

# The human oncoprotein MDM2 arrests the cell cycle: elimination of its cell-cycle-inhibitory function induces tumorigenesis

Doris R. Brown, Charles A. Thomas and Swati Palit Deb<sup>1</sup>

Department of Microbiology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284, USA

<sup>1</sup>Corresponding author  
e-mail: debs@uthscsa.edu

**The human oncoprotein MDM2 (hMDM2) overexpresses in various human tumors. If amplified, the *mdm2* gene can enhance the tumorigenic potential of murine cells. Here, we present evidence to show that the full-length human or mouse MDM2 expressed from their respective cDNA can inhibit the G<sub>0</sub>/G<sub>1</sub>-S phase transition of NIH 3T3 and normal human diploid cells. The protein harbors more than one cell-cycle-inhibitory domain that does not overlap with the p53-interaction domain. Deletion mutants of hMDM2 that lack the cell-cycle-inhibitory domains can be stably expressed in NIH 3T3 cells, enhancing their tumorigenic potential. The tumorigenic domain of hMDM2 overlaps with the p53-interaction domain. Some tumor-derived cells, such as Saos-2, H1299 or U-2OS, are relatively insensitive to the growth-inhibitory effects of hMDM2. These observations suggest that hMDM2 overexpression in response to oncogenic stimuli would induce growth arrest in normal cells. Elimination or inactivation of the hMDM2-induced G<sub>0</sub>/G<sub>1</sub> arrest may contribute to one of the steps of tumorigenesis.**

**Keywords:** cell-cycle arrest/human MDM2/oncoprotein/tumorigenesis

## Introduction

The mouse double minute-2 (*mdm2*) gene is evolutionarily conserved among eukaryotes (Fakharzadeh *et al.*, 1991), which suggests that the gene product (MDM2) serves an important function in eukaryotic cells. As is the case for many other oncogenes, the dysfunction of the *mdm2* gene was discovered before its normal function. Originally, the gene was identified as an amplified and overexpressed gene in a spontaneously transformed derivative of mouse BALB/c cell line 3T3DM (Fakharzadeh *et al.*, 1991). Since 3T3DM cells overexpress several splice variants of MDM2, it was not clear which form of the protein is capable of inducing tumorigenesis. Amplification of the entire *mdm2* gene capable of expressing all the spliced forms was shown to enhance tumorigenic potential of murine cells (Fakharzadeh *et al.*, 1991; Finlay, 1993). The human homolog of the *mdm2* gene (*hmdm2*) is frequently overexpressed in many human cancers (Ladanyi *et al.*, 1993; Leach *et al.*, 1993; Sheikh *et al.*, 1993; Cordon-Cardo *et al.*, 1994; Florenes *et al.*, 1994; Quesnel *et al.*,

1994; Reifenberger *et al.*, 1994; Gudas *et al.*, 1995; Baunoch *et al.*, 1996), suggesting that the genetic alteration may be one of the common causes of oncogenesis. Recently, Lundgren *et al.* (1997) reported that 16% of transgenic mice that overexpress MDM2 from a mini-gene containing the introns 7 and 8 of *mdm2* inserted within its cDNA under a bovine  $\beta$ -lactoglobulin promoter develop breast tumor. These findings suggest that MDM2 has an oncogenic function.

The *mdm2* gene product was originally detected in a complex with the tumor suppressor p53 (Momand *et al.*, 1992). Overexpression of *hmdm2* gene was found in many cancer cells with wild-type p53 (Oliner *et al.*, 1992; Leach *et al.*, 1993). These findings led to the hypothesis that hMDM2 may induce oncogenesis by inactivating the tumor suppressor p53 (Oliner *et al.*, 1992; Leach *et al.*, 1993). Later studies reveal alteration in the expression of both *p53* and *hmdm2* genes (Cordon-Cardo *et al.*, 1994), suggesting that abnormal expression of the two genes may confer an additive effect on cell growth. Besides p53, MDM2 also interacts with the retinoblastoma gene product pRb (Xiao *et al.*, 1995), the transcription factors E2F1/DP1 (Martin *et al.*, 1995), ribosomal L5 protein (Marechal *et al.*, 1994), simian virus 40 (SV40) T antigen (Brown *et al.*, 1993) and human TATA-binding protein (Leng *et al.*, 1995a).

Among all the interactions of MDM2 with cellular or viral proteins, the interaction of human or mouse MDM2 with p53 has been investigated the most. Our laboratory, as well as others, have shown that human or mouse MDM2 can interact with the tumor suppressor p53 in cell-free systems (Brown *et al.*, 1993; Chen *et al.*, 1993) or in the whole cell (Brown *et al.*, 1993; Oliner *et al.*, 1993; Haines *et al.*, 1994; Leng *et al.*, 1995b) and inhibit transactivation by wild-type p53. Work from our laboratory showed that the interaction of hMDM2 with p53 is needed for inhibition of p53-mediated transactivation (Brown *et al.*, 1993), and only 127 amino acids (amino acids 28–154) of hMDM2, out of a total of 491, are sufficient to inhibit p53-directed transactivation (Leng *et al.*, 1995b). MDM2 regulates several functions of p53. Human or mouse MDM2 regulates p53-mediated growth suppression and apoptosis (Chen *et al.*, 1994, 1996; Haupt *et al.*, 1996). Also, the p53-regulatory function of MDM2 is required for embryonic development (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995).

Wild-type p53 induces MDM2 expression by recognizing a response element situated downstream of the first exon of the oncogene (Barak *et al.*, 1993, 1994; Juven *et al.*, 1993). Several laboratories (Perry *et al.*, 1993; Chen *et al.*, 1994; Price and Park, 1994; Bae *et al.*, 1995) have shown that ionizing radiation induces MDM2 expression in a p53-dependent manner. Thus, the presence of an autoregulatory feedback loop has been suggested (Barak

**Table I.** Non-tumor cells do not tolerate stable overexpression of full-length hMDM2

Cells	Plasmids transfected	Transient hMDM2 expression	Generation of antibiotic resistant colonies	Antibiotic resistant colonies expressing hMDM2 <sup>a</sup>
Normal human keratinocyte	pCMVneo.hmdm2	ND	+	-
MEF (normal mouse embryo fibroblast)	pHyg <sup>b</sup> + pCMVneo.hmdm2	+	+	-
MEF (p53 <sup>-/-</sup> )	pHyg <sup>b</sup> + pCMVneo.hmdm2	+	+	-
	pCMVneo.hmdm2 del 491-221	+	+	-
10(3) (p53 <sup>-/-</sup> murine fibroblast)	pCMVneo.hmdm2	+	+	-
	pCMVneo.hmdm2 del 491-221	+	+	-
NIH 3T3	pCMVneo.hmdm2	+	+	-
Saos-2 (osteosarcoma)	pCMVneo.hmdm2	+	+	+
H1299 (large cell lung carcinoma)	pCMVneo.hmdm2	+	+	+
	pCMVneo.hmdm2 del 491-221	+	+	+
21PT (human breast tumor)	pCMVneo.hmdm2	+	+	+
	pCMVneo.hmdm2 del 491-221	+	+	+

ND: not done

<sup>a</sup>At least 30 colonies were analyzed in each case.

<sup>b</sup>Plasmid that confers hygromycin resistance to the cell (pHyg).

*et al.*, 1993; Otto and Deppert, 1993; Picksley and Lane, 1993; Wu *et al.*, 1993).

At least five to seven MDM2-related polypeptides have been found in cultured mouse or human cells that overexpress MDM2 (Gudas *et al.*, 1993; Olson *et al.*, 1993; Haines *et al.*, 1994; Maxwell, 1994; Sigalas *et al.*, 1996). Two of these five forms (p90, the full length protein and p58, which harbors a deletion at the C-terminus) are capable of binding p53. The presence of alternately spliced forms of MDM2 that cannot bind p53 suggests p53-independent biochemical function(s) of hMDM2. It is not known which form(s) of MDM2 induce tumorigenesis. As stated above, several laboratories including ours have shown that full-length human or mouse MDM2 expressed from their respective cDNA can interact with p53 and inhibit p53-mediated transactivation (Momand *et al.*, 1992; Brown *et al.*, 1993; Oliner *et al.*, 1993; Haines *et al.*, 1994; Leng *et al.*, 1995b). If hMDM2 induces tumorigenesis by inactivating the tumor suppressor p53, overexpression of full-length hMDM2 from its cDNA should enhance tumorigenic potential of NIH 3T3 cells. Also, the deletion mutants of hMDM2 that interact with p53 to inactivate its transcriptional activation should harbor the same property. To test this hypothesis, we overexpressed full-length hMDM2 or its deletion mutants from their respective cDNA in NIH 3T3 cells and analyzed the growth regulatory properties of the oncoprotein. Our results show that the full-length hMDM2 inhibits the G<sub>0</sub>/G<sub>1</sub>-S phase transition of the cell cycle and cannot confer growth advantage to NIH 3T3 cells. Some tumor-derived cells are partially insensitive to the hMDM2-mediated cell-cycle inhibition. Deletion of the cell-cycle-inhibitory domains of hMDM2 activates the tumorigenic potential of the oncoprotein. The cell-cycle-inhibitory function of hMDM2 is p53-independent, but the tumorigenic domain overlaps with its p53-interaction domain.

## Results

### **Overexpression of full-length hMDM2 from its cDNA is disadvantageous to the growth of non-tumor cells**

Since full-length hMDM2 expressed from its cDNA can interact with p53 and inhibit p53-mediated transcriptional

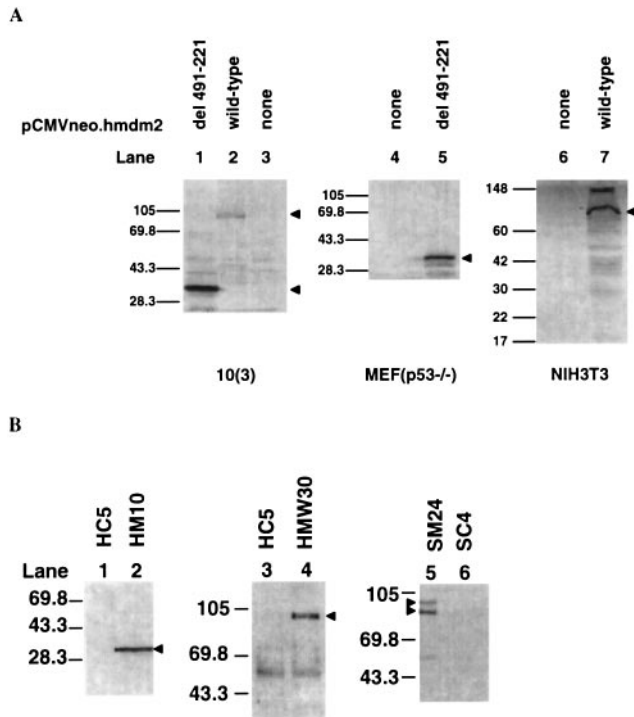
activation (Brown *et al.*, 1993; Leng *et al.*, 1995b), we wished to determine whether hMDM2 can enhance the tumorigenic potential of NIH 3T3 cells by inactivating p53. As a first step, we attempted to generate stable transfectants of NIH 3T3 cells that overexpress hMDM2. An hMDM2 expression plasmid harboring the hMDM2 cDNA (pCMVneo.hmdm2; Leng *et al.*, 1995b) was transfected into NIH 3T3 cells. Forty-eight hours after transfection, half of the cells were analyzed for transient expression and the other half was selected to generate neomycin resistant colonies. The results (summarized in Table I) showed that although hMDM2 expressed transiently (Figure 1A) and G418-resistant colonies were generated, hMDM2 expression was not detected in the pooled G418-resistant stable transfectants or in isolated colonies. Similar results were found in several other cell lines that are not derived from tumors (Table I).

Our attempts to generate stable transfectants of some tumor-derived cells overexpressing hMDM2, on the other hand, were successful. Tumor-derived cells, such as Saos-2 (Masuda *et al.*, 1987), H1299 (Mitsudomi *et al.*, 1992) and 21PT (Band *et al.*, 1990), were transfected with the hMDM2 expression plasmid (pCMVneo.hmdm2; Leng *et al.*, 1995b). G418-resistant colonies were selected and expanded. Western blot analysis of the cell extracts showed stable expression of hMDM2 in these cells (Figure 1B). These results suggest that overexpression of hMDM2 is not toxic to all of the cells.

Also, MDM2 has been stably overexpressed in NIH 3T3 and primary rat embryo fibroblast (REF) cells by amplifying a cosmid harboring the entire *mdm2* gene (Fakharzadeh *et al.*, 1991; Finlay, 1993). The MDM2 overexpressing NIH 3T3 cells have been shown to form tumors in nude mice (Fakharzadeh *et al.*, 1991). These results led us to speculate that MDM2 overexpressed from the cDNA may be functionally different from the oncoprotein overexpressed from the mouse genomic clone. The full-length hMDM2 overexpressed from the cDNA may cause growth disadvantage in cell lines that are not tumor derived.

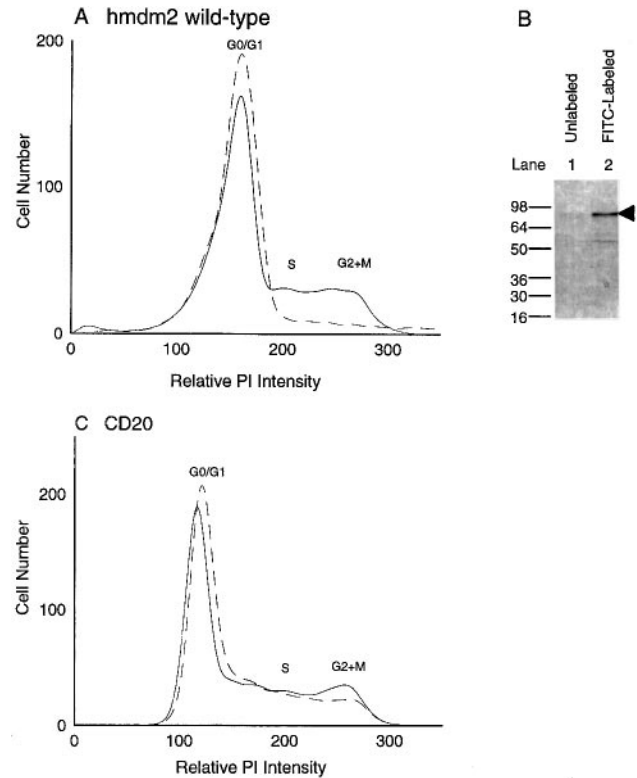
### **hMDM2 blocks the G<sub>0</sub>/G<sub>1</sub>-S phase transition of non-tumor cells**

To determine how hMDM2 modulates cell growth, we analyzed the effect of transiently overexpressed hMDM2



**Fig. 1.** (A) Transient expression of hMDM2 and/or hMDM2 del 491–221 in 10(3) (lanes 1–3), p53<sup>-/-</sup> MEF (lanes 4 and 5) and NIH 3T3 (lanes 6 and 7) cells detected by Western blot analysis. Stable transfectants isolated from the same experiments did not express either of these two proteins. ‘None’ lanes (3, 4 and 6) show Western blot analysis of vector transfected cell extracts. (B) Expression of full-length hMDM2 and hMDM2 del 491–221 in H1299 (lanes 1–4) and Saos-2 (lanes 5 and 6) stable transfectants detected by Western blot analysis. SM24 is a Saos-2, and HMW30 and HM10 are H1299 stable transfectants. SM24 and HMW30 express wild-type hMDM2 and HM10 expresses a C-terminal deletion mutant hMDM2 del 491–221. HC5 and SC4 are H1299 and Saos-2 cells, respectively, that are stably transfected with the vector. For both panels, migration of the mol. wt markers is shown on the left and migration of hMDM2 and hMDM2 del 491–221 is indicated by arrowheads.

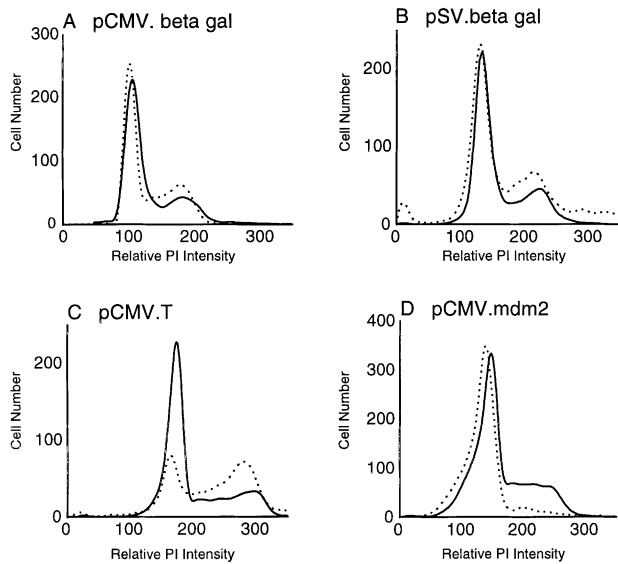
on cell-cycle progression of NIH 3T3 cells. NIH 3T3 cells were transfected with hMDM2 expression plasmid harboring the human cDNA (pCMV.hmdm2; Leng *et al.*, 1995b). Cells were harvested and fixed 48 h after transfection. The hMDM2-overexpressing cells were identified by immunostaining with an anti-hMDM2 monoclonal antibody (2A10; Chen *et al.*, 1993) and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. The cells were then stained with propidium iodide. The FITC-labeled and unlabeled cells were sorted (30 000 cells in each case) and analyzed for cell-cycle transition by flow cytometry (program PEAKS3.FTN) using a fluorescence-activated cell sorter (FAC Star<sup>plus</sup>, Becton Dickinson). Results of several (more than six) independent experiments using three different anti-hMDM2 antibodies showed that transient overexpression of full-length hMDM2 inhibits the G<sub>0</sub>/G<sub>1</sub>–S phase progression of the NIH 3T3 cells (Figure 2A). As shown in the figure, >90% of the FITC-labeled cells (shown by a broken line) were arrested at the G<sub>0</sub>/G<sub>1</sub> phase, and only 8% of the cells were cycling (S+G<sub>2</sub>+M phases). On the other hand, 38% of unlabeled cells (solid line) were found in the S+G<sub>2</sub>+M phases. hMDM2 expression in the FITC-labeled cells was confirmed by Western blot analysis of the sorted FITC-labeled



**Fig. 2.** Overexpression of hMDM2 blocks the G<sub>0</sub>/G<sub>1</sub>–S phase transition of NIH 3T3 cells. pCMV.hmdm2- or pCMV.CD20-transfected cells were identified by immunostaining with their respective monoclonal antibody and an FITC-conjugated secondary antibody. (A) DNA content of the FITC-labeled (broken line) and unlabeled (solid line) cells were analyzed after propidium iodide staining. Compared with the unlabeled cells (38%), lower percentage (8%) of FITC-labeled cells progressed to the S+G<sub>2</sub>+M phases. (B) Transient expression of hMDM2 in sorted FITC-labeled cells detected by Western blot analysis. 30 000, 91% pure, FITC-labeled cells and 60 000, 99% pure, unlabeled cells were used for this purpose. Migration of the mol. wt markers is shown on the left and migration of hMDM2 is indicated by an arrowhead. (C) pCMV.CD20-transfected NIH 3T3 cells did not undergo G<sub>0</sub>/G<sub>1</sub> arrest. Similar percentage of FITC-labeled (broken line) or unlabeled (solid line) cells progressed to the S+G<sub>2</sub>+M phases.

and unlabeled cells. Figure 2B (lane 2) shows that hMDM2 was expressed in the FITC-labeled cells.

To confirm that the hMDM2-mediated inhibition of the G<sub>0</sub>/G<sub>1</sub>–S phase transition is not an artifact due to transfection of plasmid DNA or non-specific overexpression of any protein, we transfected NIH 3T3 cells separately with three different plasmids. One of these plasmids (pCMV.CD20) expresses the surface marker CD20 under the control of cytomegalovirus (CMV) promoter, a second plasmid (pCMV.βgal) expresses the enzyme β-galactosidase under the control of CMV promoter, and the third plasmid (pSV.βgal) also expresses the enzyme β-galactosidase under the control of SV40 early promoter. Cells overexpressing CD20 or β-galactosidase were identified using their respective antibodies and an FITC-labeled secondary antibody. A monoclonal antibody against CD20 (Becton Dickinson) was used to identify pCMV.CD20 transfected cells, whereas cells overexpressing β-galactosidase were identified using a polyclonal antibody against β-galactosidase (5' to 3'). Overexpression of the surface marker CD20 (Figure 2C) or the enzyme β-galactosidase



**Fig. 3.** Human or mouse MDM2-mediated  $G_0/G_1$  arrest is not a result of non-specific protein overexpression. (A) pCMV.β-gal, (B) pSV.β-gal, (C) pCMV.T or (D) pCMV.mdm2 expression plasmid was transfected into NIH 3T3 cells. Transfected cells were identified by immunostaining with the respective antibodies and an FITC-conjugated secondary antibody. DNA content of the FITC-labeled (broken line) and unlabeled (solid line) cells were analyzed after propidium iodide staining. The figure shows that overexpression of β-galactosidase from the CMV promoter (A) or from the SV40 early promoter (B) does not block the  $G_0/G_1$ -S phase transition of NIH 3T3 cells (similar percentage of FITC-labeled or unlabeled cells progressed to the S+G<sub>2</sub>+M phases). Overexpression of T antigen (C) accelerates the  $G_0/G_1$ -S phase transition of NIH 3T3 cells. Compared with the unlabeled cells (32%), higher percentage (72%) of FITC-labeled cells progressed to the S+G<sub>2</sub>+M phases, whereas mouse MDM2 (D) blocks the  $G_0/G_1$ -S phase transition (42.5% unlabeled and 9.9% labeled cells progressed to the S+G<sub>2</sub>+M phases).

(Figure 3A and B) did not significantly alter the phase distribution of transfected and untransfected cells, suggesting that the hMDM2-mediated inhibition is not due to overexpression of any protein.

To determine whether our flow cytometric assay can recognize acceleration of the  $G_1$ -S phase transition, we used the viral oncoprotein SV40 T antigen as a positive control. As in the case of hMDM2, SV40 large T antigen is a transforming protein that interacts with the tumor suppressor p53 (Lane and Crawford, 1979; Linzer and Levine, 1979) and inhibits p53-mediated transcriptional activation (reviewed in Levine, 1993; Lane 1994). To determine the effect T antigen overexpression on the  $G_1$ -S phase transition of NIH 3T3 cells, a T antigen expression plasmid (pCMV.T; Deb *et al.*, 1995) was transfected into NIH 3T3 cells. The T antigen-expressing cells were identified using a monoclonal antibody against T antigen (Pab419; Harlow *et al.*, 1981), sorted and analyzed as described in the earlier experiment. Transient overexpression of T antigen from the CMV promoter enhanced the  $G_0/G_1$ -S phase transition of T antigen expressing cells as expected (Figure 3C).

Although human and mouse MDM2 show high degree of sequence homology (Fakhrazadeh *et al.*, 1991; Oliner *et al.*, 1992), overexpression of mouse MDM2 from the genomic clone was shown to enhance the tumorigenic potential of NIH 3T3 cells (Fakhrazadeh *et al.*, 1991; Finlay, 1993). Therefore, we tested whether the growth-

inhibitory effect is specific for hMDM2. Overexpression of mouse MDM2 from an MDM2 expression plasmid (pCMV.mdm2; Haines *et al.*, 1994) also led to the  $G_0/G_1$  arrest of NIH 3T3 cells (Figure 3D), suggesting that the hMDM2-mediated growth arrest is not species specific.

To determine whether hMDM2-mediated growth arrest is specific for NIH 3T3 cells, hMDM2 expression plasmid was transfected into two normal human diploid fibroblast cell lines, WI38 and MRC5. The untransfected and hMDM2 overexpressing cells were sorted and analyzed as described above. Transient overexpression of hMDM2 caused  $G_0/G_1$  arrest in MRC5 and WI38 cells, suggesting that the effect is not specific for NIH 3T3 cells (Table IIA). As in the case of NIH 3T3 cells, overexpression of the surface marker CD20 from the pCMV.CD20 expression plasmid did not induce  $G_0/G_1$  arrest (Table IIB).

### **hMDM2 blocks the $G_0/G_1$ -S phase transition of NIH 3T3 cells at low or high levels**

Overexpression of MDM2 from the amplified *mdm2* gene has been shown to induce tumorigenesis (Fakhrazadeh *et al.*, 1991; Finlay, 1993). Although the *mdm2* gene was amplified to elevate the normal levels of the protein, in the genomic clone, MDM2 was expressed from its own promoter. Therefore, hMDM2-mediated  $G_0/G_1$  arrest could be a result of high levels of hMDM2 overexpressed from a strong CMV promoter, while the protein may confer growth advantage at low levels. To test this possibility, we analyzed the growth-regulatory effects of hMDM2 at different levels of the protein expressed. We transiently transfected NIH 3T3 cells with 1, 5, 10 and 30 μg of an hMDM2 expression plasmid (pCMV.hmdm2; Leng *et al.*, 1995b). To detect the  $G_0/G_1$  arrest mediated by low levels of wild-type hMDM2, the cells were blocked at mitosis by treatment with nocodazole after transfection (Giunta and Carlo, 1995; Chen *et al.*, 1996). Nocodazole arrests the cell cycle at mitosis. Therefore, the cells that have completed DNA replication (untransfected cells) will not undergo cell division in the presence of nocodazole, and accumulate at the  $G_2/M$  phase. However, cells that will not be able to enter the S phase or complete DNA replication will not be able to reach  $G_2/M$  phase. Thus, hMDM2 expressing cells should not show significant number of cells in the S and  $G_2/M$  phases, even in the presence of nocodazole.

The transfected cells were identified by immunostaining with a monoclonal antibody against hMDM2 (2A10, Chen *et al.*, 1993) and an FITC-labeled secondary antibody, and then sorted. The intensity of the FITC fluorescence associated with the cells was measured at 530 nm using FACS (fluorescence activated cell sorter) and was plotted against cell numbers. The monoclonal antibody used for this experiment recognizes mouse as well as human MDM2 (Chen *et al.*, 1993). Thus, the FITC fluorescence of the untransfected NIH 3T3 cells should be indicative of endogenous MDM2. The amounts of hMDM2 expressed in the transfected and untransfected cells were estimated from the intensity of fluorescence, and compared. The transfected and untransfected cells were also stained with propidium iodide and their DNA content determined.

The result of this experiment is shown in Table III. When 1 μg hMDM2 expression plasmid was used for transfection, most of the transfected cells showed 1.7- to

Table II.

	WI38		MRC5	
	Unlabeled Cells	FITC-labeled Cells	Unlabeled Cells	FITC-labeled Cells
<b>(A) hMDM2 arrests the G<sub>0</sub>/G<sub>1</sub>-S phase transition of normal human diploid cells</b>				
%G <sub>0</sub> /G <sub>1</sub>	65.57 ± 0.37	89.65 ± 1.03	41.77 ± 0.20	79.64 ± 0.88
%S	17.15 ± 0.19	5.45 ± 0.25	35.42 ± 0.19	12.17 ± 0.34
%G <sub>2</sub> +M	20.28 ± 0.20	4.9 ± 0.24	22.81 ± 0.15	8.19 ± 0.28
<b>(B) CD20 expression does not alter cell cycle distribution of normal human diploid cells</b>				
%G <sub>0</sub> /G <sub>1</sub>	75.37 ± 0.59	77.90 ± 0.96	73.40 ± 0.63	75.13 ± 0.89
%S	6.75 ± 0.18	6.49 ± 0.28	12.13 ± 0.26	10.81 ± 0.34
%G <sub>2</sub> +M	17.53 ± 0.29	15.61 ± 0.43	14.47 ± 0.28	14.06 ± 0.38

1.95-fold higher intensity of green fluorescence than the untransfected cells, while the brightest transfected cells showed 2.5- to 3.7-fold higher fluorescence than the untransfected cells. These results suggest that the levels of hMDM2 expressed in these cells were 1.7- to 3.7-fold higher than the untransfected cells. The DNA content analysis showed that 1.7- to 3.7-fold higher expression of hMDM2 inhibited the G<sub>0</sub>/G<sub>1</sub>-S phase progression of cells (Table III).

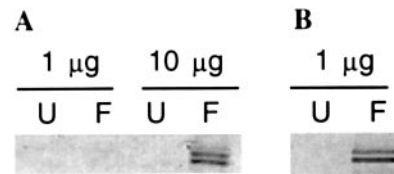
Similarly, when we used 5 µg hMDM2 expression plasmid, most of the transfected cells expressed 5-fold higher levels of hMDM2, while the brightest cells showed 6.6-fold higher expression 6- to 8.3-fold. Similarly, 10 µg hMDM2 expression plasmid enhanced hMDM2 expression. When we used 30 µg expression plasmid, most of the cells expressed 9.5-fold higher levels of hMDM2, whereas the brightest cells showed 22.6-fold higher levels of hMDM2. Since an increase in the amount of expression plasmid from 5–30 µg showed an increase in the FITC fluorescence, the amount of antibody used was not limiting for cells expressing 5- to 10- fold higher levels of hMDM2. In all the cases, overexpression of hMDM2 resulted in G<sub>0</sub>/G<sub>1</sub> arrest. The efficiency of inhibition increased with 10- to 20-fold increase in the levels of hMDM2, although not proportionally. These results indicate that 2- to 20-fold overexpression of hMDM2 from its full-length cDNA induces G<sub>0</sub>/G<sub>1</sub> arrest in NIH 3T3 cells. Therefore, the hMDM2-mediated G<sub>0</sub>/G<sub>1</sub> arrest could be physiologically relevant when hMDM2 is induced by a factor such as p53. Figure 4 shows the comparative levels of hMDM2 expression in NIH 3T3 cells transfected with 1 or 10 µg hMDM2 expression plasmid, as detected by Western analysis.

#### Some tumor-derived cells are partially insensitive to hMDM2-mediated G<sub>0</sub>/G<sub>1</sub> arrest

Studies in our laboratory and elsewhere suggest that several tumor-derived cells can tolerate stable overexpression of hMDM2 from its cDNA (Figure 1B, lanes 4 and 5; Table I) (Chen *et al.*, 1994). Therefore, we investigated how hMDM2 regulates the G<sub>0</sub>/G<sub>1</sub>-S phase transition of these cells. Since it was possible to generate hMDM2-overexpressing stable transfectants of H1299 or Saos-2 cells, these cells were transfected with the hMDM2 expression plasmid pCMV.hmdm2. The hMDM2-expressing cells were identified and analyzed by flow cytometry as described above. The results [Table IV (A)] show that overexpression of hMDM2 reduces the percentage of

Table III. Overexpression of full-length hMDM2 inhibits the G<sub>0</sub>/G<sub>1</sub>-S phase transition of NIH 3T3 cells at low or high levels

Amount of pCMV.hmdm2 transfected (µg)	Increase in FITC fluorescence in transfected cells	Percent S+G <sub>2</sub> +M cells	
		Unlabeled cells	FITC-labeled cells
1	1.74- to 2.54-fold	48.42 ± 0.49	17.65 ± 0.42
5	5- to 6.6-fold	67.03 ± 0.58	30.34 ± 0.54
30	9.55- to 22.6-fold	55.48 ± 0.53	12.05 ± 0.35
1	1.95- to 3.7-fold	62.85 ± 0.57	25.99 ± 0.51
10	5.9- to 8.3-fold	58.1 ± 0.54	18.16 ± 0.43



**Fig. 4.** Comparative Western blot analysis of hMDM2 expressed from 1 or 10 µg pCMV.hmdm2 in NIH 3T3 cells. (A) NIH 3T3 cells transfected with 1 or 10 µg pCMV.hmdm2 were sorted. 20 000 FITC-labeled (lane F) and unlabeled (lane U) cells were analyzed in each case. (B) hMDM2 expression from 1 µg pCMV.hmdm2 was confirmed by Western analysis of 40 000 FITC-labeled (lane F) and unlabeled (lane U) cells. The Western blot shown in (A) was developed for a shorter period of time than the blot shown in (B).

cycling Saos-2 cells from 54 to 32%, and H1299 cells from 57 to 47%. These results suggest that Saos-2 and H1299 cells are less sensitive to hMDM2-mediated G<sub>0</sub>/G<sub>1</sub> arrest than NIH 3T3, WI38 or MRC5 cells, which show a 3- to 5-fold reduction in the number of cycling cells under similar conditions (Figure 2; Table II). Consistent with this observation, our stable transfection analysis indicates that H1299 and Saos-2 cells can stably overexpress hMDM2 (Table I; Figure 1B). These findings suggest that hMDM2 may stably overexpress in some genetically defective cells that are partially insensitive to the negative growth regulatory effects of hMDM2.

It has been shown that MDM2 relieves p53 or Rb-mediated growth suppression in U-2OS cells (Chen *et al.*, 1996; Xiao *et al.*, 1995). Thus, the U-2OS cells may be completely resistant to hMDM2-mediated growth suppression. To test this possibility, we expressed high or low levels of hMDM2 in U-2OS cells. U-2OS cells were transfected with 1 or 20 µg hMDM2 expression plasmid (pCMV.hmdm2). Consistent with the results reported

Table IV.

(A) Some tumor-derived cells are partially insensitive to hMDM2-mediated G<sub>0</sub>/G<sub>1</sub> arrest

	Saos2		H1299	
	Unlabeled cells	FITC-labeled cells	Unlabeled cells	FITC-labeled cells
%G <sub>0</sub> /G <sub>1</sub>	45.3 ± 0.18	67.7 ± 0.63	42.5 ± 0.52	52.48 ± 0.74
%S	38.2 ± 0.17	23.3 ± 0.37	43.37 ± 0.53	38.12 ± 0.63
%G <sub>2</sub> +M	16.5 ± 0.11	9.0 ± 0.23	14.13 ± 0.30	9.4 ± 0.31

(B) U-2OS cells are tolerant to low levels but sensitive to high levels of hMDM2

	pCMV.hmdm2 (1 µg)		pCMV.hmdm2 (20 µg)	
	Unlabeled cells	FITC-labeled cells	Unlabeled cells	FITC-labeled cells
%G <sub>0</sub> /G <sub>1</sub>	32.27 ± 0.41	29.34 ± 0.50	39.12 ± 0.45	74.17 ± 0.88
%S	45.70 ± 0.48	27.80 ± 0.49	37.40 ± 0.44	19.95 ± 0.45
%G <sub>2</sub> +M	22.63 ± 0.34	42.9 ± 0.61	23.48 ± 0.35	5.88 ± 0.25

earlier (Chen *et al.*, 1996; Xiao *et al.*, 1995), U-2OS cells were tolerant to hMDM2 expressed from 1 µg pCMVhmdm2, and did show a slight increase in the G<sub>1</sub>–S phase transition. However, as in the case of Saos-2 and H1299, U-2OS cells do show G<sub>0</sub>/G<sub>1</sub> arrest at high levels of hMDM2 expressed from 20 µg pCMVhmdm2 [Table IV (B)]. Thus, levels of hMDM2 required to induce growth suppression in tumor-derived cells are higher than that for untransformed cells.

Both Saos-2 (Masuda *et al.*, 1987) and H1299 (Mitsudomi *et al.*, 1992) cells have a homozygous deletion in the p53 gene and do not express any p53 protein. Saos-2 cells express a C-terminal deletion mutant of the retinoblastoma susceptibility gene product (Rb). The protein is non-functional and does not translocate to the nucleus (Shew *et al.*, 1990). H1299 cells express wild-type Rb (Mitsudomi *et al.*, 1992). The U-2OS cell line contains wild-type p53 and Rb (Lee *et al.*, 1987; Diller *et al.*, 1990; Shew *et al.*, 1990). Our data suggest that all three cell lines are partially resistant to hMDM2-mediated growth suppression. Therefore, hMDM2-mediated growth suppression is not dependent on the status of p53 or Rb.

#### Growth-inhibitory domains of hMDM2 reside within amino acid residues 155 to 324

To identify the growth-inhibitory domains of hMDM2, a series of C- and N-terminal deletion mutants were individually expressed in NIH 3T3 cells (Figure 5). To detect growth suppression mediated by the mutants of hMDM2 that may have compromised growth-suppression ability, the cells were blocked at mitosis by treatment with nocodazole after transfection. Cells expressing each deletion mutant were identified with appropriate anti-hMDM2 antibodies, sorted and analyzed by flow cytometry. Results of our experiments show that deletion of N-terminal 58 or 190 amino acid residues did not alter hMDM2-mediated G<sub>0</sub>/G<sub>1</sub> arrest significantly (Figure 6A and B). This result suggests that the growth-inhibitory domain(s) of hMDM2 resides within amino acid residues 190–491. Since the N-terminal 58 amino acid residues are essential for interaction with p53 (Brown *et al.*, 1993; Leng *et al.*, 1995b), the growth-inhibitory effect of hMDM2 does not require an interaction with p53.

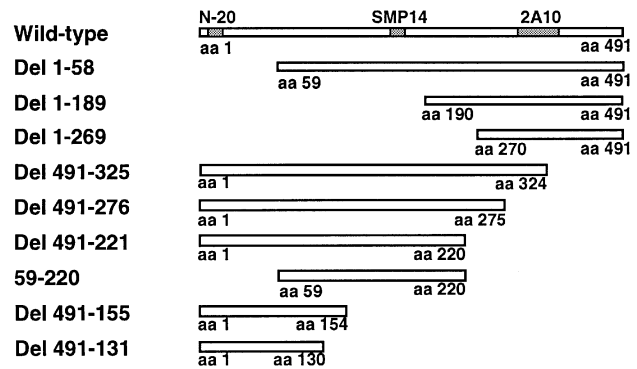
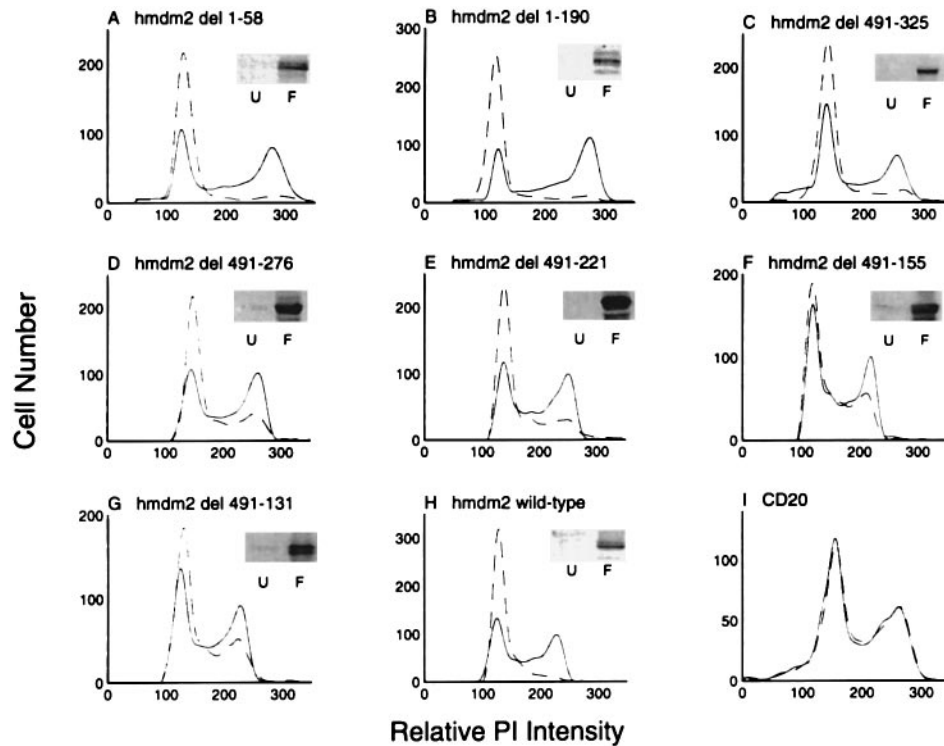


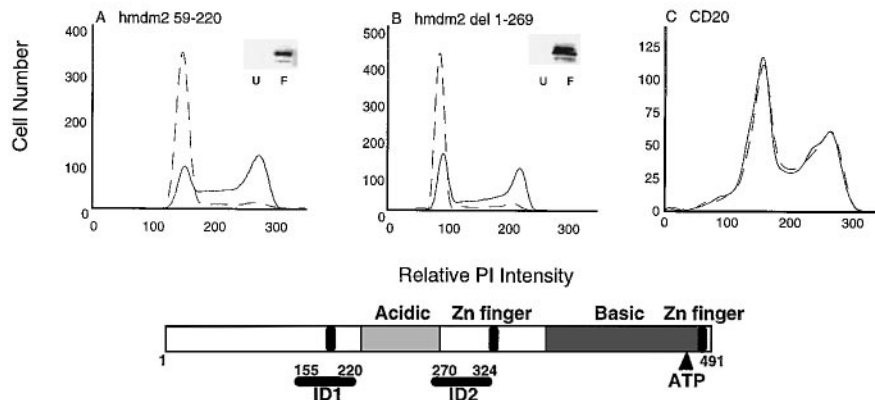
Fig. 5. Deletion mutants of hMDM2 used to determine the growth-inhibitory domains. The epitopes of three antibodies (N20, SMP14 and 2A10) are shown.

Deletion of 166 amino acids from the C-terminus (del 491–325) did not reduce the efficiency of G<sub>0</sub>/G<sub>1</sub> arrest significantly (Figure 6C). Deletion up to amino acid residue 276 from the C-terminus (del 491–276) reduced, but did not fully release, the growth-inhibitory activity (Figure 6D). Further C-terminal deletion up to amino acid 221 did not reduce growth inhibition any further (Figure 6E). These results suggest that at least one growth-inhibitory domain resides within the amino acid residues 324–276. We will refer to this domain as inhibitory domain 2 (ID2). Consistent with this observation, results of our stable transfection analysis showed that this C-terminal deletion mutant of hMDM2 (del 491–221) cannot be stably expressed in NIH 3T3 cells (Table I). Further deletion up to amino acid 155 or 131 drastically reduced the growth arrest (Figure 6F and G). Thus, another growth-inhibitory domain resides within the amino acid residues 155–221. We will refer to this domain as inhibitory domain 1 (ID1). As expected, overexpression of full-length hMDM2 induces G<sub>0</sub>/G<sub>1</sub> arrest in this experiment (Figure 6H), whereas overexpression of CD20 did not alter the cell-cycle profile (Figure 6I).

To confirm that hMDM2 harbors two non-overlapping growth suppressor domains, we generated two deletion mutants of hMDM2. The mutant hMDM2 58–220 harbors ID1, whereas the mutant hMDM2 del 1–269 harbors ID2



**Fig. 6.** The full-length hMDM2 has more than one growth-inhibitory domain. Flow cytometric analysis of NIH 3T3 cells transiently expressing wild-type and deletion mutants of hMDM2. The deletion mutants used are labelled at the top of the figure. The cell-cycle profile of the FITC-labeled cells are shown by a broken line, and that of the unlabeled cells by a solid line. The 2A10 antibody was used for experiments (A) and (B), SMP14 antibody was used for experiments (C–E) and N-20 was used for (F–H). Expression of the deletion mutants of hMDM2 in the sorted FITC-labeled (lane F) and unlabeled (lane U) cells are shown with each cell-cycle profile. (I) CD20 overexpression did not alter the cell-cycle profile of NIH 3T3 cells in the presence of nocodazole. FITC-labeled or CD20 expressing cells (broken line) showed the same pattern as the unlabeled (solid line) cells. A monoclonal antibody against CD20 (Becton Dickinson) was used to identify the transfected cells.



**Fig. 7.** hMDM2 harbors two independent growth-inhibitory domains. Flow cytometric analysis of NIH 3T3 cells transiently expressing deletion mutants of hMDM2 harboring (A) growth-inhibitory domain 1 (ID1) or (B) growth-inhibitory domain 2 (ID2). The deletion mutants used are labelled at the top of the figure. The cell-cycle profile of the FITC-labeled cells are shown by a broken line and that of the unlabeled cells by a solid line. Cells expressing hMDM2 59–220 were recognized by the antibody SMP14 (A) and the cells expressing hMDM2 del 1–269 were recognized by the antibody 2A10 (B). Expression of the deletion mutants in the sorted cells are shown by Western analysis. U: unlabeled and F: FITC-labeled cells. (C) pCMV.CD20 transfected cells did not show any significant alteration in the cell-cycle profile.

(Figure 7). Our data suggest that either of the mutants efficiently arrests the  $G_0/G_1$ –S phase transition of NIH 3T3 cells (Figures 7A and B), whereas overexpression of CD20 does not have any significant effect on the cell cycle (Figure 7C). As shown in Figure 6E, the efficiency of growth arrest induced by the hMDM2 deletion mutant del 491–221 that harbors ID1 was less than that induced by hMDM2 58–220. This difference was reproducible in several experiments suggesting that the N-terminal 58 amino acid residues may interfere with the growth arrest

function of ID1. The extent of growth arrest induced by different deletion mutants of hMDM2 is summarized in Table V.

**The deletion mutants of hMDM2 lacking the putative nuclear localization signals can localize into the nucleus**

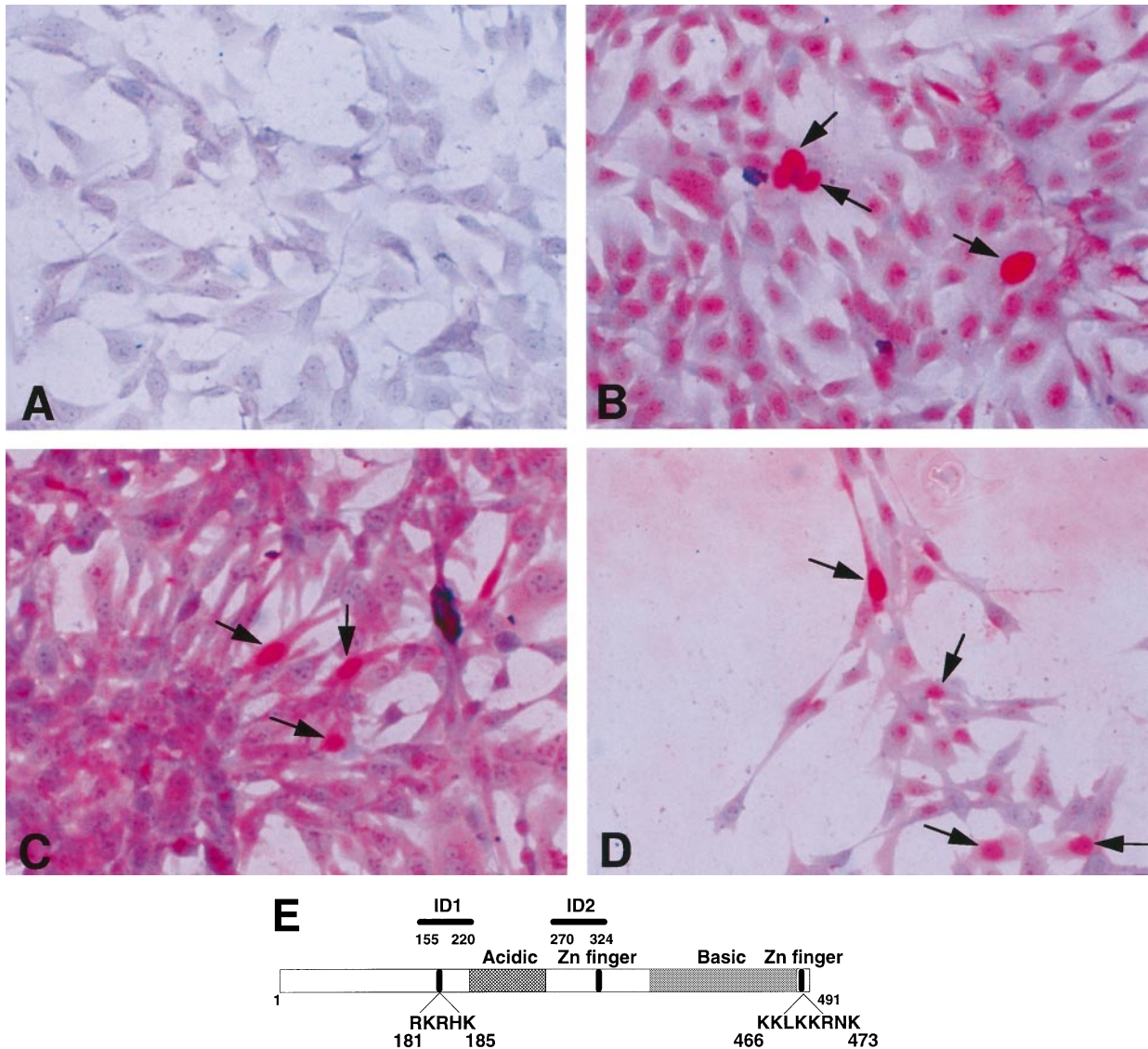
As shown in Figure 8E, hMDM2 has two putative nuclear localization signals, one (RKRHK) is located in between amino acid residues 181–185, and a second signal,



**Table V.** Growth arrest induced by expression of different mutants of hMDM2

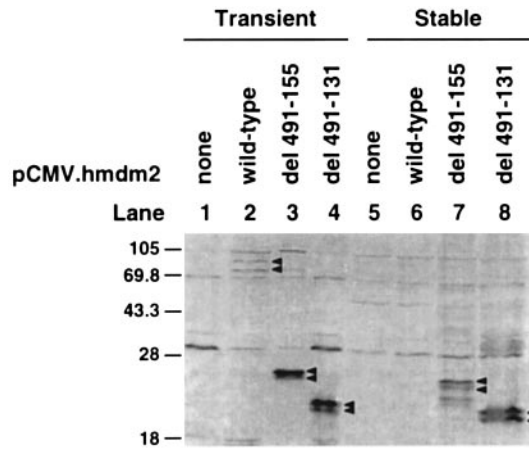
hMDM2 protein expressed	Percent S+G <sub>2</sub> +M cells	
	Unlabeled cells	FITC-labeled cells
Wild-type	58.1 ± 0.54	18.16 ± 0.43
Del 1-58	71.47 ± 0.61	18.67 ± 0.45
Del 1-189	78.89 ± 0.64	15.25 ± 0.38
Del 1-269	70.04 ± 0.60	17.49 ± 0.43
Del 491-325	65.95 ± 0.63	19.84 ± 0.46
Del 491-276	71.12 ± 0.61	40.62 ± 0.67
Del 491-221	74.04 ± 0.65	33.34 ± 0.75
59-220	84.87 ± 0.66	14.53 ± 0.30
Del 491-155	73.79 ± 0.73	65.75 ± 0.84
Del 491-131	74.20 ± 0.71	56.21 ± 0.78

KKLKKRNK is located in between amino acid residues 466-473 (Roberts, 1989; Fakharzadeh *et al.*, 1991; Oliner *et al.*, 1992; Sigalas *et al.*, 1996). Thus, all of the deletion mutants (Table V), except hMDM2 del 491-154 and del 491-130, should contain at least one nuclear localization signal. Since only the mutants hMDM2 del 491-154 and del 491-130 do not induce growth arrest of NIH 3T3 cells, it is important to know whether the mutants lacking the nuclear localization signals are localized to the nucleus. To determine the localization of transiently expressed hMDM2, NIH 3T3 cells were transfected with 1 µg of wild-type or del 491-155 expression plasmids. Cells were fixed 48 h after transfection and immunostained using a monoclonal antibody against hMDM2 (Ab1, Oncogene Science), a biotin-conjugated secondary antibody and avidin-biotin alkaline phosphatase complex (Vector Laboratories). The OSACL cells overexpress hMDM2 (Khatib *et al.*, 1993) and were used as positive controls.



**Fig. 8.** Nuclear localization of the wild-type hMDM2 and del 491-155. Wild-type or the deletion mutant of hMDM2 was immunostained to develop red color. (A) OSACL cells immunostained in the absence of primary antibody, (B) OSACL cells and (C) NIH 3T3 cells transfected with 1 µg pCMV.hmdm2, or (D) NIH 3T3 cells transfected with 1 µg pCMV.hmdm2 del 491-155 were immunostained in the presence of Ab1 (Oncogene Science). Presence of hMDM2 was detected by development of red color. Cells that were stained comparatively darker are shown by arrows. Magnification of the photograph is 200×. (E) The putative nuclear localization signals of hMDM2.





**Fig. 9.** Stable expression of hMDM2 deletion mutants that lack the growth inhibitory domains in NIH 3T3 cells. Lysates prepared from transiently or stably transfected NIH 3T3 cells were subjected to Western blot analysis using an N-terminal anti-hMDM2 antibody (N-20, Santa Cruz). Plasmids used for transfection are shown at the top. Migration of the wild-type or deletion mutants of hMDM2 are indicated by arrowheads. Positions of the mol. wt markers in kDa are shown at the side.

To determine the background staining, OSACL cells were stained in the absence of the primary antibody. As expected, the data show an insignificant amount of background staining (Figure 8A). When the cells were immunostained in the presence of a monoclonal antibody against hMDM2, presence of hMDM2 was detected as red color in the nucleus (Figure 8B). Immunostaining of the NIH 3T3 cells transfected with the wild-type or mutant (del 491–155) hMDM2 expression plasmids are shown in Figure 8C and D, respectively. In both the cases, the red stain was detected in the nuclei indicating nuclear localization of the wild-type and del 491–155.

#### **Deletion mutants of hMDM2 lacking the growth-inhibitory domains can be expressed stably in NIH 3T3 cells**

Since deletion up to the amino acid residue 155 or 131 released the growth-inhibitory property of hMDM2 (Figure 6F and G), we attempted to express these mutant proteins (del 491–155 or del 491–131) stably in NIH 3T3 cells, as described in Materials and methods. Pooled G418-resistant colonies generated from the transfected cells were analyzed for stable expression of hMDM2 or its deletion mutants. Pooled G418-resistant colonies generated from vector-transfected cells were used as control. Transient expression of the hMDM2 deletion mutants was also tested to confirm that the proteins were expressed transiently. The data obtained is shown in Figure 9. hMDM2 and its deletion mutants were expressed in transiently transfected cells (lanes 2–4). However, only the deletion mutants del 491–155 and del 491–131 were stably overexpressed in the G418-resistant pooled colonies (lanes 7 and 8). Cell extracts prepared from G418-resistant pooled colonies of vector-transfected (lanes 1 and 4) or pCMV.hmdm2-transfected (lane 6) cells did not show expression of wild-type hMDM2 or the mutants. This observation confirms that deletion of both the growth-inhibitory domains releases hMDM2-mediated growth arrest.

**Table VI.** Overexpression of hMDM2 deletion mutants lacking the growth inhibitory domains enhances the tumorigenic potential of NIH 3T3 cells

Experiment No.	Protein ectopically expressed	No. mice/No. sites injected	No. tumors generated/No. sites injected
1	None <sup>a</sup>	3/6	0/6
	Del 491–155	3/6	6/6
	Del 491–131	3/6	6/6
2	None <sup>a</sup>	3/6	0/6
	Del 491–155	3/6	6/6

<sup>a</sup>Vector transfected cells.

#### **Deletion mutants of hMDM2 lacking the growth-inhibitory domains enhance the tumorigenic potential of NIH 3T3 cells**

To determine whether deletion of the growth-inhibitory domains of hMDM2 unmasks its tumorigenic property, we analyzed the tumorigenic potential of the NIH 3T3 stable transfectants overexpressing hMDM2 deletion mutant del 491–155 or del 491–131. NIH 3T3 cells stably transfected with the vector were used as control. Cells ( $1 \times 10^7$ ) obtained from the G418-resistant pooled colonies were injected subcutaneously into flanks of athymic nude mice, as described in Materials and methods. All the mice injected with cells overexpressing hMDM2 deletion mutant del 491–155 or del 491–131 developed palpable tumors at all the injection sites in three weeks. As expected, vector transfected NIH 3T3 cells did not generate any tumor (Table VI).

These results suggest that elimination of the growth-inhibitory domains of hMDM2 activates its tumorigenic property. Also, the tumorigenic domain of hMDM2 resides within the first 130 amino acids of hMDM2. It is important to note that this domain of hMDM2 is sufficient to inactivate p53-mediated transactivation (Leng *et al.*, 1995b) suggesting that hMDM2 may induce tumorigenesis by inactivating p53. Although located within the first 130 amino acids, the tumorigenic and p53-interaction domains of hMDM2 may, however, function independent of each other. Further analyses are needed to answer this question.

## **Discussion**

Results of our experiments show that the human oncoprotein hMDM2 expressed from its cDNA can arrest the cell-cycle progression of untransformed cells at the G<sub>0</sub>/G<sub>1</sub> phase. Elimination of this growth-inhibitory function converts the protein to a tumorigenic form. These properties of hMDM2 are consistent with the general definition of oncoproteins. Proto-oncoproteins, the normal cellular counterparts of oncoproteins, are needed for normal cell-growth regulation, while the oncoproteins induce tumorigenesis if abnormally expressed (reviewed in Bishop, 1991; Hunter, 1991).

A recent report (Serrano *et al.*, 1997) has demonstrated that overexpression of oncogenic *ras* can induce G<sub>1</sub> arrest of primary human and rodent cells. Loss of the growth-arrest function activates its oncogenic property. These properties of oncogenic *ras* are remarkably similar to that of hMDM2. The growth arrest induced by *ras*, however,

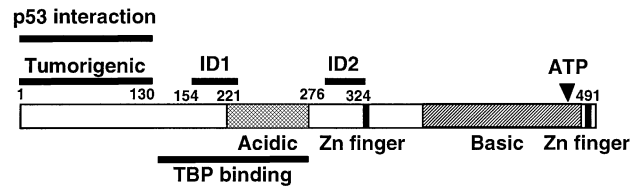
seems to follow a different pathway from hMDM2-mediated growth arrest. The ras-mediated G<sub>1</sub>-arrest requires induction of functional p53 and p16. hMDM2 can induce G<sub>0</sub>/G<sub>1</sub>-arrest in NIH 3T3 cells which have deleted p16 gene (Linardopoulous *et al.*, 1995; Quelle *et al.*, 1995) and in p53<sup>-/-</sup> cells (Table I; Figure 2).

The two apparently opposite functions of hMDM2, tumorigenesis and growth arrest, are intriguing and suggest a novel cell-growth-regulatory mechanism. The cell-cycle-inhibitory function could be a protective mechanism of normal cells against growth proliferation in response to abnormal tumorigenic signals. It is possible that both the functions are necessary at different stages of normal cell growth, and a balance of the two functions is maintained in normal cells. It is unlikely that the tumorigenic forms are derived in normal cells by structural modification of the full-length protein, because in that case we should be able to generate stable transfectants of untransformed cells expressing the full-length protein.

Since the mouse and the human *mdm2* gene expresses several splice variants (Gudas *et al.*, 1993; Olson *et al.*, 1993; Haines *et al.*, 1994; Maxwell, 1994; Sigalas *et al.*, 1996), it is possible that some splice variants code for the tumorigenic MDM2, which lacks functional growth-inhibitory domains. This hypothesis agrees with the fact that amplification of the *mdm2* gene (Fakharzadeh *et al.*, 1991; Finlay 1993) or insertion of introns within its cDNA (Lundgren *et al.*, 1997) is necessary to overexpress the protein in untransformed cells and to induce tumorigenesis (Fakharzadeh *et al.*, 1991; Finlay, 1993). Presence of the introns may be necessary to generate the tumorigenic MDM2. This hypothesis is also consistent with our observation that overexpression of deletion mutants of hMDM2 that lack the growth inhibitory domains induces tumorigenicity in NIH 3T3 cells (Table VI).

NIH 3T3 cells should express normal levels of endogenous MDM2. Also, amplification of the *mdm2* gene should generate the full-length protein with the growth inhibitory property as well as the tumorigenic form(s). Since the *mdm2* gene amplification or overexpression of the hMDM2 deletion mutants induces tumorigenesis in NIH 3T3 cells (Fakharzadeh *et al.*, 1991; Table VI), the oncogenic form of MDM2 or hMDM2 should have a dominant effect over the growth-inhibitory form. It is possible that MDM2 expression from the normal gene in normal cells is regulated differently than expression from the amplified gene, and overexpression of the tumorigenic form may be favored when the gene is amplified. Different regulatory factors may induce expression of different splice variants of hMDM2. It is known that the tumor suppressor p53 can induce MDM2 expression (Barak *et al.*, 1993, 1994; Juven *et al.*, 1993; Wu *et al.*, 1993). If the *mdm2* gene is amplified, it is likely that the amount of p53 available to induce MDM2 expression from all the templates will be limiting. The tumorigenic form of MDM2 expressed under this condition may be different from the p53-induced form. hMDM2 overexpression in breast cancer cells has been associated with the presence of estrogen receptor (Sheikh *et al.*, 1993; Gudas *et al.*, 1995; Baunoch *et al.*, 1996). Thus, the estrogen receptor may induce the overexpression of the tumorigenic form of hMDM2.

As indicated by our results, some tumor cells are



**Fig. 10.** Functional domains of hMDM2. The acidic, basic, Zn-finger and ATP-binding domains have been predicted by computer analysis. The rest of the functional domains are shown according to our studies. The two growth-inhibitory domains are labelled ID1 and ID2. As described in the text, the tumorigenic domain overlaps with the p53-interaction domain.

partially insensitive to the growth-inhibitory effect of hMDM2, and hMDM2 can be stably overexpressed in these tumor cells (Tables I and IV and Figure 1B). This observation suggests that some tumor-derived cells could be defective in the ability to respond to hMDM2-mediated growth inhibition. This property of hMDM2 is very similar to the retinoblastoma (Rb) and Rb family of proteins, which do not suppress growth of some tumor-derived cells (Zhu *et al.*, 1993). Tumor-derived cells are often polyclonal in nature. It is also possible that some of the cell populations are fully resistant to hMDM2-mediated growth arrest, while the others are susceptible. The hMDM2-resistant cell population can stably overexpress the oncoprotein (Table I and Figure 1B).

Consistent with this observation, it has been shown that hMDM2 can inhibit p53-mediated growth suppression in tumor-derived cells (Chen *et al.*, 1994, 1996; Haupt *et al.*, 1996). As the growth-inhibitory domains of hMDM2 are non-functional in such cells, the p53-interaction domain would inhibit p53-mediated growth suppression. Thus, the hMDM2 overexpression observed in cancer cells could be indicative of a genetic defect that renders the cells partially tolerant to hMDM2 overexpression, or a result of overexpression of hMDM2 that harbors inactive growth-inhibitory domains. Analysis of the splice variants of hMDM2 overexpressing in human cancer cells may reveal whether one, or both, of the mechanisms are involved in hMDM2-mediated tumorigenesis.

Our deletion analysis suggests that hMDM2 harbors more than one growth-inhibitory domain. These growth-inhibitory domains (ID1 and ID2) do not overlap with the p53-interaction domain of hMDM2 (Leng *et al.*, 1995b) (Figures 6, 7 and 10). Recently, Schlott *et al.* (1997) have looked for mutations in the *hmdm2* gene from several cancer samples that produced elevated levels of the *hmdm2* transcript in the absence of gene amplification. Using RT-PCR and nucleotide sequencing analysis, they have identified several mutations between amino acids 302 and 310 that may interfere with ID2 defined by our analyses. Mutations in the growth-inhibitory domains of hMDM2 may be an alternate pathway that allows for overexpression of the protein in cancer cells, and needs to be thoroughly explored.

The tumorigenic domain of hMDM2 overlaps with the domain that inhibits p53-mediated transactivation (Table VI and Figure 10). This finding strengthens the hypothesis that inactivation of p53 contributes to the hMDM2-induced tumorigenesis. However, our data do not exclude the possibility that both the tumorigenic domain and the p53-inactivation domain of hMDM2 reside within the

N-terminal 130 amino acid residues and have different sequence requirements or functional domains.

hMDM2 harbors two putative Zn-finger domains and an acidic activation domain (Figure 10) (Brown *et al.*, 1993; Fakharzadeh *et al.*, 1991). We have shown that the putative acidic activation domain of hMDM2 interacts with the human TATA-binding protein *in vivo* (Leng *et al.*, 1995a). Therefore, hMDM2 shows the structural characteristics of a sequence-specific transcriptional activator. We analyzed whether the hMDM2-mediated G<sub>0</sub>/G<sub>1</sub> arrest is associated with induction of one of the growth suppressors such as p53, p21 or p27. The induction of p53, p21 and p27 in sorted hMDM2-expressing and untransfected cells was tested by Western blot analysis using the respective monoclonal or polyclonal antibodies. The results (data not shown) suggest that these proteins are not induced as a consequence of hMDM2 overexpression. Consistent with this observation, the acidic activation domain of hMDM2, involved in binding the TATA-binding protein (Leng *et al.*, 1995a), is not required for the G<sub>0</sub>/G<sub>1</sub> arrest.

It has been reported that p53 regulatory functions of MDM2 are essential for embryonic development (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). Our data show that MDM2 harbors tumorigenic and growth-inhibitory functions. Both the tumorigenic and the growth-inhibitory forms of MDM2 are capable of interacting with p53. These findings raise the question of which form of MDM2 is important for embryonic development. It is possible that only the tumorigenic form of MDM2 is necessary during embryonic development. However, the full-length MDM2 should arrest growth of normal embryonic cells when induced by another factor such as p53. In normal mouse embryonic cells, the growth-inhibitory function of MDM2 may be necessary to block cell growth in response to DNA damage or similar oncogenic stimuli.

## Materials and methods

### Plasmids and hMDM2 deletion mutants

The hmdm2 cDNA, mouse mdm2 cDNA and the plasmid pCMV.CD20 were generous gifts from Bert Vogelstein (Oliner *et al.*, 1992), Donna George (Fakharzadeh *et al.*, 1991) and Ed Harlow (Zhu *et al.*, 1993) respectively. The plasmid pCMV.βgal is from Clontech and pSV.βgal is from Promega. Construction of pCMV.T and the deletion mutants of hmdm2 cDNA has been described earlier in detail (Brown *et al.*, 1993; Deb *et al.*, 1995; Leng *et al.*, 1995a,b). Deletion mutants were generated by polymerase chain reaction (PCR) using primers flanking initiation and termination codons with appropriate start and stop sites. The wild-type or the deletion mutants were cloned in pGEM3zf (-) vector (Promega) under the control of T7 promoter for *in vitro* expression, and in pHCMV Bam vector (Hinds *et al.*, 1990) under the control of CMV promoter for *in vivo* expression. The pHCMV Bam vector has been designated as pCMVneo. The pCMV vector has been derived from pHCMV Bam vector and does not harbor the neomycin resistance gene (Subler *et al.*, 1992).

### Cells and transfections

NIH 3T3, MRC-5, WI38, Saos-2, H1299 and U-2OS cells were purchased from American Type Culture Collection and were maintained in media as suggested by the suppliers. Normal human keratinocyte cells were purchased from Clonetics and were grown in complete keratinocyte growth medium (Clonetics). Normal and p53-null mouse embryo fibroblasts (MEF, gifts from Lawrence Donehower; Harvey *et al.*, 1993) and 10(3) (gift of Arnold Levine; Dittmer *et al.*, 1993) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supple-

mented with 10% fetal bovine serum. 2IPT (gift of Vimla Band; Band *et al.*, 1990) cells were maintained in alpha H and E medium (Band and Sager, 1989).

Cells were seeded 18–24 h before transfection at 0.4–1×10<sup>6</sup> cells per 10 cm dish and transfected by the calcium phosphate method (Gorman *et al.*, 1982). The cells were transfected with plasmids that express wild-type or deletion mutants of hMDM2 and confer G418 resistance. With the wild-type or p53-null MEF cells, a plasmid conferring hygromycin resistance (Sugden *et al.*, 1985) was cotransfected. Half of the cells per 10 cm dish were collected 30–48 h after transfection to test transient expression, and the other half was replated at a ratio of 1:10 or 1:5 to select for G418 (500 µg per ml) or hygromycin (100 µg per ml) resistant colonies. Colonies were isolated and expanded in the presence of appropriate antibiotic. Expression of wild-type or deletion mutants of hMDM2 in pooled and isolated colonies were detected by Western blot analysis of the cell extracts. Western blot analysis was carried out essentially as described previously (Leng *et al.*, 1995b; Shivakumar *et al.*, 1995). Four anti-hMDM2 antibodies, N-20, SMP14 (Santa Cruz), Ab-1 (Oncogene Science) and 2A10 (Chen *et al.*, 1993), recognizing three different domains of hMDM2 (Chen *et al.*, 1993), were used for this purpose.

### Flow cytometry

For flow cytometric analysis, 10 µg expression plasmid were used in most of the cases unless stated otherwise. Cells were harvested 48 h after transfection and fixed with 70% methanol for at least 2 h at -20°C. For experiments where the transiently transfected cells were blocked at mitosis, 40 h after transfection, cells were treated with 20 ng per ml of nocodazole (Oncogene Science) for 8 h. The fixed cells were rehydrated with phosphate-buffered saline (PBS) for 30 min at 4°C, blocked with horse serum to prevent non-specific staining and incubated with appropriate antibody for 12–20 h at 4°C. Cells were then washed three times in PBS and incubated with FITC-conjugated goat anti-mouse or anti-rabbit secondary antibody (Oncogene Science) for 60 min at 37°C. Following incubation, the cells were washed thrice in PBS. For cells transfected with pCMV.CD20, antibody incubations were carried out prior to methanol fixation (Zhu *et al.*, 1993). To stain DNA, the cells were incubated with 0.1 mg/ml RNase A, 50 µg/ml propidium iodide and 0.1% NP40 in a trisodium citrate buffer for 30 min. The samples were analyzed by FACS (FAC Star<sup>plus</sup>, Becton Dickinson). Relative levels of hMDM2 per cell were determined by FITC-fluorescence intensity in the green channel (530 nm). Vector-transfected cells were used to determine the background fluorescence. Cells showing higher FITC-fluorescence intensity than vector-transfected cells were sorted and collected. Expression of hMDM2 (or the deletion mutants) in the sorted cells was reconfirmed by analyzing expression of hMDM2 and its deletion mutants by Western blot analysis. The intensity of propidium iodide staining was recorded in the red channel. PEAKS.FTN or Modfit program was used to determine the percentage of G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>+M phase cells. At least 10 000 FITC-labeled, and an equal number of unlabeled, cells were analyzed in each experiment. Experiments were repeated several times.

The levels of hMDM2 expression in the transfected cells (Table III) was quantitated and compared using a method described earlier (Mittnacht and Weinberg, 1991; Pollice *et al.*, 1992; Sharro *et al.*, 1996). The FITC-fluorescence associated with the transfected cells was compared by using a protocol provided by the instrument's manufacturer (Becton Dickinson). In our flow cytometer, the difference in fluorescence intensities from 10 to 10<sup>4</sup> is divided in 512 channels (linear digitized units). We calculated the difference by plotting the data in a semi-logarithmic plot with the channel number in the x-axis and fluorescence intensities (10 to 10<sup>4</sup>) in the y-axis.

### Immunostaining

To determine localization of transiently expressed hMDM2, NIH 3T3 cells were transfected with 1 µg of wild-type or del 491–155 expression plasmids. Forty-eight hours after transfection, cells were fixed, heat treated and incubated using a monoclonal antibody against hMDM2 (Ab1, Oncogene Science), using a protocol provided by Oncogene Science. The cells were then incubated with a biotin-conjugated secondary antibody and avidin-biotin alkaline phosphatase complex (Vector Laboratories) following the supplier's protocol.

### Tumorigenicity assay

For tumorigenicity assay, 1×10<sup>7</sup> cells suspended in 0.2 ml serum free DMEM were injected subcutaneously into the flanks of 4–6-week-old

female athymic mice (National Cancer Institute). Animals were monitored regularly for tumor occurrence and size.

## Acknowledgements

We thank Dr Donna George for providing the plasmid expressing mouse MDM2, Dr Arnold Levine for the monoclonal antibody 2A10 and the 10(3) cell line, Dr Lawrence Donehower for normal and p53<sup>-/-</sup> mouse embryo fibroblasts and Dr Vimla Band for 21PT cell line. This study was supported by funds from Wendy Will Cancer Research Foundation and NIH CA70712. D.B. is supported by a predoctoral fellowship from Howard Hughes Medical Institute.

## References

- Bae, I., Smith, M.L. and Fornace, A.J. (1995) Induction of p53-, MDM2-, WAF1/CIP1-like molecules in insect cells by DNA-damaging agents. *Exp. Cell Res.*, **217**, 541–545.
- Band, V. and Sager, R. (1989) Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *Proc. Natl Acad. Sci. USA*, **86**, 1249–1253.
- Band, V., Zajchowski, D., Swisshelm, K., Trask, D., Kulesa, V., Cohen, C., Connolly, J. and Sager, R. (1990) Tumor progression in four mammary epithelial cell lines derived from the same patient. *Cancer Res.*, **50**, 7351–7357.
- Barak, Y., Juven, T., Haffner, R. and Oren, M. (1993) MDM2 expression is induced by wild-type p53 activity. *EMBO J.*, **12**, 461–468.
- Barak, Y., Gottlieb, E., Juven-Gershon, T. and Oren, M. (1994) Regulation of MDM2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. *Genes Dev.*, **8**, 1739–1749.
- Baunoch, D.A. et al. (1996) MDM2 overexpression in benign and malignant lesions of the human breast. *Int. J. Oncol.*, **8**, 895–899.
- Bishop, M. (1991) Molecular themes in oncogenesis. *Cell*, **64**, 235–248.
- Brown, D.R., Deb, S., Munoz, M., Subler, M.A. and Deb, S.P. (1993) The tumor suppressor p53 and the oncoprotein Simian Virus 40 T antigen bind to overlapping domains on the MDM2 protein. *Mol. Cell. Biol.*, **13**, 6849–6857.
- Chen, C.Y., Oliner, J.D., Zhan, Q., Fornace, A.J., Vogelstein, B. and Kastan, M.B. (1994) Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. *Proc. Natl Acad. Sci. USA*, **91**, 2684–2688.
- Chen, J., Marechal, V. and Levine, A.J. (1993) Mapping of the p53 and mdm-2 interaction domains. *Mol. Cell. Biol.*, **13**, 4107–4114.
- Chen, J., Wu, X., Lin, J. and Levine, A.J. (1996) mdm2 inhibits the G<sub>1</sub> arrest and apoptosis functions of the p53 tumor suppressor protein. *Mol. Cell. Biol.*, **16**, 2445–2452.
- Cordon-Cardo, C., Latres, E., Oliva, M.R., Pollack, D., Woodruff, J.M., Marechal, V., Chen, J., Brennan, M.F. and Levine, A.J. (1994) Molecular abnormalities of MDM2 and p53 genes in adult soft tissue sarcomas. *Cancer Res.*, **54**, 794–799.
- Deb, S., Brown, D.R., Lega, H.M. and Deb, S.P. (1995) Transcriptional antagonism between p53 and SV40 T antigen. *Int. J. Oncol.*, **6**, 243–249.
- Diller, L. et al. (1990) p53 functions as a cell cycle control protein in osteosarcomas. *Mol. Cell. Biol.*, **10**, 5772–5781.
- Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A., Moore, M., Finlay, C. and Levine, A.J. (1993) Gain of functions in mutations in p53. *Nature Genet.*, **4**, 42–46.
- Fakharzadeh, S., Trusko, R.S. and George, D.L. (1991) Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *EMBO J.*, **10**, 1565–1569.
- Finlay, C.A. (1993) The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth. *Mol. Cell. Biol.*, **13**, 301–306.
- Florenes, V.A., Maeldansmo, G.M., Forus, A., Andreassen, A., Myklebost, O. and Fodstad, O. (1994) MDM2 gene amplification and transcript levels in human sarcomas: relationship to tp53 gene status. *J. Natl Cancer Inst.*, **86**, 1297–1302.
- Giunta, M., and Carlo, P. (1995). Techniques for monitoring cell cycle phrases. In Pagano, M. (ed.), *Cell Cycle Materials and Methods*. Springer-Verlag, pp. 46–52.
- Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. *Mol. Cell. Biol.*, **2**, 1044–1051.
- Gudas, J.M., Nguyen, H., Klein, R.C., Katayose, D., Seth, P. and Cowan, K.H. (1995) Differential expression of multiple MDM2 messenger RNAs and proteins in normal and tumorigenic breast epithelial cells. *Clin. Cancer Res.*, **1**, 71–80.
- Haines, D.S., Landers, J.E., Engle, L.J. and George, D.L. (1994) Physical and functional interaction between wild-type p53 and MDM2 proteins. *Mol. Cell. Biol.*, **14**, 1171–1178.
- Harlow, E., Crawford, L.V., Pim, D.C. and Williamson, N.M. (1981) Monoclonal antibodies specific for simian virus 40 tumor antigen. *J. Virol.*, **39**, 861–869.
- Harvey, M. et al. (1993) *In vitro* growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene*, **8**, 2457–2467.
- Haupt, Y., Barak, Y. and Oren, M. (1996) Cell type-specific inhibition of p53-mediated apoptosis by mdm2. *EMBO J.*, **15**, 1596–1606.
- Hinds, P.W., Finlay, C.A., Quartin, R.S., Baker, S.J., Fearon, E.R., Vogelstein, B. and Levine, A.J. (1990) Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the 'hot spot' mutant phenotypes. *Cell Growth Differ.*, **1**, 571–580.
- Hunter, T. (1991) Cooperation between oncogenes. *Cell*, **64**, 249–270.
- Jones, S.N., Roe, A.E., Donehower, L.A. and Bradley, A. (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature*, **378**, 206–208.
- Juven, T., Barak, Y., Zauberman, A., George, D.L. and Oren, M. (1993) Wild-type p53 can mediate sequence-specific transactivation of an internal promoter within the MDM2 gene. *Oncogene*, **8**, 3411–3416.
- Khatib, Z.A., Matsushime, H., Valentine, M., Shapiro, D.N., Sherr, C.J. and Look, A.T. (1993) Coamplification of the CDK4 gene with MDM2 and GLI in human sarcomas. *Cancer Res.*, **53**, 5535–5541.
- Ladanyi, M., Cha, C., Lewis, R., Jhanwar, S.C., Huvos, A.G. and Healey, J.H. (1993) MDM2 gene amplification in metastatic osteosarcoma. *Cancer Res.*, **53**, 16–18.
- Lane, D.P. (1994) p53 and human cancers. *Br. Med. Bull.*, **50**, 582–599.
- Lane, D.P. and Crawford, L.V. (1979) T antigen is bound to host protein in SV40-transformed cells. *Nature*, **278**, 261–263.
- Leach, F.S., Tokino, T., Meltzer, P., Burrell, M., Oliner, J.D., Smith, S., Hill, D.E., Sidransky, D., Kinzler, K.W. and Vogelstein, B. (1993) p53 mutation and MDM2 amplification in human soft tissue sarcomas. *Cancer Res.*, **53**, 2231–2234.
- Lee, W.-H., Shew, J.-Y., Hong, F.D., Sery, T.W., Donoso, L.A., Young, L.-J., Bookstein, R. and Lee, E.Y.-H.P. (1987) The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. *Nature*, **329**, 642–645.
- Leng, P., Brown, D.R., Deb, S. and Deb, S.P. (1995a) The human oncoprotein MDM2 binds to the human TATA-binding protein *in vivo* and *in vitro*. *Int. J. Oncol.*, **6**, 251–259.
- Leng, P., Brown, D.R., Shivakumar, C.V., Deb, S. and Deb, S.P. (1995b) N-terminal 130 amino acids of MDM2 are sufficient to inhibit p53-mediated transcriptional activation. *Oncogene*, **10**, 1275–1282.
- Levine, A.J. (1993) The tumor suppressor genes. *Annu. Rev. Biochem.*, **62**, 623–651.
- Linardopoulos, S., Street, A., Quelle, D.E., Parry, D., Peters, G., Sherr, C.J. and Balmain, A. (1995) Deletion and altered regulation of p16<sup>INK4a</sup> and p15<sup>INK4b</sup> in undifferentiated mouse skin tumors. *Cancer Res.*, **55**, 5168–5172.
- Linzer, D.I.H. and Levine, A.J. (1979) Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell*, **17**, 43–52.
- Lundgren, K., Montes de Oca Luna, R., McNeil, Y.B., Emerick, E.P., Spencer, B., Barfield, C.R., Lozano, G., Rosenberg, M.P. and Finlay, C.A. (1997) Targeted expression of MDM2 uncouples S phase from mitosis and inhibits mammary gland development independent of p53. *Genes Dev.*, **11**, 714–725.
- Marechal, V., Elenbaas, B., Piette, J., Nicolas, J.C. and Levine, A.J. (1994) The ribosomal L5 protein is associated with MDM2 and MDM2-p53 complexes. *Mol. Cell. Biol.*, **14**, 7414–7420.
- Martin, K., Trouche, D., Hagemeler, C., Sorensen, T.S., LaThangue, N.B. and Kouzarides, T. (1995) Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature*, **375**, 691–694.
- Masuda, J., Miller, C., Koeffler, H.P., Battifora, H. and Cline, M.J. (1987) Rearrangement of the p53 gene in human osteogenic sarcomas. *Proc. Natl Acad. Sci. USA*, **84**, 7716–7719.
- Maxwell, S.A. (1994) Selective compartmentalization of different mdm2 proteins within the nucleus. *Anticancer Res.*, **14**, 2541–2548.
- Mitsudomi, T. et al. (1992) p53 gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of ras mutations and clinical features. *Oncogene*, **7**, 171–180.

- Mitnacht, S. and Weinberg, R.A. (1991) G<sub>1</sub>/S phosphorylation of the retinoblastoma protein is associated with an altered affinity for the nuclear compartment. *Cell*, **65**, 381–393.
- Momand, J., Zambetti, G.P., Olson, D.C., George, D.L. and Levine, A.J. (1992) The mdm-2 oncogene product forms a complex with p53 and inhibits p53-mediated transactivation. *Cell*, **69**, 1237–1245.
- Montes de Oca Luna, R., Wanger, R.S. and Lozano, G. (1995) Rescue of early embryonic lethality in *mdm2*-deficient mice by deletion of p53. *Nature*, **378**, 203–206.
- Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L. and Vogelstein, B. (1992) Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature*, **358**, 80–83.
- Oliner, J.D., Pietenpol, J.A., Thiagalangam, S., Gyuris, J., Kinzler, K.W. and Vogelstein, B. (1993) Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature*, **362**, 857–860.
- Olson, D.C., Marechal, V., Momand, J., Chen, J., Romocki, C. and Levine, A.J. (1993) Identification and characterization of multiple MDM2 proteins and MDM2-p53 protein complexes. *Oncogene*, **8**, 2353–2360.
- Otto, A. and Deppert, W. (1993) Upregulation of *mdm-2* expression in Meth A tumor cells tolerating wild-type p53. *Oncogene*, **8**, 2591–2603.
- Perry, M.E., Piette, J., Zawadzki, J.A., Harvey, D. and Levine, A.J. (1993) The MDM2 gene is induced in response to UV light in a p53-dependent manner. *Proc. Natl Acad. Sci. USA*, **90**, 11623–11627.
- Picksley, S.M. and Lane, D.P. (1993) The p53-*mdm2* autoregulatory feedback loop—A paradigm for the regulation of growth control by p53? *BioEssays*, **15**, 689–690.
- Pollice, A.A. *et al.* (1992) Sequential paraformaldehyde and methanol fixation for simultaneous flow cytometric analysis of DNA, cell surface proteins, and intracellular proteins. *Cytometry*, **13**, 432–444.
- Price, B. and Park, S.J. (1994) DNA damage increases the levels of MDM2 messenger RNA in wtp53 human cells. *Cancer Res.*, **52**, 896–899.
- Quelle, D.E. *et al.* (1995) Cloning and characterization of murine p16<sup>INK4a</sup> and p15<sup>INK4b</sup> genes. *Oncogene*, **11**, 635–645.
- Quesnel, B., Preudhomme, C., Fournier, J., Fenaux, P. and Peyrat, J.P. (1994) MDM2 gene amplification in human breast cancer. *Eur. J. Cancer*, **30A**, 982–984.
- Reifenberger, G., Riefenburger, J., Ichimura, K., Meltzer, P.S. and Collins, V.P. (1994) Amplification of multiple genes from chromosomal region 12q13–14 in human malignant gliomas: preliminary mapping of the amplicons shows preferential involvement in CDK4, SAS, and MDM2. *Cancer Res.*, **54**, 4299–4303.
- Roberts, B. (1989) Nuclear location signal-mediated protein transport. *Biochim. Biophys. Acta*, **1008**, 263–280.
- Schlott, T., Reimer, S., Jahns, A., Ohlenbusch, A., Ruschenburg, I., Nagel, H. and Droese, M. (1997) Point mutations and nucleotide insertions in the MDM2 zinc finger structure of human tumors. *J. Pathol.*, **182**, 54–61.
- Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D. and Lowe, S.W. (1997) Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and p16<sup>INK4a</sup>. *Cell*, **88**, 593–602.
- Sharro, S.A. (1996) Analysis of flow cytometry data. In Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M. and Strober, W. (eds) *Current Protocols in Immunology*. John Wiley and Sons, Somerset, NJ, pp. 5.2.1–5.2.10.
- Sheikh, M.S., Shao, Z.M., Hussain, A. and Fontana, J.A. (1993) The p53-binding protein MDM2 gene is differentially expressed in human breast carcinoma. *Cancer Res.*, **53**, 3226–3228.
- Shew, J.-Y., Lin, B.T.-Y., Chen, P.-L., Tseng, B.Y., Yang-Feng, T.L. and Lee, W.-H. (1990) C-terminal truncation of the retinoblastoma gene product leads to functional inactivation. *Proc. Natl Acad. Sci. USA*, **87**, 6–10.
- Shivakumar, C., Brown, D.R., Deb, S. and Deb, S.P. (1995) Wild-type human p53 activates the human proliferating cell nuclear antigen promoter. *Mol. Cell. Biol.*, **15**, 6785–6793.
- Sigalas, I., Calvert, A.H., Anderson, J.J., Neal, D.E. and Lunec, J. (1996) Alternatively spliced *mdm2* transcripts with loss of p53 binding domain sequences: transforming ability and frequent detection in human cancer. *Nature Med.*, **2**, 912–917.
- Subler, M.A., Martin, D.W. and Deb, S. (1992) Inhibition of viral and cellular promoters by human wild-type p53. *J. Virol.*, **66**, 4757–4762.
- Sugden, B., Marsh, K. and Yates, J. (1985) A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein Barr virus. *Mol. Cell. Biol.*, **5**, 410–413.
- Wu, X., Bayle, J.H., Olson, D. and Levine, A.J. (1993) The p53-MDM2 autoregulatory feedback loop. *Genes Dev.*, **7**, 1126–1132.
- Xiao, Z.H., Chen, J., Levine, A.J., Modjtchedi, N., Xing, J., Sellers, W.R. and Livingston, D.M. (1995) Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature*, **375**, 694–698.
- Zhu, L., Heuvel, S.V.D., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N. and Harlow, E. (1993) Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes Dev.*, **7**, 1111–1125.

Received July 14, 1997; revised February 9, 1998;  
accepted March 3, 1998