The MDM2 oncogene has both p53-dependent and p53-independent activities. We have previously reported that antisense MDM2 inhibitors have significant anti-tumor activity in multiple human cancer models with various p53 statuses (Zhang, Z., Li, M., Wang, H., Agrawal, S., and Zhang, R. (2008) Proc. Natl. Acad. Sci. U. S. A. 105, 11636–11641). We have also provided evidence that MDM2 has a direct role in the regulation of p21, a cyclin-dependent kinase inhibitor. Here we provide evidence supporting functional interaction between MDM2 and p21 in vitro and in vivo. The inhibition of MDM2 with anti-MDM2 antisense oligonucleotide or Short Interference RNA targeting MDM2 significantly elevated p21 protein levels in PC3 cells (p53 null). In contrast, overexpression of MDM2 diminished the p21 level in the same cells by shortening the p21 half-life, an effect reversed by MDM2 antisense inhibition. MDM2 facilitates p21 degradation independent of ubiquitination and the E3 ligase function of MDM2. Instead, MDM2 promotes p21 degradation by facilitating binding of p21 with the proteasomal C8 subunit. The physical interaction between p21 and MDM2 was demonstrated both in vitro and in vivo with the binding region in amino acids 180–298 of the MDM2 protein. In summary, we provide evidence supporting a physical interaction between MDM2 and p21. We also demonstrate that, by reducing p21 protein stability via proteasome-mediated degradation, MDM2 functions as a negative regulator of p21, an effect independent of both p53 and ubiquitination.

p21\textsuperscript{WAF1/CIP1}, which belongs to the CIP/KIP1 family of cyclin-dependent kinase inhibitors, has long been characterized as an inhibitor of cell proliferation, but increasing evidence suggests that it plays a role in cell differentiation, senescence, and modulation of apoptosis (1). The regulation of p21 also may be more complicated than previously thought (3–5). Its transcription can be regulated through p53-dependent (2) and -independent pathways (3); its degradation is also processed by ubiquitin-dependent (4) and -independent (5) pathways via proteasome-mediated mechanisms.

The MDM2\textsuperscript{1} (mouse double minute 2) oncoprotein is a negative regulator of p53 (6) that functions through blocking its transcriptional activity (7) and promoting its proteasome-mediated degradation (8). However, MDM2 now has been shown to interact with other cellular proteins including p19\textsuperscript{ARF} (9), E2F1 (10), p300 (11), ribosomal L5 protein (12), and p73 (13), suggesting that it has p53-independent activities (6). The Ring finger domain in the C terminus of MDM2, containing the ubiquitin E3 ligase activity, is responsible for p53 ubiquitination and subsequent degradation (14). More recently, this E3 ligase has also been shown to facilitate the proteasome-dependent degradation of the androgen receptor (15).

The MDM2 oncoprotein is overexpressed in many human malignancies, and high MDM2 levels are associated with a poor prognosis (6). The MDM2 oncoprotein may also have a role in cancer therapy. We have recently developed specific anti-human MDM2 antisense oligonucleotides that have in vitro and in vivo activities against several human cancer models (16–20). Following MDM2 inhibition, induction of p21 was observed in human cancer cells or xenografts, regardless of p53 status (17–20), indicating a possible role of MDM2 in p21 regulation. In the present work, we sought to attain a greater understanding of the interaction between MDM2 and p21. We demonstrated that MDM2 has direct activity in the degradation of p21 protein by directly binding to it and facilitating its interaction with the C8 subunit of 20 S proteasome, independent of both p53 and ubiquitination. We also have shown that p21 has an important role in the mechanism of action for MDM2 inhibitors.

**EXPERIMENTAL PROCEDURES**

Plasmids and Reagents—The pCMV-MDM2 and pC53-SN vectors for the expression of human MDM2 and p53 were kindly provided by Dr. J. Chen (Moffitt Cancer Center). The human MDM2 cDNA insert was digested with BamHI and XhoI and subcloned into the same sites of pcDNA3 and pGEX-5X-2 (Amersham Biosciences). The pcDNA3-Flag-MDM2 was a gift of Dr. Z. Ronai (Ruttenberg Cancer Center). Additional constructs expressing deletions of MDM2 protein were generated by proofreading PCR of pCMV-MDM2 using primers containing BamHI and NotI sites followed by ligation back into the pcDNA3-Flag-MDM2 digested with the same enzymes. The pcDNA3-p21 was kindly provided by Dr. C. Maki (Harvard University), and the plasmid expressing HA-tagged p21 protein was constructed with proofreading PCR in which the downstream primer contained a sequence encoding the HA epitope. To generate the vector expressing p21 protein in bacteria, human p21 cDNA insert was digested with BamHI and EcoRI from pcDNA3-p21 and subcloned into the same sites of pGEX-5X-2. pGEX-2T-C8 and pcDNA3-HA-ubiquitin were gifts of Dr. M. J. Allday (Imperial College) and Dr. Bert O’Malley (Baylor University), respectively. The vector for the expression of His-tagged ubiquitin was kindly provided by Dr. E. Flemington (Tulane University). To construct short interference RNA (siRNA) expression plasmids under the control of the U6 promoter, selected oligonucleotides were cloned into pBabe-U6 at BamHI and XhoI sites for expression of siRNA in vivo.

Glutathione S-transferase; Ni-NTA, nickel-nitrioltriacetic acid; AS, antisense oligonucleotide; CMV, cytomegalovirus.

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**To whom correspondence and reprint requests should be addressed:** Dept. of Pharmacology and Toxicology and Comprehensive Cancer Center, University of Alabama at Birmingham, OH 13, 1670 University Blvd., Birmingham, AL 35294. Tel.: 205-934-5558; Fax: 205-975-9350; E-mail: ruiwen.zhang@ccc.uab.edu.
One and two pairs of siRNA oligonucleotides from MDM2 and p21 were synthesized and cloned into pBabe-U6. The target sequence of the oligonucleotides for MDM2 knockdown (derived from the MDM2 gene, +937 to +955) is 5'-gaaggagctgctaggt-3'. The sequences of the oligonucleotides selected for p21 gene knockdown, +400 to +418 and +460 to +478, respectively, are 5'-gacttggagcttcgccag-3' and 5'-gatgtgctgctgctgctg-3'. Puromycin, neomycin, cycloheximide, MG115, MG132, 5-fluorouracil (5-FU), and cisplatin were purchased from Sigma. Anti-human MDM2 antisense oligonucleotide and its control were described previously (20).

**Cell Lines**—PC3 cells were maintained as described previously (20). COS-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. To establish MDM2, p21, and MDM2 plus p21 stable knockdown PC3 cell lines, PC3 cells were transfected with pBabe-U6-MDM2 and/or pBabe-U6-p21. Positive cell clones were selected, maintained with puromycin (1.2 μg/ml), and confirmed by immunoblotting. For the stable MDM2 overexpressing PC3 cell line, PC3 cells were transfected with pcDNA3-MDM2 followed by the addition of a selective reagent, neomycin (450 μg/ml). The positive clones were confirmed by immunoblotting and maintained in medium containing neomycin.

**Co-immunoprecipitation**—Cells were lysed in lysis buffer containing 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40, 20 mM Tris, pH 8.0, and 5% (v/v) protease inhibitor mixture (Sigma). After pre-clearance by centrifugation at 14,000 rpm for 10 min, the extracts were incubated with appropriate antibodies as indicated in the figures at 4 °C overnight. The immunocomplex was captured by protein G-Sepharose beads (Amersham Biosciences), which were washed five times with lysis buffer. The bound proteins were eluted by 5× SDS sample buffer at 100 °C for 5 min, resolved on SDS-PAGE, and detected by the appropriate antibodies indicated in the figures.

**In Vitro Translation**—MDM2 and p21 protein were synthesized in vitro from corresponding vectors in reticulocyte lysates using the TnT T7 polymerase quick-coupled transcription/translation system (Promega).

**Recombination Protein Preparation and Glutathione S-Transferase (GST) Pull Down Assay**—The plasmids pGEX-2T-C8, pGEX-5X-2, and pGEX-5X-2-p21, or empty vector were transformed into Escherichia coli BL-21 (DE3) (Promega) and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma). Bacterial pellets were lysed in lysis buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 5% (v/v) protease inhibitor mixture (Sigma) and then were sonicated for 2 min. The fusion proteins and GST protein were purified with glutathione-Sepharose beads, eluted with elution buffer (50 mM Tris, pH 8.0, and 5% (v/v) protease inhibitor mixture) and quantified with a BCA protein assay kit (Pierce).

To determine whether there is a direct physical binding between MDM2 and p21 protein in vitro, GST-MDM2 or GST-p21 protein (1 μg) was incubated with in vitro transcribed and translated p21 or empty vector protein in the binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 5% (v/v) protease inhibitors) at 4 °C for 2 h. The bound proteins were captured by glutathione-Sepharose beads, which were washed five times with phosphate-buffered saline containing 0.1% Nonidet P-40, eluted with 5× SDS sample buffer at 100 °C for 10 min, resolved on SDS-PAGE, and detected with p21 antibody (Oncogene) or MDM2 antibody (Oncogene). The same procedures were performed to detect p21 protein binding to GST-C8 protein. The cell lysates, with the same amounts of proteins, were incubated with identical amounts of GST-C8 or GST protein at 4 °C overnight. The bound protein also was detected by p21 antibody.

**Ubiquitination Assay**—PC3 cells were transfected with various vectors (indicated in the figures). After 48 h, cell lysates were immunoprecipitated with p21 or p53 antibody (Oncogene), and the bound proteins were purified with protein G-Sepharose beads, resolved on SDS-PAGE, and detected by ubiquitin antibody (Santa Cruz).

**Ni-NTA-Agarose Purification**—Cells were lysed in buffer A (50 mM guanidinium chloride, 0.1 M NaHPO₄, Na₂HPO₄, pH 8.0, 100 mM imidazole, 0.5% (v/v) protease inhibitors) and incubated with 50 μl of Ni-NTA beads (Qiagen) for 3 h at room temperature; this was followed by the intense washing of beads with buffer A and 25 mM Tris-HCl, pH 6.8, 20 mM imidazole. The bound proteins were eluted by 5× SDS sample buffer supplemented with 200 mM imidazole at 100 °C for 5 min, resolved on SDS-PAGE, and detected by the appropriate antibodies.

**Aminoacylation and Transaminase Cell Proliferation Assay**—The methods described previously (20) were utilized.

**Clonogenic Assay**—Cells were treated with cisplatin or 5-FU at various concentrations for 36 h. After being rinsed with fresh medium, cell colonies were allowed to grow for 10–14 days to form colonies.

**RESULTS**

**MDM2 Protein Reduces p21 Protein Stability Independent of p53 and Ubiquitination**—The effect of MDM2 on p21 protein was first investigated in PC3 (p53 null) cells by using anti-MDM2 antisense oligonucleotide (AS) or siRNA to knock down MDM2 expression. Following MDM2 inhibition, p21 was acti-
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Fig. 2. MDM2 facilitates p21 degradation independent of its E3 ligase function. A, overexpressed MDM2 induces ubiquitination of p53 protein but not p21 protein. A1, PC3 cells were transiently transfected with expression vectors for MDM2 and ubiquitin for 40 h. The cell lysates were immunoprecipitated with ubiquitin antibody or p21 antibody. A2, PC3 cells were co-transfected with MDM2 and ubiquitin with or without p53 for 40 h, and cell lysates were immunoprecipitated with p53 antibody; this was followed by immunoblotting with ubiquitin or p53 antibody. B1, PC3 cells were transfected with vectors of p21, MDM2, or His-ubiquitin (Ub) or in various combinations of the three vectors for 40 h. This was followed by incubation with MG132 (15 μM) for an additional 8 h. Whole cell lysates were incubated with Ni-NTA beads. Bound proteins were eluted, resolved on SDS-PAGE, and detected with p21 or MDM2 antibody. B2, PC3 cells were transfected with MDM2 and His-ubiquitin (Ub) with or without p53 for 48 h; whole cell lysates were incubated with Ni-NTA beads. Bound proteins were eluted and resolved on SDS-PAGE, and ubiquitinated MDM2 or p53 was detected by MDM2 or p53 antibody. C, MDM2 protein facilitates p21 protein binding to the proteasome C8 subunit in vitro. C1, PC3 cells were co-transfected with MDM2 and M2 or p53 for 48 h; the cell lysates were incubated overnight with equal amounts (4 μg) of bacterially expressed GST-C8 fusion protein. The bound p21 protein was captured by glutathione-Sepharose beads, resolved on SDS-PAGE, and detected with p21 antibody (upper panel). The p21 protein expression levels in the whole cell lysates (Input) were also detected by immunoblotting (lower panel). C2, cell lysates of PC3 cells transfected with MDM2 and p21 expression vectors for 48 h were incubated overnight with equal amounts (4 μg) of GST-C8 fusion protein or GST protein. The bound p21 protein was resolved on SDS-PAGE (15%) and detected by MDM2 or p53 antibody.

D, the capacity of MDM2 protein to decrease p21 protein stability is independent of its E3 ligase function. D1, the schematic shows the comparison of the schematic structures of full-length MDM2 protein and its ring finger domain-deleted protein (MDM2 Deletion A). D2, endogenous p21 protein level in PC3 cells overexpressed with MDM2 or MDM2 Deletion A (40 h) followed by the treatment with proteasome inhibitors MG115 (10 μM, 8 h) was detected by immunoblotting. D3, PC3 cells overexpressed with MDM2 or MDM2 Deletion A with p53 for 40 h. MDM2, p53, and β-actin proteins were detected by immunoblotting.
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Fig. 2, B1, followed by the treatment with proteasome inhibitor MG132. Ubiquitinated p21 was purified by Ni-NTA beads and probed by p21 antibody. Consistent with the results above, MDM2 did not facilitate p21 ubiquitination (Fig. 2, B1). However, ectopically expressed p53 was observed to be ubiquitinated in PC3 cells with co-transfected MDM2 (Fig. 2, B2).

Because p21 protein can bind directly to the C8 subunit of the 20 S proteolytic core of the 26 S proteasome and is degraded by purified 20 S proteasome in vitro (21), we hypothesized that MDM2 has a direct effect on this process. When cell lysates of MDM2 and/or p21 overexpressed PC3 cells were incubated with GST-C8 in vitro, p21 bound to C8 only in the lysates of cells co-transfected with MDM2 and p21 (Fig. 2C), supporting the notion that MDM2 facilitates p21-C8 interaction. To establish that the effect of MDM2 on p21 protein stability is independent of its E3 ligase function, an MDM2 deletion protein lacking the Ring finger domain (where E3 ligase activity resides) was constructed (Fig. 2, D1). This deletion protein (Deletion A) retained the capacity to reverse endogenous p21 stabilization by MG132 (Fig. 2, D2) but failed to decrease the co-transfected p53 protein level (Fig. 2, D3).

There Is Direct Physical Binding between MDM2 and p21 Proteins—In the immunoprecipitation experiments with endogenous proteins, whole cell lysates of PC3 cells were precipitated by p21 antibody (Fig. 3, A1) or MDM2 antibody (Fig. 3, A2) in parallel with immunoprecipitates with the non-immune IgG was performed with MDM2/p21 antibody (A1) or p21/MDM2 antibody (A2). B, COS-1 cells were overexpressed with MDM2-Flag protein with or without p21-HA protein for 24 h. Immunoblotting for whole cell extracts or immunoprecipitates with the Flag antibody (B1) or HA antibody (B2) was performed with HA/MDM2 antibody (B1) or Flag/p21 antibody (B2). C, the Flag-tagged MDM2 protein and its truncated forms bind to the p21 protein in COS-1 cells. C1, this representation shows the schematic structures of MDM2 and its deletion proteins. C2, COS-1 cells were transfected with p21-HA alone or with Flag-tagged MDM2 full-length protein or its deletions for 24 h followed by immunoprecipitation with Flag antibody. The bound protein was resolved on 12.5% SDS-PAGE and detected by immunoblotting with HA antibody. D, MDM2 deletion mutant (Deletion D), lacking the binding site for p21 (amino acids 180 to 298), is not capable of inducing p21 degradation. PC3 cells were transfected with full-length MDM2 or A180–298 mutant for 40 h, which was followed by the incubation with MG132 for an additional 8 h. Target proteins were detected by immunoblotting. E, MDM2 protein binds to the p21 protein in vitro. E1, GST-MDM2 (upper panel) or GST-p21 (lower panel) expressed in bacteria, purified with glutathione-Sepharose beads, resolved on SDS-PAGE, and detected by MDM2 antibody (upper panel) or p21 antibody (lower panel).
ing, MDM2-Flag- and/or p21-HA-expressing vectors were transfected into COS-1 cells. To detect bound proteins, the whole cell lysates were immunoprecipitated with anti-Flag antibody (Fig. 3, B1) or anti-HA antibody (Fig. 3, B2). The bound proteins were detected by anti-HA antibody (Fig. 3, B1) or anti-Flag antibody (Fig. 3, B2), confirming that the interaction of the two proteins was detected only following co-transfection (Fig. 3, B1 and B2).

The binding domains on the MDM2 protein were mapped by immunoprecipitation with full-length and a series of truncated MDM2 proteins. The region between amino acids 180 and 297 was involved in MDM2 binding to the p21 protein (Fig. 3, C1 and C2). To further stress the importance of the binding between MDM2 and p21 proteins in the process of p21 degradation, the Δ180–298 MDM2 mutant was constructed. This mutant MDM2 protein was not capable of inducing p21 degradation.

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**FIG. 4.** p21 has a role in the tumor inhibitory and chemosensitization effects induced by MDM2 inhibition. A, p21 knockdown (p21 KD) PC3 cells were resistant to the decreased cell proliferation effects of MDM2 inhibition compared with parent cells. p21 knockdown and parent PC3 cells were treated with various concentrations of AS for 48 h, followed by immunoblotting (A1) or cell proliferation assay (A2). B, C, D, and E, chemosensitization effects were reduced in MDM2 and p21 double knockdown PC3 cells compared with MDM2 knockdown (MDM2 KD) PC3 cells. The p21 and MDM2 protein levels were detected in MDM2 knockdown, p21 knockdown, MDM2 and p21 double knockdown, and parent PC3 cells treated with cisplatin (B1) or 5-FU (B2) for 24 h followed by immunoblotting. Assays for cell viability (C), apoptosis (D), or clonogenicity (E) were performed in these four cell lines exposed to cisplatin or 5-FU. C, D, and E: , parental cells; , p21 knockdown cells; , MDM2 knockdown cells; and , MDM2 and p21 knockdown cells.
degradation (Fig. 3D). To exclude the possibility that the observed interaction between p21 and MDM2 proteins occurs in a complex form involving multiple protein partners in the cell, a pull down assay was accomplished by using bacterially expressed GST-MDM2 or GST-p21 protein and in vitro synthesized p21 or MDM2 protein, respectively. Direct binding between the two proteins was detected (Fig. 3E), indicating that the binding does not require participation of other proteins.
p21 Has an Important Role in MDM2 Knockdown-induced Cell Growth Inhibition and Chemosensitization—Our previous studies demonstrated that, in cell culture and in xenograft models of human cancers, MDM2 inhibition resulted in significant anti-tumor activity and chemosensitization effects (17–20). In a dose-dependent manner, p21 was elevated as a result of MDM2 knockdown. In this work, we attempted to clarify the role that p21 may play in the biological activity of MDM2 knockdown. The parental and p21 knockdown PC3 cells were treated with the anti-MDM2 antisense oligonucleotide (Fig. 4, A1). The p21 knockdown cells were less sensitive to the MDM2 antisense treatment than parental cells. At a concentration of 200 nM AS, the proliferation index was 120% higher than that of parental cells (Fig. 4, A2).

The possible role of p21 in chemosensitization effects induced by MDM2 knockdown was investigated by using p21 knockdown, MDM2 knockdown, MDM2 and p21 double knockdown, and parental PC3 cells that were treated with various concentrations of cisplatin or 5-FU. MDM2-knockdown PC3 cells, which had the highest p21 expression level (Fig. 4B), were most sensitive to the drugs as measured by cell viability (Fig. 4C), apoptosis index (Fig. 4D), and clonogenicity (Fig. 4E). These effects were reversed by p21 knockdown, indicating that p21 plays a critical role in MDM2 inhibition.

**DISCUSSION**

Our previous observations of p21 up-regulation following MDM2 knockdown by antisense oligonucleotides in tumor cells with varying p53 status (wild type, mutant, or null) indicate an important role of MDM2 in the regulation of p21 independent of p53. We now have provided evidence that MDM2 is a negative regulator of p21, directing its degradation. In the present study, we have generated at least three novel and important results. First, the MDM2 protein directly binds to p21 protein and facilitates its interaction with the C8 subunit of 20 S proteasome. Second, MDM2 then facilitates proteasome-mediated p21 protein degradation, independent of both ubiquitin and p53. Third, p21 has an important role in tumor inhibition and chemosensitization induced by MDM2 knockdown, indicating that negatively regulating p21 is one of the important p53-independent pathways contributing to MDM2 tumorigenic activity.

Because the p21 protein acts as both a negative and positive regulator of cyclin-CDK complex function, it must be under tight regulation both transcriptionally and post-translationally (5). Although the transcriptional control of p21 has been well established (22), questions remain about the mechanism of p21 protein proteasome-mediated proteolysis. Until recently, it has been accepted that the ubiquitination of p21 is required for its degradation through the proteasome (5, 23). There was some evidence supporting the involvement of ubiquitin E3 ligase SCF<sub>Skp2</sub> in p21 protein proteolysis during S phase progression (24). However, p21 is still a short-lived protein in the absence of SCF<sub>Skp2</sub> (22). In the present work, several lines of evidence indicate that MDM2 promotes p21 proteasome-mediated degradation. The direct physical binding between these two proteins, as shown in our in vivo and in vitro experiments, may be required for the process. The mutant of MDM2 protein without the binding site (Δ180–298) failed to promote p21 protein degradation. Although the E3 ligase activity of MDM2 protein plays a major role in p53 degradation, apparently it is not required to promote p21 degradation. We also have observed that proteasome-mediated p21 protein degradation was ubiquitin-independent. Based on this observation, important questions arose about possible mechanisms by which MDM2 promotes p21 degradation. Because p21 protein lacks stable secondary and tertiary structures (24) and ubiquitination for its proteasome-mediated degradation is not necessary, it is possible that the p21 protein can be recognized by the proteasome without modification (23). The p21 protein can directly bind to the C8 subunit of the 20 S proteasome and is degraded by purified 20 S proteasome in vitro (23). Although the C8 subunit lacks proteolytic activity, it is important for 20 S proteasome assembly (25). In the present study, the binding between p21 and C8 in vitro was detected in lysates of cells co-transfected with MDM2 and p21, suggesting that MDM2 promotes p21 interaction with the proteasome to facilitate its degradation.

The p53-DMM2 feedback loop is a natural pathway regulating p53 protein degradation and its functions (6) in which MDM2 acts as a p53 negative regulator. However, increasing evidence suggests that MDM2 has p53-independent activities associated with its tumorigenic properties (6). Based on our results, inactivating p21 by promoting its protein degradation may play a critical role in MDM2 tumorigenic activity.

Although it is generally accepted that p21 confers a survival advantage to cells, the role of p21 in apoptosis still is controversial. There are increasing data showing that p21 can induce apoptosis (19) and sensitize tumor cells to chemotherapy (26–29). The p21-induced apoptosis may be independent of p53 (19) and has been demonstrated in androgen-independent prostate cancer cells (30, 31). Our results are in agreement with those reports. In our previous studies (18–20), we observed that MDM2 knockdown by antisense technology resulted in concomitant p21 elevation, increased apoptosis, decreased cell proliferation, and chemosensitization in various cancer cells, regardless of p53 status (20). In the present study, we have established p21 knockdown, MDM2 knockdown, and MDM2 plus p21 double knockdown PC3 cell lines using siRNA technology. We have now found that p21 knockdown cells were resistant to the apoptosis and anti-proliferation effects induced by antisense MDM2 knockdown, indicating that p21 plays a critical role in the tumor inhibitory effects of MDM2 inhibition. Furthermore, MDM2 knockdown cells were the most sensitive to the chemotherapy agents such as 5-FU and cisplatin. Compared with the MDM2 knockdown gene background, additional knockdown of p21 reduced the sensitivity to the level of parent cells. Taken together, these data further point to the importance of p21 in the chemosensitization induced by MDM2 knockdown.

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