The Growth Arrest Function of the Human Oncoprotein Mouse Double Minute-2 Is Disabled by Downstream Mutation in Cancer Cells

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Abstract

We have reported earlier that ectopic expression of mouse double minute-2 (MDM2) induces G1 arrest in normal cells. To explain occasional overexpression of MDM2 in cancer cells, we searched for deletion or substitution mutation in the growth suppressor domains of MDM2 in several breast cancer cell lines that overexpress the oncoprotein. Our results suggest the absence of alteration (deletion or substitution) in the open reading frame of MDM2 transcripts in such cells. Because the breast cancer cell line MCF-7 overexpresses MDM2, we isolated the full-length MDM2 transcript from this cell line. The MDM2 cDNA synthesized from transcripts isolated from MCF-7 cells inhibited G1 to S phase transition in normal human diploid cells such as WI38, suggesting that the genetic alterations in breast cancer cells that overexpress MDM2 disable the growth arrest function of the oncoprotein. Consistently, overexpression of full-length MDM2 in MCF-7 cells over its high endogenous level did not inhibit G1-S transition efficiently. Although MDM2 overexpression was accompanied by CDK4 overexpression or absence of cdk4 inhibitor p16 in most breast cancer cells, we found remarkably high levels of cyclin A rather than cyclin E in these cells. Ectopic expression of cyclin A released MDM2-mediated inhibition of G1-S transition in normal human diploid WI38 cells. We propose that cancer cells expressing high levels of cyclin A escape MDM2-mediated G1 arrest, which may account for a selective growth advantage over normal cells. (Cancer Res 2005; 65(5): 1839-48)

Introduction

The human homologue of the mouse double minute-2 (mdm2) gene is frequently overexpressed in many human breast tumors and carcinomas, soft tissue sarcomas, and other cancers, suggesting that MDM2 overexpression may be one of the common causes of oncogenesis (reviewed in refs. 1–4). Frequent overexpression of MDM2 in advanced breast tumors suggests that the oncoprotein may be used as an indicator for breast cancer prognosis (5). Frequent overexpression of MDM2 in advanced breast tumors suggests that the oncoprotein may be used as an indicator for breast cancer prognosis (5).

Amplification of the mdm2 gene enhances tumorigenic potential of murine cells (6, 7). Targeted overexpression of MDM2 in breast epithelial cells of transgenic mice did not induce direct cell proliferation. However, multiple rounds of DNA replication were found in some breast epithelial cells. Some of these transgenic mice have been reported to show tumor formation later in life (8).

Targeted overexpression of MDM2 in the basal layer of epidermis increases papilloma formation by chemical carcinogens (9). These findings suggest the requirement of added genetic damage for MDM2-induced tumorigenesis.

MDM2 recognizes the transactivation domain of p53 and inactivates p53-mediated transcriptional activation (10–14). Work from our laboratory showed that the interaction of MDM2 with p53 is needed for inhibition of p53-mediated transactivation (12, 14). MDM2 degrades p53 by targeting p53 to ubiquitination (15–18). However, mutants of MDM2 lacking the E3 ubiquitin ligase activity can efficiently bind with wild-type p53 and inhibit p53-mediated transcriptional activation (14). Also, the p53 degradation domain of MDM2 is dispensable for its oncogenic function (19). MDM2 interacts with several growth suppressors other than p53. Its interaction with the retinoblastoma susceptibility gene product Rb and the growth suppressor p14/p19 has been implicated in the oncogenic function of MDM2 (reviewed in refs. 2–4). Recent reports suggest that many of these interactions may be dispensable for MDM2-mediated oncogenesis (20, 21).

In view of all the oncogenic functions of MDM2, one would expect that overexpression of MDM2 would confer growth advantage in cultured cells. In contrast, MDM2 can only be stably expressed in cells harboring genetic defects. Overexpression of MDM2 efficiently arrests normal human diploid cells at G1 (19).

Inhibition of normal development of the mammary gland together with the absence of apoptotic cells after targeted overexpression of MDM2 in transgenic mice strongly suggests that overexpression of MDM2 has growth arrest function. Overexpression of MDM2 in the compound eyes of transgenic Drosophila showed small or rough eye phenotype with disorganization of the bristles. There was no evidence of apoptosis or cell proliferation in MDM2 overexpressing cells (22). Apart from these reports, the ring domain of MDM2 has been shown to induce growth arrest (23). Consistent with these observations, several laboratories reported that overexpression of MDM2 is a favorable prognostic marker in cancer (24–27).

More than one region of MDM2 is involved in G1 arrest (19). One of the major growth-inhibitory domains of MDM2 is nonfunctional in immortalized or transformed cells that overexpress cyclin A.1 Elimination of the growth-inhibitory domains releases MDM2-mediated growth suppression and induces tumorigenesis in murine cells. Thus, MDM2 harbors a potentially tumorigenic domain (19). MDM2 may therefore be potentially oncogenic in a cell that is defective in sensing the growth-inhibitory domains of MDM2.

At least five to seven MDM2-related polypeptides have been found in cultured mouse (28, 29) or human (30, 31) cells that...
overexpress the protein. Several splice variants of MDM2 mRNA have been detected in brain, ovarian, and bladder carcinomas along with the full-length cDNA (32–34). It is not clear whether the spliced mRNAs are translated in cancer or normal cells or what their comparative levels are.

Because MDM2 overexpresses in many human cancers, particularly in breast cancer, it is possible that a mutant of MDM2 defective in inducing growth arrest coded by a mRNA splice variant or a mutant gene overexpresses in cancer cells. Thus, we looked for mutation or deletions in the growth-inhibitory domains of MDM2 that overexpresses in cancer cells. Our data suggest that MDM2 overexpressed in cancer cells is predominantly unmutated, and is capable of inducing G1 arrest in normal diploid cells. Cancer cells that bear genetic alterations leading to deregulated expression or inactivation of cell cycle proteins that regulate G1 to S phase transition are tolerant to MDM2 overexpression.

Materials and Methods

Plasmids and MDM2 Deletion Mutants

The MDM2 cDNA was a generous gift from Bert Vogelstein (Oncology Center, The Howard Hughes Medical Institute and the Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University Medical Institutions, Baltimore, MD) (11). Construction of plasmids expressing the full-length MDM2 has previously been described in detail (12, 14, 19).

Isolation of Cytoplasmic RNA, Northern Blot Analysis, Generation of cDNA and PCR

Total RNA was isolated from exponentially growing MCF-7 cells using TRIzol reagent (Life Technologies, Invitrogen, Carlsbad, CA) following a protocol supplied by the manufacturer. Equal amounts (25 µg) of total RNA were resolved by formaldehyde agarose gel electrophoresis, blotted on nylon membranes (Schleicher and Schuell, Whatman, Clifton, NJ), and were probed with MDM2 cDNA following a protocol supplied by the manufacturer. The cDNA was labeled with [32P]-UTP by random primer labeling method using a kit from Ambion (Austin, TX). The method has been described previously (35).

Flow Cytometry

Cell Cycle Analysis. Methods for cell cycle analysis have previously been described (19,37). Cells were harvested 40 to 48 hours after transfection and fixed with 70% ethanol for overnight at 4°C. The fixed cells were incubated with FITC conjugated anti-MDM2 antibody (N20, Santa Cruz, Santa Cruz, CA) for 2 hours in PBS, 0.5% bovine serum albumin, and 0.5% Tween 20. Cells were then washed thrice in PBS and 0.5% bovine serum albumin. To stain DNA with propidium iodide (PI), the cells were incubated with 0.1 mg/mL RNase A, 50 µg/mL PI, 0.1% NP40 in a trisodium citrate buffer for 30 minutes. The samples were analyzed in a fluorescence-activated cell sorter (Elite, Beckman Coulter, Fullerton, CA) in our flow cytometry core facility. Relative levels of MDM2 expression per cell were determined by FITC-fluorescence intensity in the green channel (525 nm).

Vector-transfected cells or cells transfected with a plasmid expressing an irrelevant protein did not induce growth arrest (19). Mock-transfected cells were used to determine the background fluorescence. The boundary of the mock-transfected cells was drawn to achieve 0.1% background contamination. Cells showing higher FITC-fluorescence intensity than mock-transfected cells were gated. The intensity of PI staining was recorded in the red channel.

Analysis of Bromodeoxyuridine Incorporation. Bromodeoxyuridine (BrdUrd) incorporation studies were done as reported earlier (37). Cells transfected with MDM2 expression plasmids or mock-transfected cells were incubated with BrdUrd (10 µmol/L) for 30 to 40 minutes. Cells were then harvested by trypsinization, washed, and fixed as described. Fixed cells were rehydrated and treated consecutively with HCl (2 N) and sodium borate (0.1 mol/L) to partially denature DNA because the antibody could recognize BrdUrd in ssDNA. A phycoerythrin (PE)-coupled MDM2 antibody and an FITC-coupled anti-BrdUrd antibody were used to detect MDM2 and BrdUrd, respectively.

Comparison of Cyclin Induction. To estimate and compare levels of cyclin A, cells were incubated with FITC-coupled anti cyclin A antibody or matched isotype after incubation with PE-coupled anti-MDM2 antibody. Intensities of the fluorescent dyes were plotted against cell number and compared as shown in the figures.

Immunoprecipitation and Western Blot Analysis

Cells were washed with ice-cold PBS and harvested in a lysis buffer containing 50 mmol NaCl, 4 mmol sodium PPI, 200 mmol EDTA, 10 mmol NaF, 1 mmol phenylmethylsulfonyl fluoride, 2 µg/mL aprotinin, and 1% Triton X as described previously (14). Briefly, MDM2 was immunoprecipitated using a polyclonal antibody (12) overnight at 4°C in the presence of protein A agarose. The immunoprecipitate was then pelleted by microcentrifugation. The pellet was washed thrice with immunoprecipitation buffer [10 mmol Tris (pH 7.4), 150 mmol NaCl, 0.2% sodium orthovanadate, 1 mmol EDTA, 1 mmol EGTA, 0.2 mmol phenylmethylsulfonyl fluoride, 1% Triton X, and 0.5% Nonidet P-40]. The entire immunoprecipitate was boiled for 10 minutes in 20 µL of 2× lamella loading buffer dye [50 mmol Tris (pH 6.7), 2% SDS, 2% β-mercaptoethanol, and bromophenol blue] and analyzed SDS-PAGE, followed by Western blot analysis using an antibody against MDM2 (Ab-1, Calbiochem, San Diego, CA).

Western blot analysis was carried out after SDS-PAGE essentially as previously described (14, 19). An enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ) was used for detection of the protein following the protocol prescribed by the manufacturer.

Antibodies

Antibody against p16 was purchased from PharMingen (San Diego, CA). CDK4 (Ab-2) from Oncogene, Rb and cyclin A antibody was purchased from Santa Cruz, and β-actin antibody was purchased from Sigma (St. Louis, MO). Cyclin E antibody was raised against glutathione S-transferase–fused cyclin E in rabbit (12).

Results

To estimate the comparative levels of MDM2 protein expressed in several breast cancer cell lines, actively growing cells were lysed and cell lysates containing equal amounts of $^{35}$S-labeled protein were immunoprecipitated with a polyclonal anti-MDM2 antibody (12). Cell lysates of WI38 and OsA-CL containing equal amount of labeled proteins were also subjected to immunoprecipitation to estimate levels of MDM2 in normal human diploid cells and a non–breast cancer cell line known to overexpress MDM2 (36), respectively. The immunoprecipitates were resolved by SDS-PAGE and subjected to autoradiography. The presence of MDM2 was also visualized by Western blot analysis using a monoclonal antibody (Fig. 1A). Level of MDM2 in extracts prepared from normal HMEC (passage 7) cells was compared with extracts prepared from WI38 cells and T47D breast cancer cells by Western blot analysis (Fig. 1B). After densitometric scanning, the MDM2 band intensities of the Western blot were normalized with the corresponding levels of actin. As evident from the columns (Fig. 1A, bottom) the levels of MDM2 were higher in cells (OsA-CL, MCF-7, and ZR-75-1) that are known to overexpress the protein as opposed to WI38, MDA-MB-435, and MDA-MB-231 cells that do not overexpress MDM2 (30, 38–40). Densitometric scanning indicated that OsA-CL cells expressed $\sim$25- to 28-fold, MCF-7 cells expressed 10- to 16-fold, ZR-75-1 cells expressed 11- to 15-fold, and BT-20 cells expressed 4- to 6-fold higher levels of MDM2 over WI38 cells. However, the immunoreactivity of the polyclonal and the monoclonal antibody showed minor variation in detecting levels of MDM2 in BT-20 cells. The level of MDM2 in WI38 and HMEC cells was comparable, whereas the level in T47D cells was about 3-fold higher than WI38 (Fig. 1B).

**Breast Cancer Cells That Overexpress MDM2 Primarily Overexpress the Full-Length Transcript.** Because overexpression of full-length MDM2 induces growth arrest, we investigated whether MDM2 expressed in breast cancer cells is coded by an mRNA splice variant that has deletion or mutation in the growth-inhibitory domains of MDM2. To investigate this possibility, we analyzed the sizes of MDM2 transcripts by Northern blot analysis. Breast cancer cells that overexpress MDM2 (MCF-7, ZR-75-1, T47D, BT20, and BT474) and the breast cancer cells that do not (MDA-MB-231, MDA-MB-435, Hs578T, and 21PT; 30, 38–41) were used for this experiment. We also compared mRNA from the normal human diploid cell line WI38 that does not overexpress MDM2, and an osteosarcoma cell line (OSA-CL) that does (42). Northern analysis of cytoplasmic RNA isolated from the aforementioned cell lines showed that all the cell lines express MDM2 mRNA of size $\sim$ 5.5 kb (Fig. 2) as reported earlier (42). Although transcripts of size bigger than 7.4 kb and a smaller form of $>$4.4 kb were visible, the 5.5 kb mRNA was the predominant form. All the sizes are large enough to encode full-length MDM2. This fact was confirmed in our subsequent analysis of MDM2 transcripts. As expected, the MDM2 overexpressing cell lines showed higher levels of MDM2 mRNA. Band intensities of MDM2 from different cell lines were normalized with the respective glyceraldehyde-3-phosphate dehydrogenase band intensities. Normalized band intensities relative to that of WI38 RNA are shown by the column at the bottom of the figure. However, the difference in the MDM2 protein levels was higher than the respective mRNA levels. This observation is consistent with the earlier reports that enhanced translation is partly responsible for MDM2 overexpression (43, 44). As reported earlier (30, 38, 40, 45), MDM2 overexpression did not show any dependence on p53 status.

**Breast Cancer Cells That Overexpress MDM2 Predominantly Express mRNA Coding the Unmutated Protein.** Because we did not detect any predominant forms of mRNA of smaller sizes, we investigated for the presence of small internal deletions or mutations in MDM2 mRNA from the transcripts isolated from the breast cancer cell lines. Breast cancer cell lines that overexpress MDM2 (MCF-7, ZR-75-1, and BT-20), the osteosarcoma cell line OSA-CL that overexpresses MDM2, and the normal human diploid cell line WI38 were used for this purpose. Five overlapping regions of MDM2 mRNA spanning from the 5′-nontranslated region to 3′-nontranslated region were amplified by reverse transcription and PCR. The primers and the exon junctions are shown in Fig. 3. The amplified fragments were analyzed by agarose gel electrophoresis, cloned, and sequenced. In each case, the amplified fragments did

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**Figure 1.** MDM2 often overexpresses in cancer cells. Equal amounts of proteins from the indicated cell extracts were either (A) subjected to immunoprecipitation or (B) directly analyzed by SDS-PAGE and Western blot analysis. MDM2 band intensities were normalized with the corresponding actin band intensities. **Bottom,** normalized band intensities relative to that from WI38 cell extracts; arrows, migration of MDM2 and actin.
not show any reduction in the expected size. Sequence analysis of the cloned cDNA showed the absence of mutation or sequence variation in MDM2 mRNA. These results suggest that MDM2 overexpressed in breast cancer cells is predominantly unmutated, although the presence of a nonabundant splice variant cannot be ruled out. Thus, the inability of MDM2 to arrest growth of breast cancer cells overexpressing MDM2 is perhaps due to one or more than one genetic defect downstream of MDM2.

Ectopic Expression of MDM2 Using cDNA Isolated from Breast Cancer Cell MCF-7 Can Induce G1 Arrest in Normal Human Diploid WI38 Cells. If the growth-inhibitory function of MDM2 has been disabled by a downstream mutation in breast cancer cells, expression of MDM2 from the MDM2 mRNA expressed in breast cancer cells would induce G1 arrest in cells that are responsive to MDM2-mediated G1 arrest. To test this possibility, cDNA was made by reverse transcription of mRNA from MCF-7 cells and was amplified by MDM2-specific oligonucleotide primers by PCR. The cDNA was cloned in pCMV expression vector and sequenced (14, 19). Plasmid expressing MDM2 from MDM2 cDNA generated from MCF7 cells was transfected into WI38 cells. Forty-eight hours after transfection, the cells were fixed and immunostained with a FITC-coupled anti-MDM2 antibody (N20, Santa Cruz) to detect and distinguish MDM2-expressing cells from untransfected cells. The mock-transfected cells were immunostained similarly to estimate background. The method has been previously reported (37).

WI38 cells successfully transfected with the MDM2 expression plasmid expressed higher levels of MDM2 than the untransfected cells and thus were more intensely labeled with PE (Fig. 4B, transfected). As estimated by the PE intensity, the transfected cells showed ~3.4-fold increase in PE intensity over the untransfected cells. The transfected and untransfected were gated. The percentage of transfected (MDM2 overexpressing) and untransfected cells incorporating BrdUrd was then determined using flow cytometry. The mock-transfected cells were immunostained similarly to estimate background. The method has been previously reported (37).

In a separate experiment, 48 hours after transfection with the MDM2 expression plasmid, cells were incubated with BrdUrd, washed, harvested, and fixed. Cells that were successfully transfected with MDM2 expression plasmid were identified with a PE-coupled anti-MDM2 antibody. BrdUrd incorporation was detected with a FITC-coupled anti-BrdUrd antibody. The percentage of transfected (MDM2-overexpressing) and untransfected cells incorporating BrdUrd was then determined using the PE channel. BrdUrd incorporation was drastically inhibited in full-length MDM2 expressing cells. Less than 0.5% (background level) of the full-length MDM2-expressing WI38 cells showed BrdUrd incorporation, whereas >14% of the untransfected cells actively incorporated BrdUrd (Fig. 4C). These results suggest that at least 14% of the untransfected WI38 cells crossed the G1 boundary to enter the S phase, whereas MDM2-expressing cells gated from the same sample did not cross the boundary. Thus, MCF-7-MDM2 is capable of inducing G1 arrest in WI38 cells. These results strongly suggest that downstream genetic damage(s) present in MDM2 overexpressing breast cancer cell line MCF-7 disable the cell cycle inhibitory function of MDM2.

Genetic Alterations in Cancer Cells May Inactivate MDM2-Mediated G1 Arrest. To explain how the G1 arrest function of MDM2 is disabled in cancer cells that overexpress the protein, we have investigated possible alterations in the cell cycle regulatory proteins that function during the G1 to S phase transition. It has
been reported earlier that the CDK4 gene often coamplifies with MDM2 gene in cancer cells (36, 46). The levels of CDK4 and the CDK4 inhibitor p16 was analyzed by Western blot analysis of extracts prepared from cancer cells that do or do not overexpress MDM2 and were compared with the respective levels present in normal diploid WI38 and HMEC cell extracts. As shown in Fig. 5, cells expressing higher levels of MDM2 had a distinctly higher level of CDK4. Also, most of the breast cancer cells did not express the cyclin-dependent kinase inhibitor p16, suggesting that deregulated cyclin-dependent kinase activity of CDK4 in MDM2 overexpressing cells may contribute to inactivation of MDM2-mediated G1 arrest. To test this possibility, we compared the levels of phosphorylated and underphosphorylated forms of Rb by using phosphorylation-specific antibody. Consistent with the

Figure 3. Five sets of primers were designed to amplify five overlapping regions of MDM2 mRNA spanning the 5′-to 3′-untranslated regions. Vertical arrows, intron-exon junctions; the primer sets are indicated as p1, p2, etc. Long horizontal arrows, directions of chain elongation; u, upstream; d, downstream.

Figure 4. The MDM2 coded by the mRNA expressed in MCF-7 cells induces G1 arrest, and inhibits BrdUrd incorporation in WI38 cells. FITC- or PE-stained MDM2-expressing cells are indicated as transfected and FITC- or PE-unlabeled cells from the same sample as untransfected. A, comparison of the DNA histograms of transfected (FITC-labeled) and untransfected WI38 cells. The peaks for the cells in G1, S, or G2-M phases are shown. B, MDM2 expression in gated transfected and untransfected WI38 cells separated after transfection with a MDM2 expression plasmid and staining with a PE-coupled anti-MDM2 antibody. Comparative PE intensities of transfected or untransfected cells are shown by a single parameter histogram. The ratio of PE intensity of the transfected and untransfected cells at the respective peak position is 3.4. C, BrdUrd incorporation in transfected and untransfected cells.

A.  

B.  

C.  

Percent of BrdU-positive Cells

Untransfected  0  10  20  30  40  50  60  70  80  90  100  110  120  130  140  150  160  170  180  190  200  210  220  230  240  250  260  270  280  290  300  310  320  330  340  350  360  370  380  390  400  410  420  430  440  450  460  470  480  490  500

p1  p2  p3  p4  p5

G1 Arrest Function of MDM2 is Disabled in Cancer Cells

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presence of higher levels of CDK4 and the absence of p16, all the cancer cells showed significantly higher levels of phosphor-
ylated Rb than in WI38 cells. Consistent with the earlier reports, we could not detect p16 expression in commercially available passage 7 HMEC cells (47). However, the levels of CDK4 expression in these cells were comparatively less than other cell lines, and Rb phosphorylation was comparable to WI38 cells (Fig. 5).

Because phosphorylated Rb would lead to cyclin E and cyclin A expression, levels of cyclin E and cyclin A were also analyzed. Interestingly, all the cancer cells showed remarkably higher levels of cyclin A, whereas the variation in the cyclin E level was minimal (Fig. 6). These results suggest that deregulated expression of cyclin A functioning at the G1 to S boundary may disable G1 arrest mediated by MDM2. Our earlier observation that cancer cells are less sensitive to MDM2-mediated growth suppression strongly supports this observation (19).

MCF-7 Cells Disable MDM2-Mediated G1 Arrest. MCF-7 cells overexpress MDM2 (30, 38, 40). Our immunoprecipitation analysis of cell extracts prepared from MCF-7 and WI38 cells suggested that the endogenous level of MDM2 in MCF-7 cells is ~10-fold higher than WI38 cells (Fig. 1). Because our Western blot analysis showed higher levels of CDK4 expression, lack of p16, and higher levels of cyclin A expression in MCF-7 cells, we tested whether further increase in the levels of MDM2 in MCF-7 cells would lead to G1 arrest. A plasmid expressing MDM2 (pCMV MDM2, 14, 19) was transfected in MCF-7 cells. Forty-eight hours after transfection, cells were fixed and immunostained with a FITC-coupled anti-MDM2 antibody to detect and distinguish MDM2-expressing cells from untransfected cells as described in the previous experiment. The mock-transfected cells were immunostained similarly to estimate background. The cells were then stained with PI to determine cellular DNA content. The transfected and untransfected MCF-7 cells were gated and their PI intensities were plotted against cell count to generate histograms. Comparison of DNA histograms of transfected and untransfected MCF-7 cells shows that MDM2 overexpression did not cause remarkable accumulation of cells in the G1-phase (Fig. 7A).

To further confirm the lack of MDM2-mediated G1 arrest in MCF-7 cells, we tested whether MDM2 prevents transition of cells to the S phase. In a separate experiment, 48 hours after transfection with the MDM2 expression plasmid, cells were incubated with BrdUrd, washed, harvested, and fixed. As described above, cells that are successfully transfected with MDM2 expression plasmid were identified with a PE-coupled anti-MDM2 antibody. BrdUrd incorporation was detected with a FITC-coupled anti-BrdUrd antibody. The percentage of transfected (MDM2-overexpressing)
and untransfected cells incorporating BrdUrd was then determined using flow cytometry.

As estimated by the PE intensity, the transfected MCF-7 cells showed ~4.7-fold increase in PE intensity over the untransfected cells as shown by the shift of the curve for transfected cells (Fig. 7B, transfected). The transfected and untransfected cells were gated. The percentage of transfected (MDM2-overexpressing) and untransfected cells that incorporated BrdUrd was estimated by counting FITC-coupled anti-BrdUrd antibody-labeled cells (Fig. 7C). The results show that MDM2 expression reduced the percentage of cells actively incorporating BrdUrd from 33.4% to 13.5% (Fig. 7C). However, the reduction was not as drastic as in the case of WI38, where the MDM2-expressing cells did not incorporate BrdUrd (Fig. 4).

The results of our flow cytometric analysis (Figs. 4B and 7B) compare MDM2 expression by measuring increase in PE intensity of each transfected or untransfected cell after immunostaining with a PE-coupled anti-MDM2 antibody. Western blot analysis, on the other hand, estimates an average of MDM2 expression in the cells by immunostaining with anti-MDM2 antibody. We next compared the endogenous levels of MDM2 with the levels of MDM2 expressed by transfection of MDM2 expression plasmid in WI38 and MCF-7 cells (Fig. 8) by Western blot analysis. The data suggest that both WI38 and MCF-7 cells transfected with equal amounts (10 μg) of MDM2 expression plasmid elevated MDM2 expression to a similar extent (~10- to 12-fold) from the corresponding endogenous levels. Densitometric analysis of the MDM2 band intensities revealed that the endogenous levels of MDM2 in MCF-7 cells is ~8-fold higher than that of WI38 cells, whereas transfection of WI38 cells with 10 μg MDM2 expression plasmid showed 12-fold increase in band intensity (Fig. 8B, columns). Thus, the data shown in Figs. 7 and 8 suggest that endogenous levels of MDM2 expressed in MCF-7 cells should be enough to induce growth suppression in WI38 cells. However, further increase in the levels of MDM2 in MCF-7 cells did not show efficient replication block (Fig. 7).

Therefore, in comparison with WI38 cells, MCF-7 cells are insensitive to MDM2-induced G1 arrest. These results strongly suggest that MDM2-induced G1 arrest pathway functions

Figure 7. MCF-7 cells are insensitive to MDM2-mediated G1 arrest or inhibition of BrdUrd incorporation. FITC- or PE-stained MDM2-expressing cells are indicated as transfected and FITC- or PE-unlabeled cells from the same sample as untransfected. A, comparison of the DNA histograms of transfected (FITC-labeled) and untransfected MCF-7 cells. The peaks for the cells in G1, S, or G2-M phases are shown. B, MDM2 expression in gated transfected and untransfected MCF-7 cells separated after transfection with a MDM2 expression plasmid and staining with a PE-coupled anti-MDM2 antibody. Comparative PE intensities of transfected and untransfected cells are shown by a single parameter histogram. The ratio of PE intensity of the transfected and untransfected cells at the respective peak position is 4.7. C, BrdUrd incorporation in transfected and untransfected cells.

Figure 8. Comparison of the endogenous levels of MDM2 in MCF-7 and WI38 cells with MDM2 expression by transfection of MDM2 expression plasmid. Equal amounts (10 μg) of MDM2 expression plasmid were transfected in WI38 and MCF-7 cells. A, equal amounts of proteins from the indicated cell extracts were analyzed by SDS-PAGE and Western blot analysis. Bottom, actin levels, MDM2 band intensities were normalized according to the actin band intensities; columns, increase in normalized MDM2 band intensities relative to endogenous levels of MDM2 in WI38 (B) and MCF-7 (C) cells. Because the levels of MDM2 in MDM2-transfected MCF-7 cells are ~80-fold higher than the endogenous levels of MDM2 in WI38 cells, the fold increases are shown by two different columns, although they were analyzed in the same Western blot.
in MDM2-overexpressing MCF-7 cells. Thus, the pathway of MDM2-induced G1 arrest is defective in MCF-7 cells.

**Overexpression of Cyclin A Rescues Cells from MDM2-Mediated G1 Arrest.** Western blot analysis of the extracts prepared from breast cancer cell lines show that most breast cancer cells do not express p16. MCF-7, ZR-75-1, and OsA-CL that overexpressed MDM2 also overexpressed cyclin D–dependent kinase CDK4 (Fig. 6). CDK4 overexpression has been shown to attenuate the cyclin kinase inhibitory role of p16 (48), and p16 has been implicated in regulating cyclin A expression (49). Consistent with these findings, most of the cancer cells that we tested showed remarkably higher levels of cyclin A, whereas the variation in the cyclin E level was minimal (Fig. 6). Because cyclin A expression is required for DNA replication (50, 51), we tested whether overexpression of cyclin A in breast cancer cells prevents MDM2-mediated growth arrest.

To determine whether cyclin A overexpression can relieve WI38 cells from the MDM2-mediated G1 arrest, WI38 cells were transfected with the MDM2 expression plasmid in the presence or absence of a plasmid expressing human cyclin A. Forty-eight hours after transfection, the cells were incubated with BrdUrd, washed, harvested, and fixed. Cells that were successfully transfected with MDM2 expression plasmid were identified with a PE-coupled anti-MDM2 antibody and gated as described above. BrdUrd incorporation was detected with a FITC-coupled anti-BrdUrd antibody. The percentage of transfected (MDM2-overexpressing) and untransfected cells incorporating BrdUrd was then determined using flow cytometry.

The percentage of transfected (MDM2-overexpressing) and untransfected cells that incorporated BrdUrd was estimated by the FITC intensity of FITC-coupled anti-BrdUrd antibody and is shown in Fig. 9A. The results show that MDM2 expression alone drastically inhibited BrdUrd incorporation. Only 1.4% of the transfected cells showed BrdUrd incorporation as opposed to 16.6% of the untransfected cells. Coexpression of cyclin A with MDM2 resisted MDM2-mediated inhibition of BrdUrd incorporation and did not significantly lower the number of transfected cells that can actively incorporate BrdUrd (Fig. 9A).

Coexpression of cyclin A with MDM2 was confirmed by immunostaining a portion of the samples with a PE-coupled anti-MDM2 and a FITC-coupled anti-cyclin A antibody. The PE and FITC intensities indicating MDM2 and cyclin A levels of the transfected and untransfected cells were estimated and were plotted against cell number (Fig. 9B and C).

WI38 cells successfully transfected with the MDM2 and cyclin A expression plasmids showed ~6- to 7-fold increase in PE intensity by immunostaining with PE-coupled anti-MDM2 antibody over the untransfected cells indicating MDM2 expression (Fig. 9B). Immunostaining with FITC-coupled anti-cyclin A antibody in WI38 cells generated a curve with two peaks (Fig. 9C, untransfected curve). The first peak is close to the curve generated by the FITC-coupled isotype (data not shown), suggesting that the cells in this peak expressed low cyclin A. The second peak showed ~3- to 5-fold higher FITC-intensity over the peak of lower FITC intensity, suggesting that these cells expressed increased levels of cyclin A. Most of the gated MDM2-expressing cells showed cyclin A expression as evidenced by the FITC staining by FITC-coupled anti-cyclin A antibody, resulting in a shift of peak towards cyclin A expressing untransfected cells (Fig. 9C, transfected and untransfected curves). The data also show that the level of cyclin A expression in MDM2 and cyclin A expression plasmid–transfected cells are similar to endogenous levels of cyclin A in untransfected cells in most cases (Fig. 9C). Therefore, MDM2 fails to induce G1 arrest in cells after cyclin A expression. These results strongly suggest that cyclin A expression prevents MDM2 from establishing a G1 block.

**Discussion**

We have reported earlier that MDM2 induces cell cycle arrest in nontransformed cells (19). Thus, cancer cells that overexpress MDM2 may overexpress mutated nonfunctional protein. In this study, we present evidence to show that MDM2 transcripts overexpressed in several breast and other cancer cell lines are predominantly the unmutated full-length version. This data is consistent with several studies which show the absence of mutations in the functional domains of MDM2, including its growth suppressor domains in non–small cell lung carcinomas.
(52), lack of splice variants in frozen sections of human breast cancers (53), or lack of mutation at the codon 17 of the MDM2 gene in human primary tumors that overexpress MDM2 (54). However, our results do not exclude the possibility of the presence of a nonabundant splice variant of MDM2.

We also show that MDM2 cDNA constructed from MDM2 transcripts overexpressed in MCF-7 cells are capable of inducing growth arrest in a normal human diploid WI38 cell line. MCF-7 cells are known to overexpress MDM2 (30, 38, 40). Both our Western analysis and immunostaining and flow cytometric analyses showed that the endogenous level of MDM2 in MCF-7 cells is remarkably higher than that of WI38 cells (Figs. 1, 4, 7, and 8). In recent years, there have been concerns that MDM2-mediated inhibition of DNA replication could be a result of very high levels of MDM2 expression in transfected WI38 cells. To estimate and compare endogenous levels as well as levels of MDM2 expressed in transfected cells, we have immunostained MDM2 in intact WI38 and MCF-7 cells and estimated the amount of MDM2 in untransfected and cells by determining the fold increase in immunostaining of MDM2 over the background (staining with PE-coupled isotype of MDM2 antibody). Our analysis suggests that expression of endogenous MDM2 in MCF-7 (or WI38) cells is not uniform, and varies from one cell to another. More than 75% of MCF-7 cells express 1- to 1.8-fold higher levels of MDM2 than WI38 cells, whereas ~21% of MCF-7 cells express at 4- to 5-fold or higher levels than WI38 cells (data not shown). This data is also in agreement with the data presented in Figs. 4B and 7B which suggests that endogenous levels of MDM2 (measured by PE intensity) in WI38 or MCF-7 cells are not uniform, and may vary at least 10-fold from one cell to another. This amorphous expression is also true for transfected cells. Immunostaining and flow cytometric detection of transfected cells show that expression of MDM2 in the majority of transfected WI38 or MCF-7 cells do not exceed 3- to 5-fold over their respective endogenous levels (Figs. 4B and 7B, compare mean distribution of cells in transfected and untransfected plots).

A 3- to 4-fold increase in MDM2 expression induced efficient replication block in WI38 cells. However, MCF-7 cells that naturally overexpress 7- to 15-fold higher levels of MDM2 than WI38 cells are obviously not growth-arrested (Fig. 7). Furthermore, these cells showed remarkably poor replication block even when we further elevated the endogenous level of MDM2 by transfecting MDM2 expression plasmid (Fig. 7). These observations suggest that the cancer cells that overexpress MDM2, such as MCF-7, are defective in sensing MDM2-mediated G1 arrest. This observation is consistent with our earlier finding that most cancer cells are relatively insensitive to MDM2-mediated growth suppression (19). Therefore, if MDM2 overexpresses in such cells either due to gene amplification or deregulation of translation, the genetically altered cells would be incapable of inducing G1 arrest and thus of preventing the growth of MDM2 overexpressing cells. This observation led us to investigate the status of G1-regulatory proteins in the breast cancer cell lines.

Coamplification of CDK4 and MDM2 genes has often been reported, particularly in osteosarcoma cells (36, 55). Consistent with the earlier reports, our data showed higher levels of CDK4 in MCF-7, ZR75-1, and Osa-CL cells, which overexpress MDM2. However, most of the breast cancer cell lines revealed the absence of the CDK4 inhibitor p16. Mutation in the p16 gene is a frequent event found in various cancers. Western blot analysis revealed higher levels of cyclin A in breast and other cancer cells. Interestingly, the variation in the levels of cyclin A in cancer cells compared with the WI38 or HMEC cells was remarkably higher than the respective cyclin E levels. The elevated level of cyclin A could be due to lack of p16 expression which ensures timely induction of cyclin A (49).

Consistent with this hypothesis and with earlier reports (56, 57), we found overexpression of cyclin A in all the breast cancer cells (Fig. 6). Furthermore, ectopic expression of cyclin A released MDM2-mediated replication block (Fig. 9). Because MDM2 induces G1 arrest in nontransformed cells, and tumor-derived cells are mostly insensitive to MDM2-mediated G1 arrest, high levels of cyclin A present in cancer cells may inactivate growth arrest function of MDM2 by cyclin A–specific phosphorylation (58). It is also possible that MDM2 may induce G1 arrest by controlling a biochemical step leading to cyclin A expression and the presence of high levels of cyclin A in cancer cells abrogates MDM2-mediated G1 arrest.

It is known that oncogenic challenges induce MDM2. In view of the G1 arrest function of MDM2, the protein may be induced in response to growth proliferation consequent to oncogenic challenges. Thus, the oncoprotein may confer a selective growth advantage to the cells that are resistant to MDM2-mediated G1 arrest by down-regulating growth of normal cells.

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The Growth Arrest Function of the Human Oncoprotein Mouse Double Minute-2 Is Disabled by Downstream Mutation in Cancer Cells

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