

MDM2 is a Central Node in the p53 Pathway: 12 Years and Counting

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Abstract: Twelve years ago, the Mdm2 oncogene was shown to bind to and inhibit the tumor suppressor protein, p53. During the past 12 years, both genetic and biochemical studies have demonstrated that Mdm2 is a key negative regulator of the tumor suppressor p53. Mdm2 and p53 form an oscillating auto-regulatory feedback loop, which is tightly controlled to allow the appropriate response to environmental stresses in order to suppress tumor formation. When Mdm2 activity is inappropriately heightened, as it is in many human tumors, p53 activity is attenuated and tumor susceptibility arises. The p53 gene is mutated in 50% of all human tumors, but in those tumors that retain wild type p53, inhibiting Mdm2 activity could activate p53 tumor suppression and therefore provide a therapeutic strategy for the treatment of cancer.

INTRODUCTION

MDM2 Binds and Inhibits p53: The First Years

The tumor suppressor protein, p53, is activated upon cellular stresses such as DNA damage and oncogene activation, and initiates a transcriptional program which leads to DNA repair, cell cycle arrest, and in some cases, apoptosis [1]. The p53 stress response pathway has been shown to be crucial for the prevention of tumor formation. For example, both mice and humans harboring a germ-line inactivating mutation in one allele of the p53 gene develop tumors very early in life and at dramatically high frequencies [2-4]. Somatic inactivating mutations of the p53 gene are also found in over 50% of all human tumors [5].

In the early 1990's it became clear that p53 is a tumor suppressor, and is important in the prevention of many types of cancer in humans. It had been identified in 1979 as an interacting protein of a DNA tumor virus protein [6, 7]. This interaction was shown in the subsequent ten years to inhibit p53's ability to bind DNA, activate transcription and function as a tumor suppressor, thereby playing a crucial role in the viral transformation of the host cell. With these observations in mind, Momand *et al.* set out to search for cellular proteins which bind to and regulate p53 function in the hope of gaining insight into this important tumor suppressor [8]. Hinds *et al.* had already identified a cellular protein of approximately 90 kd which co-immunoprecipitated with either mutant or wild-type human p53 [9]. Momand *et al.* took advantage of this and purified the 90kd protein using a p53 immunoaffinity column and extracts from a rat cell line, which overexpressed a temperature-sensitive p53. A partial protein sequence was obtained, which was used to search for a homologous sequence in the GenBank/EMBL data banks. The peptides showed high homology to a putative amino acid sequence of an open reading frame of the murine double minute 2 gene (*mdm2*) cDNA. Momand *et al.* went on to demonstrate that the 90 kd protein was indeed rat Mdm2 and that it could

inhibit a known activity of p53. Specifically, Mdm2 was shown to inhibit the ability of p53 to trans-activate a reporter plasmid driven by a p53 response element.

Interestingly, *mdm2* had just been identified as an oncogene that conferred an enhanced tumorigenic potential on cells when the gene was amplified or over-expressed [10, 11]. In fact, this well-described cellular phenotype was shown to be very similar to cells which expressed mutant p53 protein [12]. The similarity of cellular over-expression phenotypes of Mdm2 and mutant p53, together with the observed strong physical interaction of Mdm2 and p53 and the inhibition of a known activity of p53 by Mdm2, led the authors to note the potential importance of this interaction in the regulation of this important tumor suppressor.

THE MDM2-p53 Interaction: In Mice

The importance of the Mdm2 and p53 interaction was underscored through elegant mouse genetics. It has been shown that when Mdm2 is knocked out, the mouse embryo dies before implantation in the uterus. This lethal phenotype is rescued by knocking out the p53 gene, clearly demonstrating an important genetic interaction between these two genes in murine development [13, 14]. It is thought that the lethality of the Mdm2 knockout is due to inappropriate p53-dependent apoptosis due to the hypoxic embryonic environment.

To assess the importance of this interaction in the adult mouse, Mendrysa *et al.* genetically altered mice to express reduced levels of Mdm2 [15]. The resulting genetically altered mice were small. In fact, at five weeks of age a 15 to 20% reduction in body weight was evident, and all examined organs (kidney, liver, spleen and thymus) were significantly lighter. The thymus showed the most significant weight difference when compared to wild type mice (40% smaller), which was consistent with an observed major defect in lymphopoiesis. These mice also were more sensitive to radiation. Ten Gy of whole body ionizing radiation killed 100% of the altered mice in 7 to 9 days, while only 50% of wild type mice died by day 22 post-treatment. Increased apoptosis in both lymphocytes and epithelial cells was also observed. Interestingly, all these observations were shown to be p53 dependent, as crosses

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with p53 null mice caused reversal of the phenotypes, demonstrating that less Mdm2 results in more p53 activity.

Although heightened p53 activity brought about by lower Mdm2 levels leads to many defects in mice, it is a logical assumption that more p53 tumor suppressor activity should protect against cancer. This turned out to be the case as shown in a model using E μ -myc transgenic mice, which develop B-cell lymphomas [16]. Alt *et al.* observed that crossing these mice with mice that only have one allele of mdm2 dramatically suppressed lymphoma development when compared to E μ -myc mice with both mdm2 alleles. Specifically, they survived twice as long, and 20% of the mice never developed lymphomas, compared to only 5% of E μ -myc mice with both alleles of mdm2 remaining disease-free. The suppression of lymphoma development by decreasing Mdm2 was shown to be p53 dependent, as crosses with p53 null mice led to "normal" lymphomagenesis. Together, these studies in mice further demonstrate that Mdm2 is critical in regulating p53 in both the developing and mature mouse, and that alterations in Mdm2 levels can affect cancer.

THE p53-MDM2 Autoregulatory Feedback Loop

While data was accumulating that Mdm2 negatively regulates p53, Wu *et al.* and Barak *et al.* provided data that p53 can positively regulate Mdm2 [17, 18]. Wu *et al.* worked off of a previous observation that when wild type p53 activity was present in cells, more Mdm2/p53 complex was seen compared to the levels found in cells with mutant p53 protein, even though both mutant and wild type p53 had been shown to bind equally well to Mdm2 [9]. Wu *et al.* went on to explain this observation by demonstrating that wild type p53, but not mutant p53, could bind to specific DNA elements in the mdm2 promoter and activate transcription, thereby increasing overall Mdm2 levels in the cell. Interestingly, Wu *et al.* went on to show that Mdm2 was able to inhibit its own transcriptional activation by p53, thereby completing this interesting auto-regulatory feedback loop.

To date, it has been shown that Mdm2 can inhibit p53 by regulating its stability, cellular localization and ability to activate transcription [19]. One well-studied mechanism is its function as an E3 ubiquitin ligase, which targets the p53 protein for proteasomal degradation, decreasing the level of p53 in the cell [20-22]. When Mdm2 activity is artificially inhibited by small molecules, peptides, or Mdm2-antisense oligodeoxynucleotides, p53 levels rise, and the pathway is activated [23-25]. After certain cellular stresses, p53 levels also rise due to the increase in the half-life of p53. The rise in p53 levels has been attributed to the inability of Mdm2 to target p53 for degradation after stress. This inhibition of Mdm2 after cellular stress has been well studied. Post-translational modifications, protein-protein interactions and transcriptional/translational regulation have all been implicated in the inhibition of Mdm2 after stress.

For instance, immediately following DNA damage, p53 is phosphorylated at its N-terminus. The N-terminus of p53 harbors the Mdm2 interaction domain and is crucial for Mdm2 binding, therefore phosphorylation in this region may negatively affect the p53-Mdm2 interaction, resulting in the inhibition of the ability of Mdm2 to target p53 for

proteasomal degradation, leading to a rise in p53 protein and activity. Indeed, phosphorylation of threonine 18 or serine 20 of p53 has been shown to inhibit Mdm2 binding to p53 peptides *in vitro* [26-31]. However, *in vivo*, p53 mutants which cannot be phosphorylated on these residues are still stabilized in cells after stress, suggesting that other mechanisms may account for the inhibition of Mdm2 and subsequent rise in p53 levels after stress [32, 33].

An Mdm2 inhibitory protein, ARF, is induced after inappropriate oncogenic stimuli from E2F, Ras or Myc activation. ARF can bind directly to Mdm2 and prevent Mdm2-mediated p53 degradation, resulting in p53 stabilization and activation [34,35]. The exact mechanism(s) of Mdm2 inhibition by ARF is not yet fully understood, however, it has been shown that ARF can directly bind to the RING domain of Mdm2 and inhibit its E3 ubiquitin ligase activity [24, 36]. Other studies have found that over-expression of ARF leads to accumulation of ARF-Mdm2 complexes in the nucleolus of cells, possibly separating Mdm2 and p53 [37, 38]. ARF mutants that can no longer localize to the nucleolus have been shown to be impaired in their inhibition of Mdm2, resulting in higher nuclear Mdm2 levels and decreased p53 [39]. However, nucleolar sequestration of Mdm2 may not always be essential for Mdm2 inhibition, as ARF-dependent p53 stabilization has been observed without Mdm2 relocation to the nucleolus, and various truncated ARF proteins can still stabilize p53, but do not accumulate in the nucleolus [19, 40, 41].

Mdm2 protein levels also decrease dramatically after cellular stress. Immediate reduction in Mdm2 levels has been observed after a variety of cellular stresses. In response to high-dose UV irradiation and some DNA alkylating agents, both protein and mRNA levels of Mdm2 decrease, which coincides with decreased levels of ubiquitinated p53 and fewer Mdm2/p53 complexes. Nitric oxide and hypoxia also result in an initial decrease in Mdm2 protein levels, although the mechanism of the Mdm2 reduction seems to be different because there is a reduction in protein, but not mRNA [42-45]. A recent report implicates the E3 ligase activity of Mdm2 in its own destabilization after DNA damage [46]. The data illustrated that Mdm2 degradation is accelerated through Mdm2 auto-ubiquitination as a result of signaling by stress-activated PI3 kinase family members. This accelerated Mdm2 degradation correlates with p53 transcriptional activation after DNA damage.

MDM2 AND P53 OSCILLATE

As p53 and Mdm2 can regulate each other, the immediate inhibition of Mdm2 should result in an increase in the level of p53 expression or activity, which in turn should result in increased Mdm2 expression, thereby reducing p53 levels. In fact, such oscillations of Mdm2 and p53 levels have been reported in response to various stresses including ionizing and UV radiation [47-50]. Analysis of cell populations showed that p53 and Mdm2 undergo dampened oscillations after strong, but not weak, DNA damage [47]. In a recent report, the oscillations of p53 and Mdm2 were analyzed in single cells by using fluorescently labeled proteins and time-lapse microscopy [51]. Interestingly, the amount of DNA damage was also shown to be important. Specifically, when the amount of DNA damage

increased, so did the average number of oscillations, although the average height and duration of each oscillation were fixed, regardless of the amount of DNA damage. The authors described the oscillations as “digital” as opposed to “analog.” This suggests that Mdm2 and p53 would be produced in the cell in a pulsatile manner over time (digital) rather than continuously (analog) after stress, suggesting that when p53 levels are low, the cell will only incite the next pulse if the stress is still present. They went on to suggest that the digital behavior of the p53 and Mdm2 oscillations could result in a reasonably defined amount of repair enzymes being produced in response to stress, reducing the risk of having too much p53 and leading to inappropriate death, for example, in cases where the stress should be dealt with successfully without resulting in an irreversible biological outcome (e.g., apoptosis or senescence). Thus, digital pulses of p53 might be viewed as an arrangement that allows repetitive repair efforts: a first pulse of p53 is delivered, and the system waits to see whether the damage has been properly fixed. If not, a second pulse is generated, and so forth, until the damage is effectively resolved and the signals leading to p53 activation subside. On the other hand, if the extent of damage is excessive, the number of the p53 pulses may result in an irreversible response, such as apoptosis.

High Mdm2 Levels Can Promote Tumor Formation

Shortly after the discovery that Mdm2 can bind and inhibit p53, *mdm2* gene amplification was found in 30% of osteosarcomas and soft tissue sarcomas [52-54]. This is logical because over-expression of Mdm2, and therefore repression of the tumor suppressor p53, would give an obvious advantage for the development of a tumor. In fact, in a subset of these tumors, over-expression of Mdm2 is mutually exclusive to p53 mutation, which suggests that over-expression of Mdm2 can substitute for the inactivation of p53 by mutation. This hypothesis was further supported in a later study of Mdm2 amplification in over 3000 tumors from 28 tumor types [55]. Of these, 7% showed Mdm2 amplification, with soft tissue sarcomas and osteosarcomas showing the highest rate of amplification at 20% and 16%. When the authors considered only those tumor types which showed either p53 mutation or Mdm2 mutation, 65% had p53 mutations and 35% *mdm2* amplification, but only 4% had both, showing a statistically significant negative association between the occurrence of p53 mutations and *mdm2* amplification ($p=0.038$). In some cancers, Mdm2 over-expression is associated with accelerated cancer progression and lack of response to therapy [56]. These studies, together with numerous accounts of Mdm2 over-expression or amplification in a variety of human cancers, support the idea that increased Mdm2 levels could positively impact tumor formation [52-54, 57-63].

Mouse genetics is providing further convincing evidence to support the claim that high Mdm2 levels can positively impact tumorigenesis. Several groups have created transgenic mice which over-express Mdm2 to mimic the situation found in human tumors. For example, Jones *et al.* created mice which over-express Mdm2 in the whole body by using the entire *mdm2* gene, regulated by its own promoter, as a trans-gene [64]. These mice expressed an average of 4-fold

more Mdm2 in various tissues relative to non-transgenic mice. All of the Mdm2 over-expressing mice developed spontaneous tumors. The authors went on to observe that mice with two copies of the transgene developed tumors faster than mice with only one copy of the *mdm2* transgene, further demonstrating that tumor formation depends on the level of Mdm2 expression. The tumor spectrum of the Mdm2 over-expressing mice was reminiscent of p53 null mice. Malignant lymphomas made up the majority of the tumors in both mice, although the Mdm2 over-expressing mice showed a higher proportion of sarcomas when compared to the p53 null mice. Lundgren *et al.* also showed that targeted over-expression of Mdm2 in the murine mammary epithelium results in tumors, albeit with a lower penetrance (16%)[65]. In another report, Mdm2 over-expression was targeted to the basal layer of the epidermis, where p53 is known to protect against tumor formation in response to UV-damage. In this study, multiple lines of transgenic mice, expressing up to 5 fold more Mdm2 than wild type mice in the adult dorsal skin, were shown to have a greater susceptibility for tumor formation. These mice formed more papillomas after chemical carcinogenesis, and developed spontaneous hyperplastic lesions and carcinomas, further demonstrating that high levels of Mdm2 can indeed promote tumor formation [66].

Mechanisms to Increase Mdm2 Activity in Tumors

Much work has been done to try to understand the molecular mechanisms by which Mdm2 activity is increased to promote tumor formation. As mentioned previously, one well-characterized mechanism to increase Mdm2 activity in a tumor is to amplify the copy number of the *mdm2* gene, which occurs in many tumor types. However, many studies have reported heightened expression of either Mdm2 protein or mRNA in tumors in the absence of gene amplification [67], thereby suggesting that other mechanisms may exist to increase Mdm2 levels in tumors.

One proposed mechanism to increase Mdm2 levels is to enhance the translation of the *mdm2* message. Several groups have reported that some tumor cell lines, which over-express Mdm2 without gene amplification, translate the *mdm2* message efficiently through very efficient polysome loading on the message [35,68,69]. Differences in translation efficiency have been linked to differences in the 5' UTR of the *mdm2* messages. Specifically, the *mdm2* gene can be transcribed from two distinct promoter regions (P1 and P2), which produce two different messages, which have two different 5'UTRs, but share the same translation initiation site [70]. Transcripts from the P1 promoter have been shown to contain sequence elements in their 5'UTRs which inhibit translation, while transcripts from the P2 promoter have been shown to contain sequence elements that enhance translation [34, 35]. Specifically, the P1-transcript 5'UTR sequence elements reduced ribosome loading efficiency, and when linked to reporter genes, repressed translation. Trotta *et al.* demonstrated that P2-transcript 5'UTR sequence elements enhanced translation in a BCR/ABL-expressing myeloid precursor cell line. They went on to show that enhanced translation of the *mdm2* transcript required an RNA binding protein, the La antigen, which recognizes a 27-nucleotide segment specific to the 5'UTR of the P2 transcript.

Together, the above observations suggest that by increasing transcription of *mdm2* from the P2 promoter, instead of from the P1 promoter, the cell could increase Mdm2 levels by increasing the translational efficiency of the *mdm2* message. Interestingly, two pathways often activated in tumors have been shown to do just that. First, constitutively activated Ras can activate *mdm2* transcription via Ap1 and Ets binding elements in the P2 promoter [71]. Second, forced expression of the estrogen receptor (ER) induces heightened *mdm2* transcription from the P2 promoter, while inhibition of ER represses *mdm2* P2-transcription, possibly explaining the observed over-expression of Mdm2 in ER positive breast tumors [72, 73].

CONCLUSION: TARGETING THE MDM2-P53 INTERACTION

In summary, during the past 12 years, both genetic and biochemical studies have demonstrated that Mdm2 is a key negative regulator of the tumor suppressor p53. Mdm2 and p53 form an oscillating auto-regulatory feedback loop, which is tightly controlled to allow the appropriate response to environmental stresses in order to suppress tumor formation. When Mdm2 activity is inappropriately heightened, as is the case in many human tumors, p53 activity is attenuated and tumor susceptibility increases. The p53 gene is mutated in 50% of all human tumors, but in those tumors that retain wild type p53, inhibiting Mdm2 activity could activate p53 tumor suppression and therefore provide a therapeutic strategy for the treatment of cancer. A genetic analysis of p53 and Mdm2 assessed the amino acid residues needed for the p53-Mdm2 contacts, which are phenylalanine-19, tryptophan-23 and leucine-27 in p53 [74, 75]. In 1996, the structure of p53 bound to Mdm2 was solved by X-ray crystallography, which confirmed these residues as contacts and revealed that the amino terminal domain of Mdm2 forms a deep, rather small hydrophobic cleft into which the trans-activation domain of p53 binds [76]. Using this information, Vassilev *et al.* identified a family of synthetic compounds (Nutlin), which bind Mdm2 in the small hydrophobic cleft, thereby antagonizing p53 binding [25]. Nutlin was shown to trigger p53 activation, cell cycle arrest and apoptosis in tumor cells with wild type p53. Importantly, Nutlin was also shown to inhibit tumor growth in animal xenograft models without apparent toxicity to normal tissues. These data demonstrate the potential of targeting the Mdm2-p53 interaction, which over a decade of research has shown to be a critical node in this important tumor suppressor pathway.

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