Alternative and aberrant splicing of *MDM2* mRNA in human cancer

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MDM2 has been characterized as a protein that binds to and facilitates degradation of the tumor suppressor p53. Interestingly, more than 40 different splice variants of *MDM2* transcripts have been identified both in tumors and normal tissues, and the majority of these variants do not contain sequence encoding the p53 binding site. This review describes the different splice forms, the tissues in which they have been identified, and their association with tumor progression and prognosis. In addition, we discuss the potential functions of these variants and how they interact with full-length MDM2 protein.

Introduction

The first alternatively spliced *MDM2* transcripts in human tumors were identified in 1996 (Sigalas et al., 1996), but only during the past year have any potential functions been suggested. A problem that has developed is one of consistency. Even though most of the splice variant sequences are in GenBank, some investigators have chosen to give different names to previously published isoforms. This makes it very difficult to determine the frequency of occurrence of specific variants or to compare the variants expressed within different tumor histiotypes. This review briefly summarizes the functions of full-length MDM2 and then describes all the known *MDM2* splice variants and their potential role in human cancer.

Full-length MDM2

The *MDM2* gene was originally isolated from the spontaneously transformed murine cell line 3T3DM (Cahilly-Snyder et al., 1987; Fakharzadeh et al., 1991). The human homolog of *MDM2*, also referred to as *HDM2*, was cloned by Oliner et al. (1992) from the colon carcinoma cell line CaCo-2. Human *MDM2* is amplified and overexpressed in approximately one-third of human sarcomas, including those of soft tissue and bone. Combined analysis of data obtained from 3889 tumor samples from 28 tumor types revealed an overall *MDM2* amplification frequency of 7% (Momand et al., 1998). To date, *MDM2* amplification has been identified in 19 tumor types with varying frequency. In addition, MDM2 expression can be upregulated independent of gene amplification (Landers et al., 1994; Cordon-Cardo et al., 1994), and oncogenic splice variants have been identified (Sigalas et al., 1996; Matsumoto et al., 1998; Kraus et al., 1999; Pinkas et al., 1999; Bartel et al., 2001; Lukas et al., 2001). Therefore, simple analysis of gene amplification underestimates the involvement of MDM2 in human cancer.

The function of MDM2 was unclear until it was demonstrated to bind to the tumor suppressor p53 and inhibit p53-mediated transactivation (Momand et al., 1992; Figure 1A). Overexpression of MDM2 is a mechanism, independent of gene mutation, by which wild-type p53 function can be inactivated (Chen et al., 1996; Argentini et al., 2001). MDM2 itself can be upregulated by p53, generating a negative feedback loop (Wu et al., 1993; Zauberman et al., 1993). Once MDM2 binds to p53, MDM2 acts as a ubiquitin ligase and facilitates proteasomal degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997; Honda and Yasuda, 2000; Figure 1A).

As well as its p53-dependent functions, MDM2 is part of a complex network of interactions through which MDM2 affects the cell cycle, apoptosis, and tumorigenesis. Specific regions of MDM2 that interact with p53, CBP/p300, pRB, p73, E2F1, DP1, the L5 ribosomal ribonucleoprotein particle, $p14^{ARF}$, and RNA are indicated in Figure 1B. The specific functions of the MDM2 oncoprotein related to the binding of these different proteins have been discussed in detail in numerous excellent reviews (Freedman et al., 1999; Momand et al., 2000; Juven-Gershon and Oren, 1999).

Human tumors containing both mutant *p53* and *MDM2* amplification are rare (Cordon-Cardo et al., 1994), but the fact that both modifications can occur within the same tumor and that two mechanisms to inactivate wild-type p53 are redundant provides support for a p53-independent function of MDM2. In addition to its p53-associated functions, MDM2 has been reported to transform cells, independent of p53. Dubs-Poterszman et al. (1995) demonstrated transformation of *p53* null cells by expression of MDM2, and Jones et al. (1998) showed that sarcomas develop in the presence or absence of p53 in *MDM2* transgenic mice. The identification of oncogenic splice variants of human *MDM2* transcripts that lack the p53 binding site (Sigalas et al., 1996) provides further evidence of p53-independent functions of MDM2.

Although considerable evidence suggests that MDM2 has transforming potential, conflicting data have generated confusion with respect to the oncogenic function of this protein. Two groups have reported difficulty in transfecting and expressing full-length *MDM2* cDNA in various cell types (Brown et al., 1998; Kubbutat et al., 1999). These results were unexpected and inconsistent with the hypothesis that *MDM2* is exclusively an oncogene. Brown et al. (1998) characterized two growth inhibitory domains within the human MDM2 protein (ID1 and ID2 in Figure 1B). Deletion of either region allowed stable expression of MDM2 in NIH3T3 cells and enhanced the tumorigenic potential of the cells (Brown et al., 1998). Folberg-Blum et al. (2002) demonstrated that MDM2 overexpression targeted to the *Drosophila* wing induced apoptosis of wing imaginal discs, and Dilla et al. (2000) showed that expression of this protein promoted apoptosis in medullary thyroid carcinoma cells. Furthermore, MDM2 is expressed at high levels in terminally differentiated tissues such as skin, brain, and muscle (Piette et al., 1997); such findings are consistent with a growth-inhibitory antioncogenic phenotype. Potentially, some MDM2 proteins

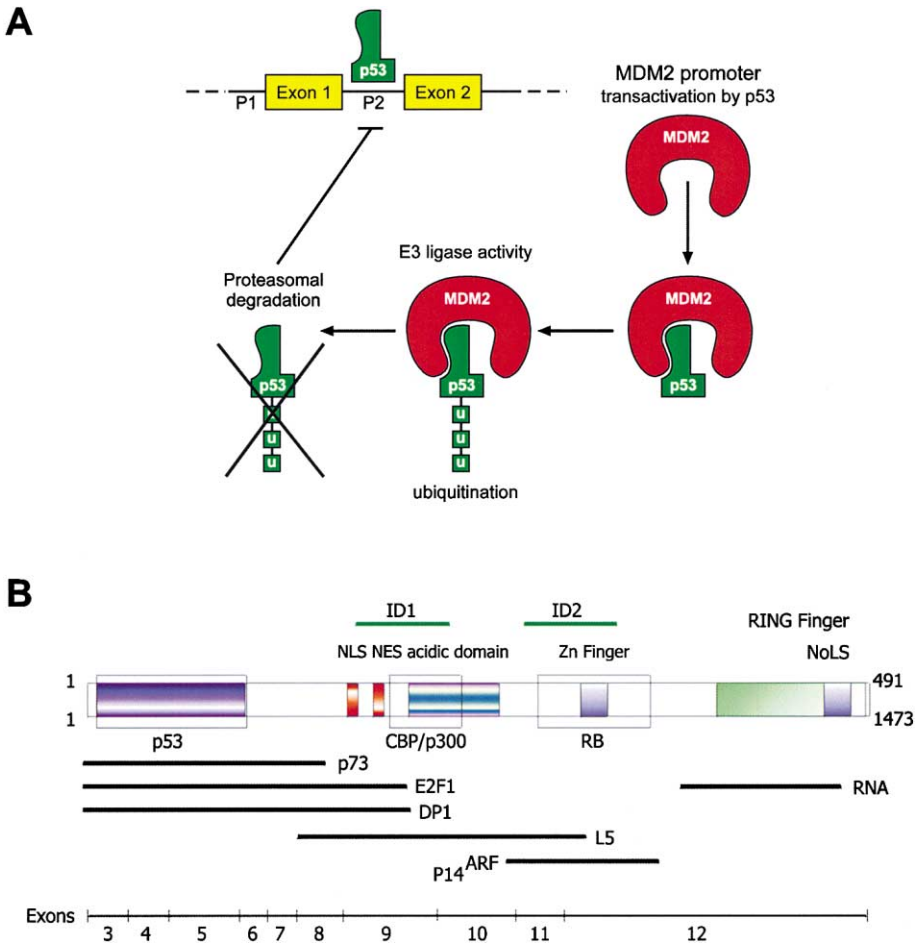

Figure 1. MDM2—structure and function

A: Model depicting the well characterized interactions between MDM2 and p53. p53 activates transcription of MDM2. MDM2 protein in turn binds to p53 and acts as an E3 ubiquitin ligase that targets p53 for degradation by the proteasome, thereby inhibiting its own transcription.
B: Structure of full-length human MDM2 protein and the cDNA that encodes it. The domains of the human MDM2 protein and the binding sites of various proteins are indicated. ID1, ID2: growth inhibitory domains 1 and 2 (Brown et al., 1998). NES: nuclear export sequence. NLS: nuclear localization sequence. NoLS: cryptic nucleolar localization signal.

encoded by splice variants could display an oncogenic function that overrides the growth-inhibiting phenotype and/or the apoptotic phenotype of full-length MDM2 protein. However, data that suggest a completely opposite function have been described for several murine MDM2 splice variants, as discussed below (Dang et al., 2002).

Alternatively and aberrantly spliced variants of *MDM2*

In addition to mutations and chromosomal aberrations, splicing of multiple pre-mRNAs is a mechanism by which gene expression can be regulated or altered. Splicing can occur either "alternatively" at intron-exon borders by using genuine donor and acceptor splice sites, or "aberrantly" at cryptic splice sites within introns or exons. The usage of different promoters can also generate alternative transcripts. For example, *MDM2* gene transcription can occur from two independent promoters. Transcripts that arise from the constitutive P1 promoter lack exon 2, whereas the p53-sensitive P2 promoter generates transcripts lacking exon 1 (Zauberman et al., 1995; Figure 1A). These mRNAs are identical except for the 5′-untranslated region (Barak et al., 1994). However, transcription from P2 is approximately 6-fold greater than that from P1 (Landers et al., 1997). The proteins derived from these two mRNAs are identical, as translation begins in exon 3, but the translation of the shorter P2-derived transcript is more efficient in tumor cells (Brown et al., 1999).

The analysis of expression of the *MDM2* oncogene has revealed the presence of multiple, different sized *MDM2* transcripts and MDM2 protein isoforms in NIH3T3 cells (Haines et al., 1994). After the determination of the exon-intron structure of the murine *MDM2* gene (Oca Luna et al., 1996), it was confirmed that two of these transcripts were alternatively spliced. Different MDM2 isoforms have also been identified in B cell lymphomas isolated from Eμ-Myc transgenic mice (Eischen et al., 1999). Six of these variants have been characterized, and five were shown not to encode portions of the p53 binding domain because of deletions of exons 3, 5, or 4–8 (Dang et al., 2002). The sixth variant did not contain exon 8 and generated no protein when a retrovirus containing its cDNA was used to transduce MEFs. An alternate translation start site at amino acid 198 was utilized by two of the variants generating identical N-terminal truncated MDM2 proteins (Dang et al., 2002). Although none of these murine variants were identical to any found to date in human tumors, the murine variant V3 demonstrated the most similarity to the splice variant MDM2-A (Figure 2). *MDM2* mRNA transcripts of 3.3, 1.6, and 1.5 kb have been detected in tumors derived from a murine mammary tumor model, the shorter transcripts having lost a portion of the C-terminal coding region (Pinkas et al., 1999).

In human breast carcinoma tissue, transcripts of 6.7, 4.7, and 1.9 kb have been detected (Pinkas et al., 1999), with the 1.9 kb mRNA lacking exon 12. Western blot analysis of a panel of human breast carcinomas demonstrated that truncated MDM2 isoforms of 85, 76, and 57 kDa were expressed in addition to the full-length 90 kDa protein (Bueso-Ramos et al., 1996).

A detailed analysis of the *MDM2* mRNA in ovarian and bladder cancers revealed alternative as well as aberrant splicing (Sigalas et al., 1996). Sigalas and coworkers have described five *MDM2* transcripts (MDM2-A, -B, -C, -D, and -E in Figure 2) that lack sequences that encode at least part of the p53 binding domain, the nuclear localization and export sequences, and the acidic domain. In vitro expression studies confirmed that the protein isoforms encoded by four of these splice variants (MDM2-A, -B, -C, and -D in Figure 2) are unable to bind p53. Individual or multiple splice variants have also been detected in glioblastomas (MDM2-A, -B, -C, -D, and -E in Figure 2;

Figure 2. Summary of all known *MDM2* mRNA splice variants and the domains that they encode.

MDM2-FL refers to full-length *MDM2* mRNA. At least some of the splice variants, labeled with "*", can be translated into protein in vitro.
The sequence that is omitted in most splice forms is highlighted in gray. The number in parentheses indicates the reference: (1) Sigalas et al., 1996; (2) Bartel et al., 2001; (3) Bartel et al., 2002; (4) Lukas et al., 2001; (5) Tamborini et al., 2001; (6) Kraus et al., 1999; (7) Schlott et al., 2001; (8) Hori et al., 2000; and (9) F.B., unpublished data.

Matsumoto et al., 1998) and glioblastoma cell lines (MDM2-LN229a, -LN229b, -LN18, -G116, and -G150 in Figure 2; Kraus et al., 1999). In breast carcinomas, previously described splice variants and five additional shorter isoforms (MDM2-281bp, -219bp, -254bp, -DelE, and -DelF in Figure 2) have been detected (Lukas et al., 2001; Hori et al., 2000). Another splice variant, MDM2-Del.G (Hori et al., 2000), which lacks the sequence between nucleotides 182 and 1432 of the coding region of the *MDM2* mRNA, exactly corresponds to the 219 bp form described by Lukas et al. (2001).

Screening 87 adult soft-tissue sarcomas (STS), 85 of which expressed the complete MDM2 coding region, revealed at least 14 additional *MDM2* transcripts in 55% of the cases (Bartel et al., 2001). Of these shorter transcripts, two (MDM2-A and -B in Figure 2) had been described previously (Sigalas et al., 1996), whereas the others, to date, are unique to STS (e.g., MDM2-PM2 and -EU2 in Figure 2). Six additional novel *MDM2* splice variants were identified in primary pediatric rhabdomyosarcoma tumors and cell lines (MDM2-FB25, -26, -28, -29, -30, and -55 in Figure 2; Bartel et al., 2002). These variants include alternatively as well as aberrantly spliced forms.

Some of the aberrantly spliced *MDM2* transcripts (e.g., MDM2-PM2, -EU2, -KB3, and -219bp) have a common splicing pattern that is illustrated in Figure 3. Splicing occurs at cryptic splice donor and acceptor sites in regions with high sequence homology and which occur as many as four times in the coding region of the *MDM2* mRNA. For example, a 10-base repeat sequence (TGGCCAGTAT in exon 5; TGCCCAGTAT of exon 12) is involved in the splicing of the KB3 variant (a splice variant detected in STS; Bartel et al., 2001) and the MDM2-219bp variant first described by Lukas et al. (2001). In addition to the splicing at repetitive sequences, the splice variant *MDM2*-DS3 (Bartel et al., 2001) shows another interesting feature. This splice form contains a novel 87 bp sequence inserted between exons 4 and 5. Although the function is not known, this sequence is similar to an α-exon found in the canine *MDM2* mRNA (Veldhoen et al., 1999). Therefore, MDM2-DS3 represents the first identified example of an expressed human MDM2 splice variant that contains α-exon sequence.

In summary, at least 40 alternatively and aberrantly spliced transcripts of *MDM2* mRNA have been identified in tumors, but it is currently unknown how many of these are actually expressed as protein. Most variant transcripts lack sequence that encodes at least part of the p53 binding domain and the p300 binding domain. The fact that some splice variants have been detected only in a particular tumor type suggests that they might contribute to the transformed phenotype of these tumors, whereas others (e.g., *MDM2*-B) may be associated with tumorigenesis in general or be generated as a consequence of the malignant phenotype.

Relationship of MDM2 expression to tumor stage and prognosis

Results of clinical studies investigating the association between MDM2 expression and tumor prognosis are contradictory. Overexpression of MDM2 in acute lymphoblastic leukemia and STS is associated with an unfavorable prognosis (Cordon-Cardo et al., 1994; Gustafsson et al., 1998; Wurl et al., 1998). In contrast, no relationship has been observed between MDM2 expression in glioblastomas and survival of patients (Newcomb et al., 1998), and *MDM2* gene amplification is a favorable prognostic marker in non-small cell lung carcinoma and STS

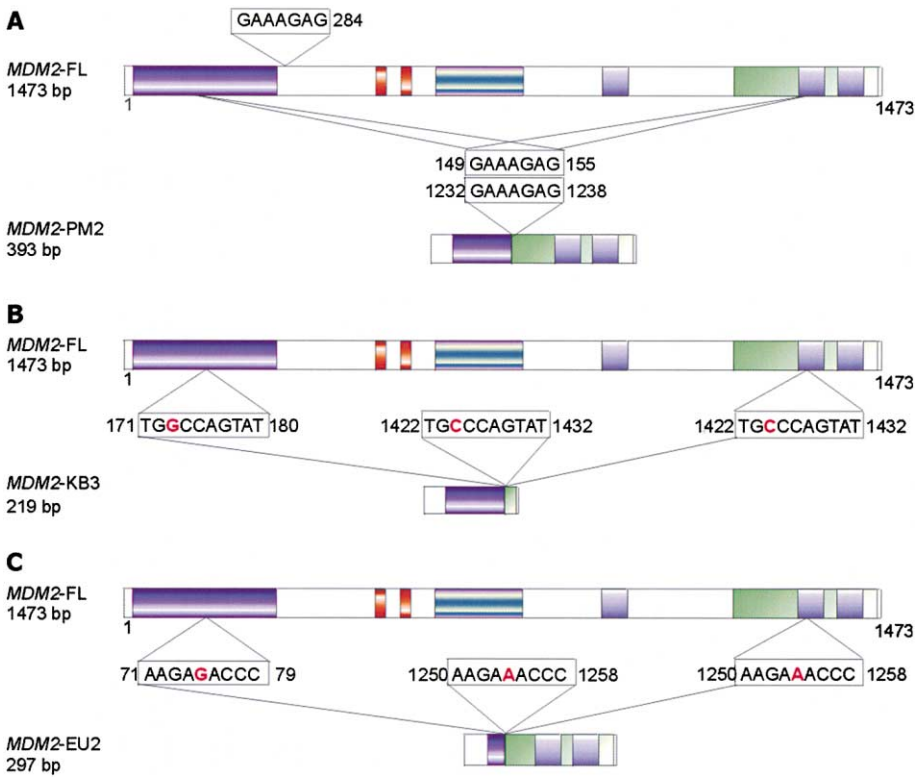

Figure 3. Mechanism of aberrant splicing at repetitive sequences.

MDM2-FL refers to full-length *MDM2* mRNA. The different shaded areas represent the domains of MDM2 as shown in Figure 1 (see text for details).

(Higashiyama et al., 1997; Bartel et al., 2001). In tumors of the head and neck region, the loss of MDM2 expression is associated with a poor prognosis (Millon et al., 2001). A potential explanation of these conflicting findings may be that alternatively or aberrantly spliced MDM2 variants are expressed in certain tumors and, when present, influence prognosis.

Expression of oncogenic splice forms of *MDM2* occurs more frequently in high-grade than low-grade tumors (Matsumoto et al., 1998; Bartel et al., 2001). Expression of aberrantly but not alternatively spliced mRNA in breast carcinoma was associated with a shortened overall patient survival (Lukas et al., 2001), but there was no association with outcome or survival time for patients with STS that expressed either alternatively or aberrantly spliced MDM2 isoforms (Bartel et al., 2001). In giant cell tumors of the bone, splice variants were expressed only in stromal cells, not in the giant cells (Evdokiou et al., 2001). This finding supports the hypothesis that stromal cells comprise the tumor element in these giant cell tumors.

Unfortunately, studies measuring MDM2 protein expression have been carried out using different antibodies, several of which recognize multiple nonspecific proteins in addition to MDM2. Therefore, the information obtained by measuring total MDM2 protein in tumors may be of little value, particularly if the antibodies used for analyses were raised against epitopes not present within the mutant MDM2 proteins. In order to determine whether splice variants are expressed, and to evaluate their relationship to tumorigenesis and prognosis, specific reagents must be developed.

One could speculate that splice variants of MDM2 exhibit an antiapoptotic function, which would help explain why some patients whose tumors contain *p53* mutations and overexpress MDM2 have a worse prognosis than do those whose tumors have only one of these modifications (Wurl et al., 1998; Figure 4). However, as discussed above, MDM2 has been shown to exhibit a p53-independent tumorigenic potential that could generate an additive growth advantage in cells expressing mutant *p53* (Dubs-Poterszman et al., 1995; Jones et al., 1998; Figure 4). It is possible that there are no splice variants expressed in tumors where *MDM2* amplification is associated with a good prognosis (Higashiyama et al., 1997). However, p53 may also facilitate chemotherapy-induced apoptosis in such tumors if MDM2-p53 binding is disrupted (Shieh et al., 1997; Khosravi et al., 1999; Mayo et al., 1997). Additional work will be required to determine which of these hypotheses are correct.

Functions of the MDM2 isoforms

Although more than 40 splice variants of *MDM2* mRNA have been identified, little is known about their functions, and it is important to remember that it is unknown whether all the variant transcripts are translated into protein. Although most variants have lost internal sequences, there does not appear to be a common deleted region that could suggest a common function. Many examples of alternative splicing force the region encoding the C terminus to be out-of-frame. This change potentially leads to the generation of novel amino acid sequences, but because these new sequences differ in every case, they are unlikely to contribute to a common novel function of the proteins. The open reading frames of many variants contain premature stop codons, whose presence suggests the generation of truncated proteins containing only the N-terminal portion of MDM2.

It is important to note that alternatively and aberrantly spliced *MDM2* mRNAs are usually found together with full-length *MDM2* transcripts. This finding is of interest because Evans et al. (2001) have demonstrated that at least one splice variant (MDM2-B, ALT1) can bind to full-length MDM2 protein and sequester it in the cytoplasm. The splice variant MDM2-B is the most frequently expressed form in numerous types of cancer, including ovarian and bladder cancers (Sigalas et al., 1996), breast cancer (Matsumoto et al., 1998; Lukas et al., 2001), STS (Bartel et al., 2001; Tamborini et al., 2001), and giant cell tumors of the bone (Evdokiou et al., 2001). However, Evans et al. (2001) also demonstrated that the binding of MDM2-B to full-length MDM2 increased wild-type p53 activity. A similar observation was made using the murine splice variants identified by Dang et al. (2002). Binding of these variants to full-length MDM2 in MEFs resulted in a release of wild-type p53 such that p53 was not targeted to the proteasome (as shown in Figure 1A), and was free to mediate growth inhibition as depicted in Figure 4. Expression of splice variants is also associated with p53 stabilization in glioblastoma cell lines despite amplifi-

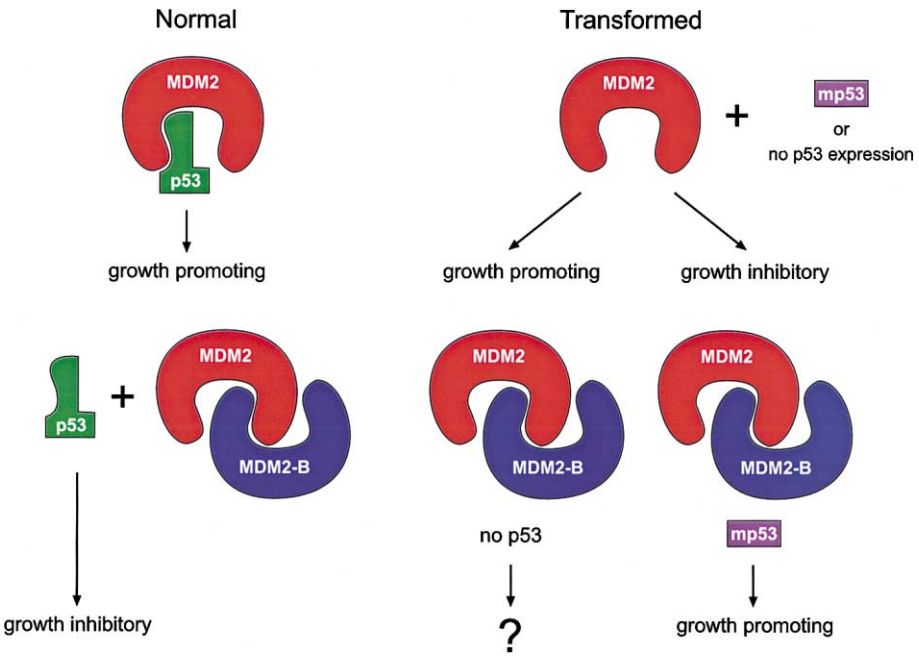

Figure 4. A model suggesting how MDM2 splice variants with an intact C terminus, for example MDM2-B, might exhibit contrasting functions.

In "normal" cells, MDM2 binds to and inhibits the function of wild-type p53, promoting cell growth. When MDM2 splice variants are expressed, they bind to full-length MDM2 protein, releasing p53, resulting in growth inhibition. In transformed cells when p53 may be mutated or not expressed, MDM2 can still promote cell growth. Under these circumstances, there may be no consequence of splice variant expression, because splice variant binding to full-length MDM2 would not release any wild-type p53. Under certain conditions, full-length MDM2 expression in a transformed cell results in apoptosis or growth inhibition. If splice variants are expressed in these cells, they may bind and inhibit the growth-inhibitory function of full-length protein, resulting in an increased rate of proliferation.

cation of the *MDM2* gene (Kraus et al., 1999), and in STS we have found a similar correlation between the expression of *MDM2* splice forms and overexpression of p53 (Bartel et al., 2001). These findings suggest that p53 accumulation arises as a consequence of alternative as well as aberrant MDM2 splicing independent of the mutational status of p53.

Most *MDM2* splice variants described to date lack sequence that encodes at least part of the p53 binding domain. In vitro binding assays have shown that the splice forms MDM2-A, -B, -C, and -D (Figure 2) lack the ability to bind to and inactivate p53 (Sigalas et al., 1996). Despite the fact that the p53 binding domain is retained in a couple of MDM2 isoforms (MDM-E, -FB26) which still have the ability to bind p53, MDM2-mediated degradation of p53 may be inhibited because of the loss of p300 binding (Zhu et al., 2001). Nevertheless, an increased amount of wild-type p53 is inconsistent with a potential transforming phenotype of *MDM2* splice variants.

Sigalas et al. (1996) demonstrated that NIH3T3 cells transfected independently with several splice variants could grow as colonies in soft agar. In support of this transforming function of MDM2 splice variants, unpublished data from S. Jones et al. show that MDM2-B can cause tumors in a transgenic mouse model. In contrast, MDM2-B is expressed in both malignant and normal mammary tissue (Lukas et al., 2001). It appears that MDM2 splice variants display different characteristics in different cellular backgrounds, as shown in Figure 4. Because it is unknown under what conditions alternative functions of full-length MDM2 are active, it is impossible to predict the function of the splice variants in each model system evaluated.

Each of the numerous MDM2 splice forms could be responsible for a distinct phenotype. For example, some isoforms encoded by splice variants may lack the ability to bind to $p14^{ARF}$, whereas other isoforms might bind to this protein and be sequestered in the nucleolus (Weber et al., 2000). Furthermore, the addition of different novel amino acid sequences at the C terminus might generate novel tumorigenic functions. However, many splice variants lack the C terminus, a finding that suggests the importance of the N-terminal portion of the protein. Much work remains before the functions of MDM2 and its isoforms encoded by alternatively spliced variants are understood and before the relationship of this new information to the MDM2-p53-$p14^{ARF}$ pathway is clarified.

Conclusions

The MDM2-p53-$p14^{ARF}$ pathway has been elegantly mapped out by many investigators (including Sherr, 1998), but it now appears that the pathway may not be as straightforward as originally thought. Full-length MDM2 displays both oncogenic and growth inhibitory properties (Figure 4), but how these different functions are controlled is unknown. It appears as though MDM2 function may parallel that of MYC. MYC expression induces apoptosis in cells where the MDM2-p53-$p14^{ARF}$ pathway is functional (Packham and Cleveland, 1995; Zindy et al., 1998). Yet, when this pathway is disrupted, the oncogenic phenotype of MYC is displayed (Sherr, 1998). Similarly, the contrasting functions of full-length MDM2 are probably dependent upon the genetic background of the cells in which they are expressed. At least some MDM2 splice variants encode proteins that possess a transforming function both in vitro and in vivo, but in different model systems, they function to release p53 from full-length MDM2, inducing wild-type p53 activity (Figure 4). Additionally, p53-independent functions of MDM2 and its isoforms encoded by alternatively and aberrantly spliced transcripts might be important in tumorigenesis, but it remains to be seen how these functions are integrated within the well-characterized MDM2-p53-$p14^{ARF}$ tumor surveillance pathway.

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