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Mdm2-mediated pRB downregulation is involved in carcinogenesis in a p53-independent manner

Seiichi Miwa [a,b], Chiharu Uchida [a], Kyoko Kitagawa [a], Takayuki Hattori [a], Toshiaki Oda [a], Haruhiko Sugimura [c], Hideyo Yasuda [d], Hirotoshi Nakamura [b], Kingo Chida [b], Masatoshi Kitagawa [a,*]

[a] *Department of Biochemistry 1, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan*
[b] *Second Department of Internal Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan*
[c] *Department of Pathology 1, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan*
[d] *Division of Bioscience, Central Laboratory, Nippon Flour Mills Co., Ltd., 5-1-3 Midorigaoka, Atsugi, Kanagawa 243-0041, Japan*

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Abstract

Mdm2 promotes ubiquitination of the tumor suppressor p53 and can function as an oncogene by largely downregulating p53. Although a p53-independent role of Mdm2 has been reported, the underlying mechanism remains unclear. In the present study, we indicated that Mdm2 is involved in p53-independent carcinogenesis via downregulation of pRB. Expression of pRB showed an apparent inverse correlation with Mdm2 expression in 30 patients with non-small cell lung cancer. There were some cases with the *p53* mutations in which a high level of Mdm2 and a low level of pRB were expressed. Mdm2 promoted ubiquitination of pRB in cells without wild-type p53. Furthermore, pRB-mediated G1 arrest in a p53-deficient cell line, SRB1, was significantly enhanced by a mutant Mdm2 that lacks pRB ubiquitination activity. Soft-agar colony formation activity of p53-knockout MEF was increased by wild-type Mdm2 but not mutant Mdm2. These findings suggest that overexpression of Mdm2 can perturb a RB pathway regardless of the *p53* gene status, promoting carcinogenesis.

Keywords: Mdm2; pRB; Lung cancer; Ubiquitin–proteasome; Cell-cycle

In normal cells, the tumor suppressor p53 is rapidly activated by phosphorylation in response to various types of genotoxic stress signals such as DNA damage or hypoxia [1]. Activation of p53 promotes the transcription of its target genes, resulting in cell cycle arrest or induction of apoptosis. In addition, it is known that Mdm2, a RING-finger type ubiquitin ligase for p53 protein, is overexpressed in a variety of human cancers including sarcoma [2], brain tumors [3], and lung cancer [4]. Mdm2 promotes ubiquitination of p53 and subsequently its degradation by the proteasome [5], thereby functioning as an oncogene largely by regulating the level of p53. A p53-independent role of Mdm2 has been reported in cell cycle control in cultured cells [6] as well as in carcinogenesis in animal models [7], although the mechanism remains unclear.

Recently, we reported that Mdm2 facilitated ubiquitination of retinoblastoma protein (pRB) and promoted its degradation of pRB via the ubiquitin–proteasome pathway [8]. The *RB* gene, located on chromosome 13q14.2, is a major tumor-suppressor gene that encodes the approximately 110-kDa nuclear phosphoprotein, pRB. pRB plays a role in controlling G1 phase and cell proliferation [9]. In late G1 phase, pRB is mainly regulated via phosphorylation by the cyclin D1/cyclin-dependent kinase 4/6 complex [10]. It is known that inactivation of $p16^{ink4}$ or enhanced degradation of $p27^{Kip1}$ activates G1 cyclin-Cdk kinases

* Corresponding author. Fax: +81 53 435 2322.
E-mail address: kitamasa@hama-med.ac.jp (M. Kitagawa).

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and induces inactivation of pRB in some types of human tumors. In regard to abnormalities in the *RB* gene, deletion of the *RB* gene and altered expression of pRB have been observed in several human cancers [11]. In addition to these reports, our previous study showed that enhanced expression of Mdm2 induced acceleration of pRB degradation and may be involved in abrogation of the RB tumor suppressor pathway.

However, it has not yet been determined whether enhanced degradation of pRB by Mdm2 is involved in carcinogenesis and whether Mdm2-mediated degradation of pRB is p53-independent. In the present study, we investigated Mdm2 and pRB expression as well as gene status of *p53* and *RB* in human non-small cell lung cancer. We also evaluated p53-independent roles of Mdm2 in pRB ubiquitination, cell cycle control, and carcinogenesis.

Materials and methods

Patient characteristics and tissue samples. Specimens were taken from 30 patients with non-small cell lung cancer (21 men and 9 women; median age: 62.4 ± 12.4 years (mean ± SD)) after informed consent was obtained. The tissues were formalin-fixed and paraffin-embedded. The patients, who had received no preoperative treatment, underwent surgery at Hamamatsu University School of Medicine. Tissue samples were obtained from 11 squamous cell carcinomas and 19 adenocarcinomas. Among the patients, 20 were in stage I, 1 in stage II, 7 in stage IIIA, and 2 in stage IIIB. Genomic DNA was extracted using DEXPAT (TaKaRa) from tumorous lesions or non-tumorous lesions of formalin-fixed and paraffin-embedded tissue attached to glass slides.

PCR-SSCP analysis. Primers for the *p53* gene were designed as follows: (exon 5) F 5′-TTCCTCTTCCTGCAGTACTC-3′, R 5′-GCCCC AGCTGCTCACCATCG-3′; (exon 6) F 5′-CTGATTGCTCTTAGGTC TGCG-3′, R 5′-AGACCTCAGGCGGCTCATAG-3′; (exon 7) F 5′-GT TGTCTCCTAGGTTGGCTC-3′, R 5′-AGTGTGCAGGGTGGCAAG TG-3′; and (exon 8) F 5′-GAGTAGTGGTAATCTACTGG-3′, R 5′-T CCTGCTTGCTTACCTCGCT-3′. PCR amplification was performed for 35 cycles with each primer set using 2.5 μl of genomic DNA. The denatured PCR products were subjected to electrophoresis in 8% non-denaturing polyacrylamide gels with or without 5% glycerol at room temperature or 4 °C and then detected by silver staining.

PCR-based LOH analysis. D13S153, the (CA)*n* repeat in intron 2, was used as a microsatellite marker for the RB locus. The primers were designed as follows: F 5′-CAGGGCTAT GTATAACCGAC-3′ and R 5′-AGTGAAGGTCTA AGCCCTCG-3′. PCR amplification was performed in the same manner as for the SSCP analysis. The denatured PCR products were analyzed by 6% denaturing polyacrylamide gel electrophoresis at room temperature and then detected by silver staining.

Plasmids. The pcDNA4/HisMax-wild-type Mdm2, pcDNA4/HisMax-mutant Mdm2 (C438A), pcDNA4/HisMax-full-length pRB, pCGN-HA-ubiquitin, and FLAG-p53 plasmids were described in our previous report [8]. pcDNA4/HisMax-Mdm2Δ273–321, which lacks the pRB-binding region reported by Hsieh et al. [12], was generated by PCR. pcDNA4/HisMax-Mdm2Δp53, which expresses a p53-binding-defective mutant Mdm2, was generated by removing the *Kpn*I–*Ppu*MI fragment from pcDNA4/HisMax-wild-type Mdm2.

Cell lines, cell culture, and transfection. The cell line SRB1 is a pRB-inducible clone of Saos2 cells regulated by tetracycline (Tet-off system) [13]. SRB1-wt-Mdm2 cells were established by transfection of pcDNA4/HisMax-wild-type Mdm2 into SRB1 cells and cloning using zeocin. SRB1-wt-Mdm2 and SRB1-mt-Mdm2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 0.5 mg/ml G418, 0.3 mg/ml hygromycin, 1000 ng/ml tetracycline, and 0.3 mg/ml zeocin. HEK293 cells, HCT116 cells, and the human adenocarcinoma cell lines A549, ABC-1, and VMRC-LCD were cultured in DMEM containing 10% FBS. H1299 and NIH3T3 cells were maintained in RPMI 1640 containing 10% FBS and DMEM containing 10% fetal calf serum (FCS), respectively. $p53^{-/-}$ and $p53^{-/-}$ $Mdm2^{-/-}$ MEFs were cultured in DMEM supplemented with 10% FBS, non-essential amino acids, and sodium pyruvate. H1299 cells were transfected using the Lipofectamine Reagent and Plus Reagent (Invitrogen), 293 cells and NIH3T3 cells by the calcium phosphate method, and HCT116 cells and $p53^{-/-}$ MEFs by the FuGENE 6 reagent (Roche Molecular Biochemicals).

In vitro kinase assays using immunoprecipitated CDK4 from SRB1 cells. SRB1, SRB1-wt-Mdm2, and SRB1-mt-Mdm2 cells were cultured in the presence of tetracycline. Cell lysates were prepared from these cells by sonication in IP buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 1 mM NaF, 0.1 mM Na_2VO_4, 10 mM β-glycerophosphate, and protease inhibitor mix) and centrifuged at 13,000 rpm for 15 min. The lysates were incubated with an anti-CDK4 antibody (C-22, Santa Cruz Biotechnology) at 4 °C for 45 min and then mixed with protein G–Sepharose 4 Fast Flow (Amersham Biosciences) for 1 h. After centrifugation, the immunoprecipitates were washed three times with IP buffer and rinsed once with 50 mM Hepes-buffer containing 1 mM DTT. The precipitated CDK4 were incubated with recombinant pRB (QED Bioscience) at 30 °C for 30 min in R buffer (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 4.5 mM 2-mercaptoethanol, 1 mM EGTA, and 100 μM ATP). The reaction was terminated by addition of SDS-sample buffer, and the reaction mixture was subjected to SDS–PAGE and immunoblotting with an anti-Ser807-phosphorylated pRB antibody (Cell Signaling Technology), anti-pRB antibody (G3-245, BD PharMingen) or anti-CDK4 antibody (C-22).

Colony growth in soft-agar assay. NIH3T3 cells (4×10^5/60 mm dish) or $p53^{-/-}$ cells (3×10^5/60 mm dish) were transfected with wt-Mdm2, mt-Mdm2 (C438A), Mdm2Δ273–321, Mdm2Δp53 or empty expression vectors in the presence or absence of Ha-Ras expression vectors. Cells were harvested at 24 h after transfection and 1/10 of them was cultured in 0.36% soft-agar layered on 0.72% hard agar in a 35-mm dish. After about 3 weeks, the numbers of colonies in triplicate 35-mm dishes were counted.

Statistics. Relationships and comparisons among groups were tested by Pearson's correlation coefficient and Fisher's PLSD method, respectively. Statistical analyses were performed using the StatView J 5.0 software.

Results

Mdm2-overexpressing lung cancers reduce pRB expression regardless of p53 gene status

As we previously reported [8], immunohistochemical analysis of non-small cell lung cancers showed that a high Mdm2 expression level was correlated with a low expression level of pRB and vice versa. Specifically, the Mdm2 and pRB expression levels showed an inverse relationship not only in the 30 cases examined in total ($R = -0.625$, $p < 0.0001$) but also in the pathological types (squamous cell carcinoma: $R = -0.72$, $p < 0.01$; adenocarcinoma: $R = -0.58$, $p < 0.01$, p values: Pearson's correlation coefficient, Fig. 1A). These results suggest that a high Mdm2 expression level coincides with downregulation of pRB in human non-small cell lung cancers. However, status of the genes such as *p53* and *RB* should be clarified for precise evaluation. We therefore examined the *p53* gene status in cancers with high Mdm2 expression level using single-strand conformation polymorphism (SSCP) analysis and immunohistochemistry. In many types of human cancers, including non-small cell lung

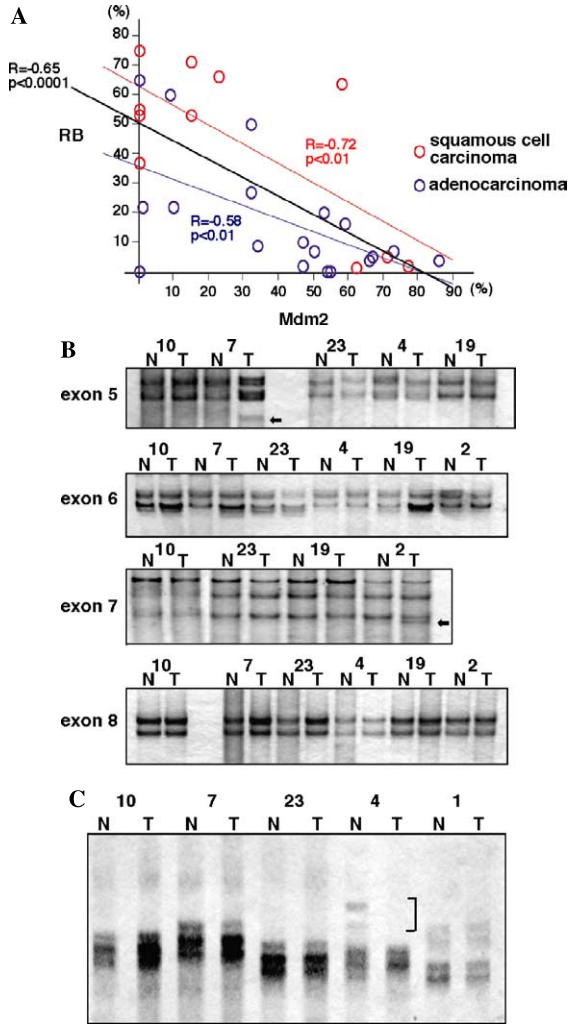


Fig. 1. Overexpression of oncoprotein Mdm2 in non-small cell lung cancers reduces pRB independent of the *p53* gene status. (A) Bivariate plots of Mdm2 versus pRB expression in 30 human non-small cell lung cancers. (Red circles) Squamous cell carcinoma; (blue circles) adenocarcinoma. The Mdm2 and pRB expression levels show an inverse relationship, as evaluated by Pearson's correlation coefficients (squamous cell carcinoma: $R = -0.72$, $p < 0.01$; adenocarcinoma: $R = -0.58$, $p < 0.01$; total: $R = -0.65$, $p < 0.0001$). (B) SSCP analysis of *p53* mutations in exons 5, 6, 7, and 8 in Mdm2-overexpressing cancers. Genomic DNA was extracted from tumorous or non-tumorous lesions of formalin-fixed and paraffin-embedded tissues and analyzed by PCR-SSCP. *p53* mutations lead to mobility shifts in the banding patterns (arrows) compared with the adjacent normal tissue (C) LOH analysis of the *RB* gene with the microsatellite marker D13S153. Loss of bands compared with the adjacent normal tissue indicates LOH (case 4). N, normal tissue; T, tumorous tissue.

cancers, point mutation within exons 5–8 of the *p53* gene has been reported [14]. Point mutation of the *p53* gene prolongs the half-life of the protein and results in accumulation of p53 protein that can be detected by immunohistochemistry. As shown in Fig. 1B, *p53* mutations in exon 5 in case 7 and in exon 7 in case 2 were found. Cancers harboring *p53* gene mutations detected by SSCP were found to exhibit a high expression level of p53 protein by immunohistochemical analysis, while cancers with the wild-type *p53* gene showed low expression level of p53 protein (data not shown). Summarized data (Table 1) showed that cancers with high Mdm2 expression level had various *p53* gene statuses, including wild-type or mutant.

It has also been reported that loss of heterozygosity (LOH) of the *RB* gene is related to absence of pRB expression in various human cancers [11]. We therefore examined whether cancers with high Mdm2 expression level had LOH in the *RB* gene using the microsatellite marker *D13S153* for the *RB* locus. As shown in Fig. 1C and Table 1, LOH was observed in only one of six cases that could be analyzed. We were only able to analyze gene status in some patients because of the low quality of DNA isolated from formalin-fixed and paraffin-embedded tissues. Taken together, the results suggest that elevated Mdm2 expression reduces pRB levels and is involved in p53-independent carcinogenesis.

Mdm2 inhibits pRB via pRB ubiquitination in a p53-independent manner

We addressed whether Mdm2 affects pRB in a p53-independent manner. The levels of Mdm2 and pRB were analyzed by Western blotting in some non-small cell lung cancer cell lines (adenocarcinoma) (Fig. 2A, left). A549 cells have wild-type *p53* gene, VMRC-LCD and ABC1 cells have mutant *p53* genes with G175A (exon 5) and C278T (exon 8), respectively, and H1299 cells have a homozygous deletion in *p53* gene (p53-null). The level of pRB was low in VMRC-LCD and ABC1 cells in which the Mdm2 level was high and, conversely, pRB was abundantly expressed in H1299 cells that have a small amount of Mdm2. Accumulation of p53 was observed in cell lines having mutant *p53* gene. Consistent with results of pathological data in 30 patients (Fig. 1 and Table 1), there was also an inverse relationship between Mdm2 protein and pRB protein

Table 1
p53 and *RB* gene status in Mdm2-overexpressing non-small cell lung cancer

Group	Case	Mdm2 protein level	RB		p53 status
			Protein level	LOH	
High Mdm2	1	High	Low	–	mt
wtRB	2	High	Low	–	mt
mtp53	7	High	Low	–	mt
High Mdm2					
wtRB	10	High	Low	–	wt
wtp53	23	High	Low	–	wt
High Mdm2					
mtRB	4	High	Low	+	mt
mtp53					
Low Mdm2	11	Low	High	ND	mt
	16	Low	High	ND	ND
	18	Low	High	ND	ND
	19	Low	High	ND	mt
	30	Low	High	ND	ND

ND, not done.

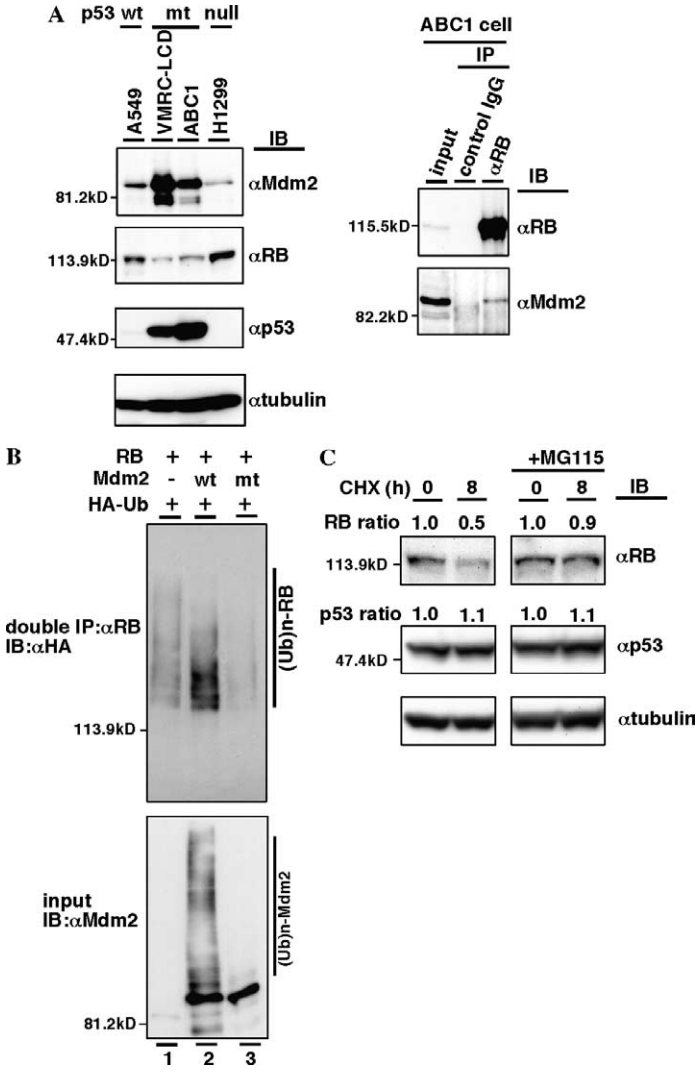


Fig. 2. Mdm2 promotes pRB ubiquitination in a p53-independent manner. (A) Western blot analysis of Mdm2 and pRB in non-small cell lung cancer cell lines (left). The human lung adenocarcinoma cell lines A549 (p53 wild-type), VMRC-LCD (p53 mutant type), ABC1 (p53 mutant type), and H1299 (p53-null) were harvested for Western blot analyses with anti-Mdm2 (SMP14), anti-pRB (G3-245), anti-p53 (DO1), and anti-α-tubulin antibodies. Endogenous pRB interacts with endogenous Mdm2 (right). ABC1 cells were immunoprecipitated with an anti-pRB antibody (G3-245) or control IgG and then immunoblotted with an anti-Mdm2 antibody (SMP14) or anti-pRB antibody. (B) Ubiquitination of pRB by Mdm2 in p53-null cells. H1299 cells (p53-null) were transiently transfected with the indicated plasmids, followed by treatment with the proteasome inhibitor MG115. Cell lysates were immunoprecipitated with an anti-pRB antibody (G3-245) and the heat-denatured immunoprecipitates were then re-immunoprecipitated with the same antibody (double-immunoprecipitation; double-IP). Ubiquitinated pRB was analyzed by Western blotting with an anti-HA antibody (12C5). Mdm2 in whole-cell lysates (input) was detected with an anti-Mdm2 antibody (SMP14). (C). Proteasome-dependent degradation of endogenous pRB. ABC1 cells were treated with 10 μg/ml of cycloheximide (CHX) for the indicated times in the presence or absence of the proteasome inhibitor MG115 (20 μM). The cell lysates were analyzed by Western blotting with the indicated antibodies. The pRB and p53 levels were normalized by the corresponding levels of tubulin and the relative ratios were determined.

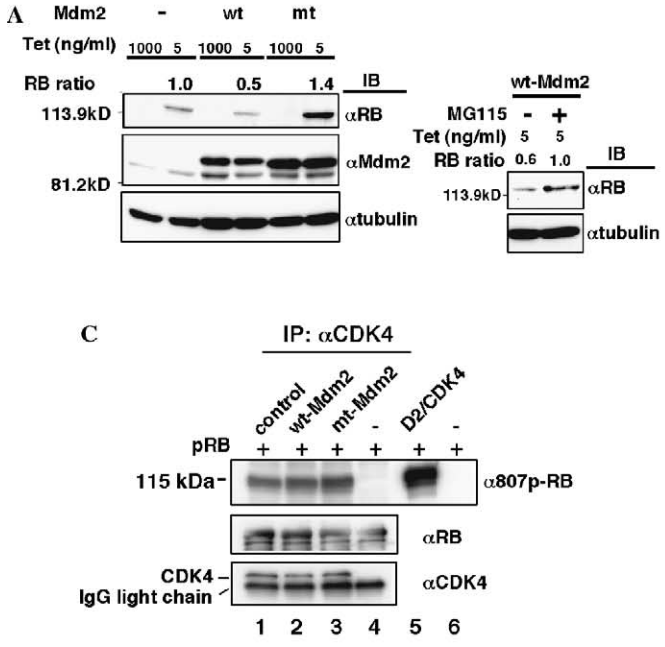
expression levels in these cell lines. Next, we investigated whether endogenous Mdm2 interacts with endogenous pRB in ABC1 cells, a mutant p53- and high-level Mdm2-expressing cell line. As shown in Fig. 2A right, Mdm2 was clearly detected in immunoprecipitates obtained with an anti-pRB antibody, but not with control IgG.

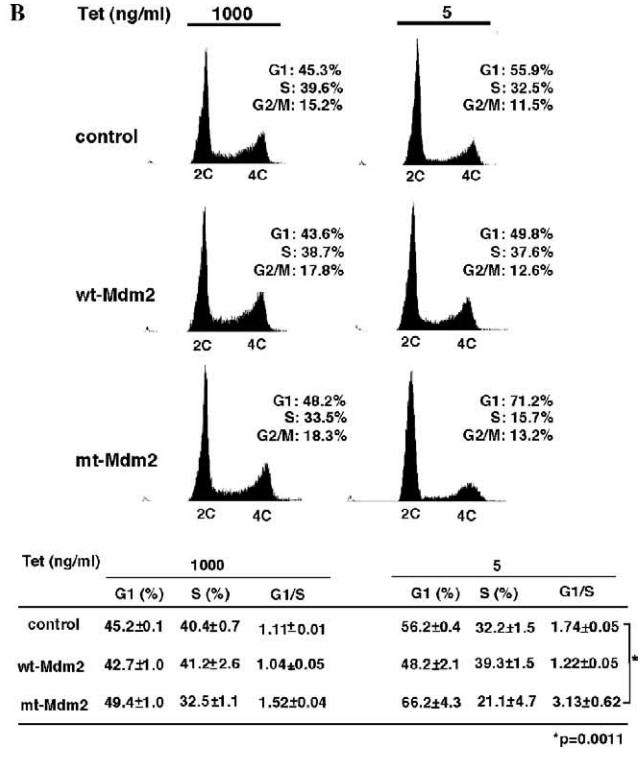
We next tested whether Mdm2 ubiquitinates pRB in a p53-independent manner. In the present study, we performed an in vivo-ubiquitination assay using the p53-null cell line H1299. As shown in Fig. 2B, wild-type (wt) Mdm2 or mutant (mt) Mdm2 (C438A RING finger domain mutant) plasmid was transfected into H1299 cells with a RB plasmid and a HA-tagged ubiquitin plasmid. Double-immunoprecipitated (IP) pRB was more strongly ubiquitinated in the presence of wt-Mdm2 (Fig. 2B, lane 2) than in the absence of wt-Mdm2 (Fig. 2B, lane 1). Furthermore, mt-Mdm2 could not facilitate ubiquitination of pRB and seemed to have a dominant-negative activity to inhibit ubiquitination of pRB (Fig. 2B, lanes 1 and 3). Mdm2 activity was monitored by its auto-ubiquitination (Fig. 2B, lower panel, lanes 2 and 3).

Finally, to examine whether pRB is degraded by enhanced Mdm2 in a proteasome-dependent manner, we performed an in vivo degradation assay by using ABC1, an Mdm2-overexpressing cell line with mutant p53 (Fig. 2A). The level of endogenous pRB clearly decreased to half of the initial levels 8 h after cycloheximide treatment, whereas degradation of mutant p53 was not observed (Fig. 2C). The degradation of pRB was completely attenuated by a proteasome inhibitor. Taken together, these findings suggest that Mdm2-overexpressing lung cancers and cell lines reduce pRB expression via the ubiquitin–proteasome system regardless of the *p53* gene status.

Mdm2 abrogates pRB-mediated cell cycle inhibition

We next examined whether Mdm2 affects the cell cycle in a p53-independent manner. We established the cell lines SRB1-wt-Mdm2 and SRB1-mt-Mdm2 by stable transfection of wild-type (wt) or mutant (mt) Mdm2 into SRB1 cells, a pRB-inducible (tet-off system) Saos-2 cell line that is p53-deficient. After the induction of pRB by exposure to a low concentration of tetracycline (5 ng/ml), the expression levels of pRB (Fig. 3A left) were measured. The level of pRB was apparently downregulated by approximately 50% in SRB1-wt-Mdm2 cells compared with that in control SRB1 cells, whereas the level of pRB increased by approximately 140% in SRB1-mt-Mdm2 cells. Furthermore, downregulation of pRB in SRB1-wt-Mdm2 cells was attenuated by a proteasome inhibitor, MG115 (Fig. 3A right). These results indicate that Mdm2 negatively regulated pRB in p53-null cells by the ubiquitin–proteasome system. To further assess the biological function of Mdm2, we investigated the cell cycle profile by flow cytometry in these SRB1 cells (Fig. 3B). Induction of pRB decreased the S population accompanied by increase in the G1 population in control SRB1 cells, while introduction of wt-Mdm2 restored the S population. On the other hand, introduction of mt-Mdm2 increased the G1 population. Data obtained from three separate experiments were statistically analyzed, and the results are shown in the lower table of Fig. 3B. The results revealed that an increase in the G1/S ratio of the cell cycle was repressed in SRB1-wt-Mdm2 cells, but significantly accelerated by the dominant-negative effect of mt-Mdm2 in SRB1-mt-Mdm2 cells compared with that





Tet (ng/ml)	1000			5		
	G1 (%)	S (%)	G1/S	G1 (%)	S (%)	G1/S
control	45.2±0.1	40.4±0.7	1.11±0.01	56.2±0.4	32.2±1.5	1.74±0.05
wt-Mdm2	42.7±1.0	41.2±2.6	1.04±0.05	48.2±2.1	39.3±1.5	1.22±0.05
mt-Mdm2	49.4±1.0	32.5±1.1	1.52±0.04	66.2±4.3	21.1±4.7	3.13±0.62

*p=0.0011

Fig. 3. Mdm2 inhibits RB-mediated G1 arrest. (A) Effects of wild-type (wt) and mutant (mt) Mdm2 on the pRB level. SRB1 cells (Saos2 cells engineered to produce pRB with reduced tetracycline) were stably transfected with wt-Mdm2 or mt-Mdm2. Cells were treated with a low dose of tetracycline (5 ng/ml) to induce pRB (left). Wt-Mdm2-expressing SRB1 cells treated with a low dose of tetracycline (5 ng/ml) were incubated with the proteasome inhibitor MG115 (right). The pRB levels were detected by Western blot analysis with an anti-pRB antibody (G3-245). The pRB levels were normalized by the corresponding levels of tubulin and the relative ratios were determined. (B) Cell cycle profiles of the stably transfected SRB1 cells used in (A) evaluated by propidium iodide (PI) staining and flow cytometry. The percentages of the G1, S, and G2/M populations were quantified using a multicycle software program. The results obtained from three separate experiments are presented in the Table. *p* value: Fisher's PLSD method. (C) Phosphorylation of pRB by CDK4 immunoprecipitated from SRB1 cells. Cell lysates were prepared from parental SRB1 (control), SRB1-wt-Mdm2 (wt-Mdm2) or SRB1-mt-Mdm2 (mt-Mdm2) cultured without pRB induction and the CDK4 assay was performed as described in Materials and methods. Phosphorylation of recombinant pRB by the immunoprecipitated CDK4 from the cells was analyzed by immunoblotting with an anti-Ser807-phosphorylated pRB (α807p-RB), anti-total pRB (αRB) or anti-CDK4 antibody (αCDK4). Phosphorylation of recombinant pRB by purified cyclin D2-CDK4 complex (D2/CDK4) expressed in insect cells was performed as a positive control.

in control cells ($p = 0.0011$). These effects on the cell cycle profile can be attributed to the amount of pRB regulated by Mdm2 shown in Fig. 3A, since p53 is not expressed in SRB1 cells. To test the possibility that Mdm2 directly affects CDK4 kinase activity, we performed in vitro kinase assay using immunoprecipitated CDK4 kinase from parental and Mdm2-expressing SRB1 cells. As shown in Fig 3C, phosphorylation of recombinant pRB by the immunoprecipitated CDK4 kinase was not changed regardless of Mdm2 expression. Moreover, not only purified GST-Mdm2 proteins but also lysates prepared from Mdm2-expressing SRB1 cells did not affect the kinase activity of purified cyclin D2-CDK4 (data not shown). These results suggest that the alteration of the cell cycle profile was due to downregulation of pRB, not because of direct modulation of CDK activity by Mdm2.

Mdm2 promotes cell transformation activity in a p53-independent manner

Next, we examined whether enhanced Mdm2 activity has cell transformation ability in a p53-independent manner. To determine whether ubiquitin ligase activity for pRB is necessary for the oncogenic activity of Mdm2, we constructed a mutant Mdm2 plasmid with a deletion in the RB-binding site (Δ273–321) or p53-binding site (Δp53) (Fig. 4A). First, we tested whether the Mdm2 mutants had binding and ubiquitination activities for p53 or pRB in vivo. As shown in Figs. 4B and C, various Mdm2 plasmids were transfected into cultured cells with a pRB or p53 plasmid in the presence (for ubiquitination) or absence (for binding) of a HA-tagged ubiquitin plasmid. Mdm2Δ273–321 lacked pRB ubiquitination activity (Fig. 4B left, lane 7) but showed p53 ubiquitination activity (Fig. 4C left, lane 5), and retained a weak-binding activity to pRB (Fig. 4B right, lane 6) despite the deletion of the reported-binding site for pRB [12]. In contrast, Mdm2Δp53 lacked both p53 binding and ubiquitination activities (Fig. 4C right, lane 5; left, lane 4) but retained pRB ubiquitination activity (Fig. 3B left, lane 6). Mt-Mdm2 was not able to ubiquitinate either pRB (Fig. 4B left, lane 5) or p53 (Fig. 4C left, lane 3). We then evaluated the effects of these Mdm2 mutants on anchorage-independent growth using a soft-agar colony formation assay (Fig. 4D). Wt-Mdm2 significantly stimulated Ras-dependent colony formation of NIH3T3 cells ($p < 0.01$ by Fisher's PLSD method), whereas Mdm2Δ273–321 did not. However, the possibility that these results reflect secondary effects through downregulation of p53 mediated by Mdm2 could not be ruled out. Therefore, to examine whether Mdm2 is also involved in cell transformation independently of p53, we assessed the colony formation ability of Mdm2 in p53-null mouse embryonic fibroblasts (MEFs). As shown in Fig. 4E, wt-Mdm2, but not mt-Mdm2 or Mdm2Δ273–321, significantly increased the colony formation of $p53^{-/-}$ MEFs in the presence ($p < 0.01$) or absence ($p < 0.05$) of Ha-Ras. Taken together, the results suggest that ubiquitin ligase activities for pRB are necessary for transformation and that Mdm2 promotes the cell transformation activity in a p53-independent manner.

Discussion

We recently proved that Mdm2 degrades pRB as well as p53 via the ubiquitin–proteasome pathway [8]. However, it has not been determined whether Mdm2 promotes ubiquitin-dependent degradation of pRB in a p53-independent manner and whether degradation of pRB by Mdm2 is involved in carcinogenesis in a p53-independent manner. In the present study, we found that wt-Mdm2 promotes ubiquitination of pRB and that mt-Mdm2 has a dominant-negative activity to inhibit ubiquitination of pRB in the p53-null lung cancer cell line H1299 (Fig. 2B). Recombinant pRB has been shown to be efficiently ubiquitinated by Mdm2 without p53 in an in vitro ubiquitination assay [8]. pRB was downregulated via the proteasome after introduction of wt-Mdm2 and upregulated after introduction of mt-Mdm2 in p53-deficient SRB1 cells (Fig. 3A). Moreover, endogenous pRB was degraded by enhanced Mdm2 in ABC1 cells (p53 mutant) in a proteasome-dependent manner (Fig. 2C). The Mdm2 domain required for pRB ubiquitination was different from that required for p53 ubiquitination, except for the RING-finger domain (Fig. 4). These results suggest that overexpression of Mdm2 facilitates ubiquitination of pRB and promoted its proteasome-dependent degradation in a p53-independent manner.

As shown in many previous studies, pRB negatively regulates cell cycle progression from G1 to S phase. pRB activity is mainly regulated by phosphorylation of pRB via G1-cyclin-CDK [9]. We found that downregulation of pRB after introduction of wt-Mdm2 abrogated the pRB-mediated cell cycle inhibition in the p53-null cell line SRB1 (Fig. 3). Moreover, introduction of mt-Mdm2 increased pRB and enhanced the RB-mediated cell cycle inhibition. These results suggest that Mdm2 negatively regulates pRB-mediated cell cycle inhibition in a p53-independent manner. These results support previous results obtained by Xiao et al. [15] showing that Mdm2 perturbs the RB-E2F pathway by promoting S-phase entry in a p53-independent manner and that Mdm2-transfected Saos2 cells (RB- and p53-negative) could not stimulate E2F reporter activity. Taken together, we speculate that Mdm2 overexpression downregulates pRB by the ubiquitin–proteasome system and that E2F are consequently activated to promote the transcription of growth-promoting genes.

A previous study of Mdm2-overexpressing transgenic mice showed a high incidence of sarcoma regardless of the *p53* status [7]. We also found p53-independent cell transformation activity of Mdm2 in NIH3T3 cells and $p53^{-/-}$ MEFs using a soft-agar colony formation assay. Mdm2Δ273–321, which ubiquitinates p53 but not pRB, did not enhance soft-agar colony formation in either

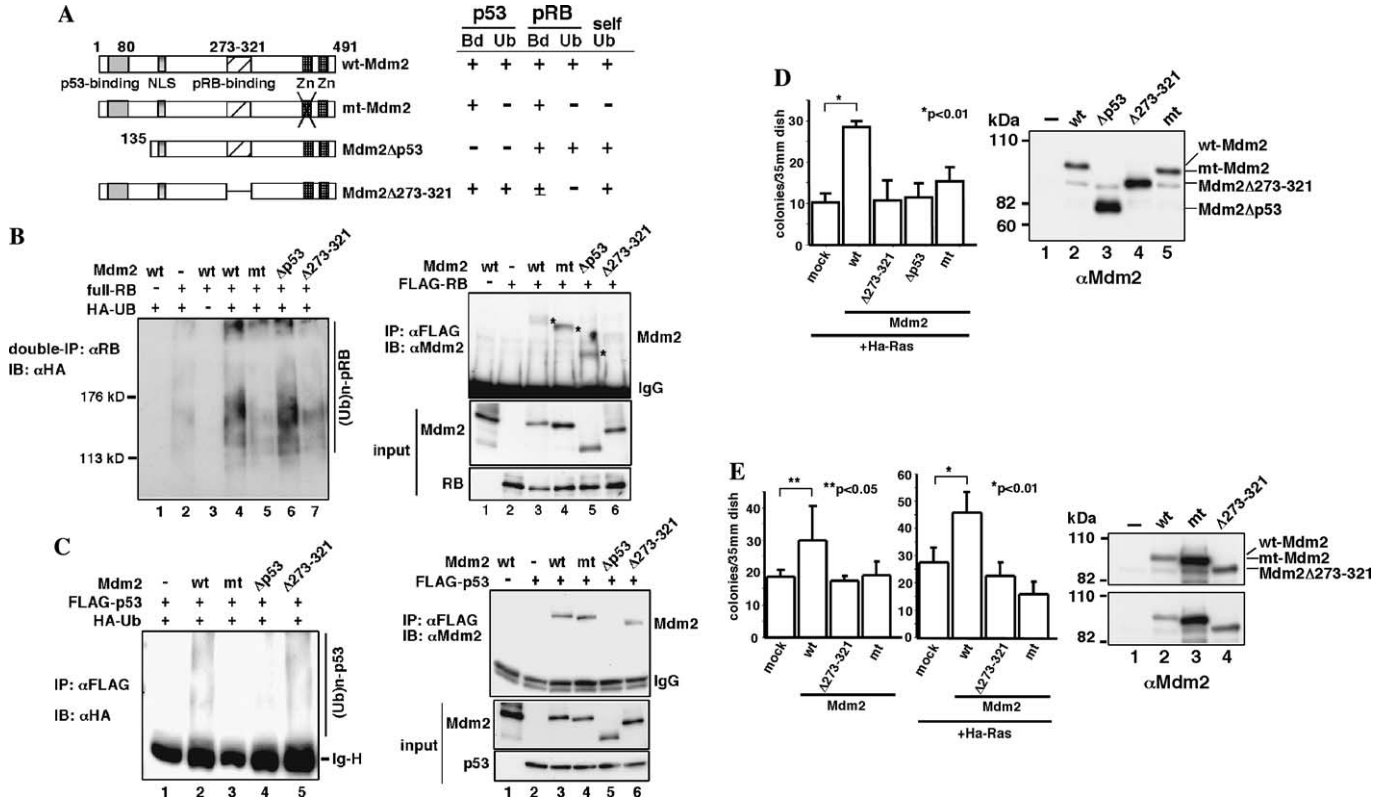


Fig. 4. p53-independent colony formation mediated by Mdm2. (A) Schematic illustrations of wild-type (wt) Mdm2 and various mutant (mt) Mdm2 constructs (left). Their binding and ubiquitination abilities for pRB and p53 tested in B and C are summarized in the Table (right). (B) Binding of Mdm2 to pRB (right) and ubiquitination of pRB (left). Various Mdm2 expression plasmids were cotransfected into 293 cells with the pRB plasmid as indicated. pRB was immunoprecipitated (IP) with an anti-FLAG (M2) antibody and the coprecipitated Mdm2 was analyzed by immunoblotting (IB) with an anti-Mdm2 antibody (SMP14). The asterisks indicate Mdm2 (right). Ubiquitination of pRB (left) by wt- and mt-Mdm2s was analyzed using an in vivo ubiquitination assay. NIH3T3 cells were transiently cotransfected with the indicated plasmids, followed by treatment with the proteasome inhibitor MG115. Ubiquitinated pRB was detected by double-immunoprecipitation (IP) with an anti-pRB antibody (G3-245) and immunoblotting with an anti-HA antibody (12CA5). (C) Binding of Mdm2 to p53 (right) and ubiquitination of p53 (left). The transfection and immunoprecipitation were performed as described in B except for the use of a p53 expression plasmid instead of the pRB plasmid. Ubiquitinated p53 was detected by IP with an anti-Flag antibody (M2) and immunoblotting with an anti-HA antibody (12CA5). (D,E) Effects of various mutants of Mdm2 on soft-agar colony formation were examined in NIH3T3 (p53 wild-type) cells (D) or $p53^{-/-}$ MEFs (E). NIH3T3 cells or $p53^{-/-}$ MEFs were transfected with the indicated Mdm2 plasmids with or without the Ha-Ras plasmid. Cells were harvested and subjected to soft-agar colony formation assays. The ability to form colonies was determined by counting the number of colonies/35 mm dish (mean ± SD in a representative experiment performed in triplicate). Mdm2 expression levels in these cells (upper panel without Ha-Ras lower panel with Ha-Ras) were detected by using anti-Mdm2 antibody (right panels). *p* value: Fisher's PLSD method.

NIH3T3 cells or $p53^{-/-}$ MEFs. These results suggest that ubiquitin–ligase activities for pRB are necessary for cell transformation by Mdm2. Taken together, overexpressed Mdm2 can promote tumorigenesis in a p53-independent manner, probably via pRB degradation.

Many studies have shown that more than 80% of abnormalities in genes involved in the RB pathway are present in human cancers and that abrogation of the RB pathway promotes carcinogenesis and cancerous growth. The tumor suppressor function of pRB is inhibited by elevated G1-cyclin/CDK activity via inactivation of $p16^{ink4a}$ and $p27^{Kip1}$ or overexpression of G1-cyclins as well as by the introduction of viral oncoproteins or LOH of the *RB* gene. Here, we have shown that downregulation of pRB caused by an elevated level of Mdm2 is involved in carcinogenesis in human lung cancers. Cancers with a high Mdm2 expression level exhibited a low pRB expression level and there was an inverse relationship between the Mdm2 and pRB expression levels in non-small cell lung cancer. Our analyses suggested that the low pRB expression level in Mdm2-overexpressing cancers was not due to LOH of the *RB* gene. Moreover, the low pRB expression level in these cancers was independent of the *p53* status as shown in Table 1. Mori et al. [16] reported that p53 alterations, $p14^{ARF}$ inactivation, and Mdm2 overexpression were detected in 63%, 14%, and 26% of non-small cell lung cancers, respectively. Gorgoulis et al. [4] reported that alterations of the $p16^{ink4a}$–pRb pathway had relationships with the p53 and Mdm2 protein expression levels in non-small-cell lung carcinomas. These findings are consistent with our results, since overexpression of Mdm2 or inactivation of $p14^{ARF}$ enhances Mdm2 activity and inhibits both the p53- and pRB-pathways, and may thereby promote carcinogenesis or malignant characters.

Since Mdm2 negatively regulates two major tumor suppressor pathways, Mdm2 is a high-potential molecular tar-

get for cancer therapy. In our previous study, knockdown of Mdm2 by siRNA negatively regulated the growth of H1299 and U2OS cells [8]. Furthermore, several studies have reported that Mdm2 inhibition by antisense oligonucleotides was effective for breast cancer [17] and prostate cancer cell lines [18]. Recently, a chemical compound that inhibits the p53/Mdm2 interaction has been identified [19]. Taken together, gene therapy by $p14^{ARF}$, knockdown of Mdm2 by siRNA/antisense, and chemical inhibitors against Mdm2 may be effective against Mdm2-overexpressing tumors to restore the impaired stability of pRB and/or p53.

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