAGAGA-3' and 5'-AGTCCCATG AAGAGATTGTACATGAC-3' Rplp0, 5'-CCCTGAAGTGCTCGACATCA-3' and 5'-TGCGGACACCCTCCAGAA-3'. In all qRT-PCR experiments, mRNA expression was normalized to the Rplp0 levels.

**Western blotting**

Proteins were extracted from MEFs or homogenized mouse tissues using radioimmunoprecipitation assay (RIPA) buffer with cOmplete proteinase inhibitors (Roche Applied Science). Proteins, resolved in SDS-PAGE and transferred to PVDF membranes, were incubated with antibodies against p53 (CM5, Vector Laboratories; 1:1000), Mdm2 (2A10, Calbiochem; 1:1000), p21 (F-5, Santa Cruz Biotechnology; 1:1000), H-Ras (sc-35, Santa Cruz Biotechnology; 1:200), β-actin (AC-74, Sigma-Aldrich; 1:5000), and γ-tubulin (Sigma-Aldrich; 1:1000), respectively. Membrane-bound proteins were then visualized with ECL Western blotting detection reagent (GE Healthcare), following incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare).

**Statistics**

All statistical analyses were performed using the Graphpad Prism 5.0 software (Graphpad Software). Differences between genotypes or treatment groups were analyzed with 2-tailed Student’s t test; and difference between survival curves
was determined via Log-rank (Mantel-Cox) Test. A \( p \) value of \( \leq 0.05 \) was deemed statistically significant.
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VITA

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