

Overexpression of MDM2 in MCF-7 Promotes Both Growth Advantage and p53 Accumulation in Response to Estradiol

Shigehira Saji,^{1,5} Shigeru Nakashima,² Shin-ichi Hayashi,³ Masakazu Toi,⁴ Shigetoyo Saji¹ and Yoshinori Nozawa²

¹2nd Department of Surgery, ²Department of Biochemistry, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500-8705, ³Saitama Cancer Center Research Institute, Ina-machi, Kitaadachi-gun, Saitama 362-0806 and ⁴Department of Surgery, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-0021

The overexpression of the oncogene product MDM2 is often observed in human breast cancer cells, especially in estrogen receptor (ER)-positive ones. To study the role of MDM2 protein in ER-positive breast cancer, we have established cell lines derived from MCF-7 which stably express increased and decreased levels of MDM2 by transfection of a mammalian expression vector containing human *mdm2* cDNA in sense and antisense orientations, respectively. Interestingly, MDM2 overexpression in MCF-7 cells afforded a remarkable growth advantage under estradiol (E₂)-supplemented condition. Then, we analyzed the expression of p53, which is an important regulator of growth and the cell cycle. Unexpectedly, the p53 accumulation induced by E₂ was remarkably higher in MCF-7 cells stably overexpressing MDM2 than in the parent MCF-7 cells. On the other hand, reduction of MDM2 suppressed the E₂-induced increase in p53 protein. Moreover, *mdm2* antisense oligonucleotides prevented E₂-induced accumulation of p53. In the steady state, the cellular levels of p53 were also correlated with those of MDM2. These interactions are not consistent with the well-known role of MDM2, which acts as a negative regulator for p53 by inhibiting its function and promoting its rapid degradation. These results suggest that MDM2 may regulate the expression of p53 in the steady state and in response to E₂ in breast cancer cells, and imply a novel and important role of MDM2 during breast carcinogenesis.

Key words: Breast cancer — MDM2 — p53 — Estrogen receptor

The *mdm2* oncogene was originally identified as a highly amplified gene on a murine double-minute chromosome in the 3T3DM cell line, a spontaneously transformed derivative of BALB/c3T3 cells.^{1,2} The corresponding human *mdm2* gene was also identified.³ Overexpression of the *mdm2* gene in NIH3T3 cells increases its tumorigenic potential, thus establishing *mdm2* as an oncogene.² The gene encodes a polypeptide consisting of 489 amino acids that contains a binding domain for the tumor suppressor p53, an acidic region, zinc finger motifs and a ring finger domain.^{2,4-6} MDM2 is believed to bind to the N-terminal region of p53 and to inhibit its transcriptional activity by masking its transactivation domain.^{3,4} Recently, it was also shown that MDM2 promoted the rapid degradation of p53.^{7,8} In contrast, p53 induces the expression of MDM2 via binding to its promoter region, suggesting that MDM2 can function as a negative feedback regulator of p53.^{4,9}

In addition to regulating p53, MDM2 has also been shown to interact with many other molecules, such as the retinoblastoma protein pRB,¹⁰ E2F transcriptional fac-

tor,¹¹ ribosomal protein L5,¹² RNA,¹³ cell fate regulator Numb,¹⁴ and cell cycle inhibitor p19^{Arf}.^{15,16} Interaction with pRB and with E2F1/DP1 promoted G1/S cell cycle progression through disrupting RB function and stimulating transcriptional activity of E2F1.^{10,11} Binding to the ribosomal protein L5 suggests the possibility that MDM2 may enhance the translation process. The association with Numb may influence processes such as differentiation and survival following reduction in overall cellular Numb levels. Based on the above results, together with the observation showing the existence of alternatively spliced forms of MDM2 that cannot bind p53 in various types of cancer cells,^{17,18} there is a possibility that MDM2 may play an important role in cell growth and differentiation in a p53-independent fashion.

The *mdm2* gene is amplified or overexpressed in 40–60% of human osteogenic sarcomas and about 30% of soft tissue sarcomas.^{3,19} Although *mdm2* gene amplification is uncommon in breast cancers, as in other epithelial tumors,^{20,21} the level of its mRNA and/or protein is up-regulated in about 40% of breast cancer specimens.^{22–25} Interestingly, there was a significant positive correlation between the levels of MDM2 and estrogen receptor (ER) in breast cancer specimen and breast cancer cell lines.^{23–26}

⁵ To whom correspondence should be addressed.
E-mail: ss-saji@wa2.so-net.ne.jp

In contrast to ER-negative cell lines, all ER-positive cells express elevated levels of *mdm2* mRNA.²⁶⁾

In order to gain further insight into the role of MDM2 in ER-positive breast cancer cells, we have established cell clones derived from MCF-7 which stably express increased and decreased levels of MDM2. To our surprise, both steady-state expression and estradiol (E_2)-induced accumulation of p53 were positively correlated to the levels of MDM2 protein, which has previously been believed to promote the rapid degradation of p53.

MATERIALS AND METHODS

Cells and cell culture A breast cancer cell line MCF-7 was obtained from the National Cancer Institute (Bethesda, MD). MCF-7 cells were maintained routinely in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 1 nM 17 β -estradiol (E_2 , Wako Pure Chemical Industries, Osaka), 100 units/ml penicillin G and 100 μ g/ml streptomycin. For experiments evaluating the effect of E_2 , cells were cultured in phenol red-free DMEM (PRF-DMEM, Gibco BRL) containing 10% FBS stripped of steroids by absorption with dextran-coated charcoal (DCC-FBS).

Establishment of MDM2-expressing transfectant Transfection into MCF-7 cells of mammalian expression vectors containing human *mdm2* cDNA, pCdm2 and pCdm2as, which were supplied by Dr. Klaus Roemer (University of California, San Diego),²⁷⁾ was performed as described previously.²⁸⁾ Briefly, cells transfected using lipofectin (Gibco BRL) were selected in the culture medium containing 600–800 μ g/ml G418 (Gibco BRL). G418-resistant colonies were cloned after 2–3 weeks and maintained in the culture medium containing 200 μ g/ml G418. The clones showing changes of MDM2 expression as compared to the parent MCF-7 cells were screened by western blotting.

Colony formation assay in soft agar Three thousand cells were suspended in 300 μ l of 0.3% Difco's noble agar in 10% DCC-FBS/PRF-DMEM and layered over 300 μ l of 0.6% agar-medium basal layer in 24-well tissue culture plates. Cells were then fed with 10% DCC-FBS/PRF-DMEM supplemented with or without 1 nM E_2 and incubated at 37°C for 16 days. Culture medium was replaced every 3 days. On day 16, the number of colonies >30 μ m in diameter was counted under a microscope.

Western blotting Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in lysis buffer (1% Triton X-100, 50 mM NaCl, 25 mM Hepes (pH 7.4), 2 mM EDTA, 1 mM PMSF, 10 μ g/ml leupeptin). Cell lysates were scraped into microcentrifugation tubes and centrifuged for 10 min at 12,000g. The resulting supernatants were transferred to fresh tubes. Protein

contents were determined by using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Proteins were separated by 7.5%, 9% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). To assess the quality of electrophoretic transfer, prestained SDS-PAGE standards (Bio-Rad) were used. The membrane was incubated for 24 h in blocking buffer (2% skimmed milk, 0.1% Tween-20 in 20 mM Tris-buffered saline pH 7.5; TBS-T) at 4°C. After having been washed with TBS-T, the membrane was incubated with the respective antibody for 1 h. MDM2 was detected using 1 μ g/ml Ab-1 (IF2) mouse monoclonal antibody (Oncogene Research Products, Cambridge, MA), p53 was detected using 0.1 μ g/ml Ab-6 (DO-1) mouse monoclonal antibody (Oncogene Research Products), p21 was detected using 1 μ g/ml C-19 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and actin was detected using 2 μ g/ml Ab-1 mouse monoclonal antibody (Oncogene Research Products). Membranes were washed with TBS-T and incubated with horseradish peroxidase-conjugated anti-mouse IgG, anti-rabbit IgG (Amersham, Buckinghamshire, UK) or anti-mouse IgM (Oncogene Research Products), then probed by the ECL chemiluminescence technique (Amersham) according to the manufacturer's recommendations. Band density was quantified by using a densitometer (Atto Densitograph, Tokyo).

UV irradiation Cells (2×10^5) were seeded in 35-mm tissue culture dishes and incubated for more than 24 h at 37°C under 5% CO₂. The medium was removed and the cells were irradiated with a 15-W germicidal lamp delivering 0.04 mW/cm²/s of UVc at a distance of 30 cm. The irradiation was continued for 5 s and resulted in 0.2 mJ/cm² UV exposure, which was quantitated with a UVR-305 UV-radiation meter (Topcon, Tokyo). After incubation for indicated periods of time with 10% DCC-FBS/PRF-DMEM in the absence of E_2 , cell lysates were prepared for western blotting.

Antisense phosphorothioate oligonucleotide treatment The antisense oligonucleotides for *mdm2* were designed according to the report of Chen *et al.*²⁹⁾ In order to evaluate sequence-specific effects, mismatch oligonucleotide, which contained the same amount of each nucleotide with a different sequence, was used as a control. The 20-mer antisense or mismatch phosphorothioate oligonucleotide was synthesized and purified by preparative reverse-phase HPLC. Cells (2×10^5) were cultured in 10% DCC-FBS/PRF-DMEM without E_2 for over 24 h in 35-mm tissue culture dishes. Before oligonucleotide treatment, 5 μ l of lipofectamine (Gibco BRL) was incubated with 200 μ l of Opti-MEM I (Gibco BRL) and various concentrations of oligonucleotide at room temperature for 40 min. The cells were washed twice with Opti-MEM I, then oligonucle-

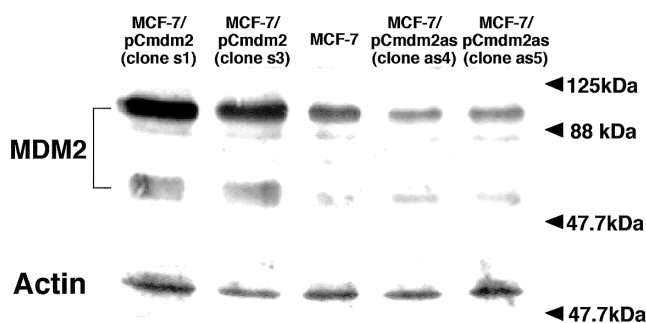


Fig. 1. MDM2 protein expression in parent MCF-7 and four types of cell clones transfected with pCMV containing human *mdm2* cDNA in sense (MCF-7/pCcmdm2) or antisense orientation (MCF-7/pCcmdm2as). After incubation for 24 h in phenol red-free DMEM containing 10% FBS stripped of steroids, cells were harvested in Laemmli's sample buffer. Proteins were separated by SDS polyacrylamide gel (9%) electrophoresis and transferred onto PVDF membrane. The membranes were probed with the anti-MDM2 antibody (Ab-1). After stripping off the anti-MDM2 antibody, actin was probed as an internal control with monoclonal antibody (Ab-1).

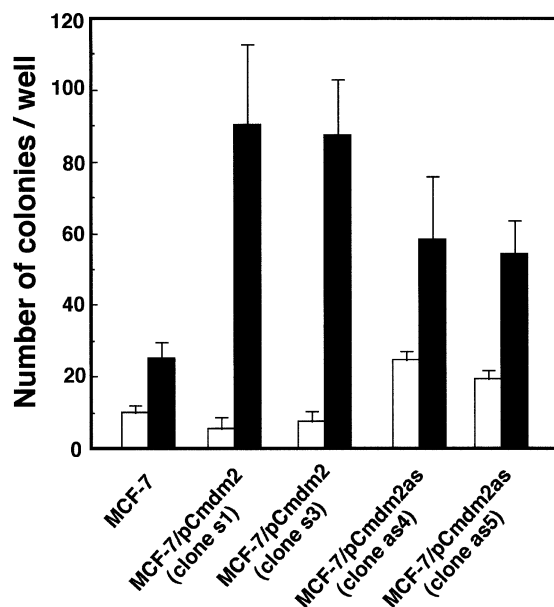


Fig. 2. Overexpression of MDM2 in MCF-7 affords a growth advantage under E₂-supplemented conditions. Three thousand cells were suspended in 0.3% Difco's noble agar containing 10% DCC-FBS/PRF-DMEM and layered over 0.6% agar medium in a 24-well tissue culture plate. Culture medium with or without 1 nM E₂ overlaid on the top layer was replaced every 3 days. On day 16, the numbers of colonies over 30 μm in diameter were counted. The results shown are means ± SD from two separate experiments, each performed in triplicate. □ E₂⁻, ■ E₂⁺.

otide-lipofectamine complexes were added to the culture dishes containing 800 μl of Opti-MEM I. After incubation of the dishes for 6 h, 1 ml of 20% DCC-FBS/PRF-DMEM was added and incubation was continued for another 18 h with or without 10 nM E₂.

Statistical analysis The statistical significance of differences in the results was evaluated by means of one-factor analysis of variance (ANOVA), and *P* values were calculated by the Bonferroni method. In the assay using AS oligo, Student's *t* test was used for evaluating the efficiency of inhibition.

RESULTS

Establishment of MCF-7 cells expressing increased or decreased level of MDM2 To study whether overexpression of MDM2 affects biological responses in breast cancer cells, we have established two types of cell clones derived from MCF-7 cells by transfecting plasmids, pCcmdm2 expressing human *mdm2* and pCcmdm2as containing *mdm2* cDNA in the antisense orientation. As shown in Fig. 1, the 90 kDa form of MDM2 protein was predominant and an additional protein with a molecular weight of about 60 kDa was also detected in all cell clones. MCF-7/pCcmdm2, clone s1 and clone s3 expressed 3.8-fold and 3.0-fold more MDM2 protein than the parent MCF-7, respectively. In contrast, MCF-7/pCcmdm2as, clone as4 and clone as5 expressed less than half as much MDM2 protein compared with MCF-7.

Overexpression of MDM2 in MCF-7 cells afforded E₂-dependent growth advantage First, we examined whether the stable expression of MDM2 in MCF-7 cells alters their proliferative ability. The soft agar colony formation assay was designed to evaluate proliferative ability under anchorage-independent conditions. In the absence of E₂, 10.0 ± 2.0, 5.3 ± 3.2, 7.3 ± 3.1, 24.6 ± 2.5 and 19.3 ± 2.5 colonies/one well of 24-well tissue culture plate were observed in MCF-7, MCF-7/pCcmdm2 (clone s1), MCF-7/pCcmdm2 (clone s3), MCF-7/pCcmdm2as (clone as4) and MCF-7/pCcmdm2as (clone as5), respectively (mean ± SD, *n* = 6) (Fig. 2). Addition of 1 nM E₂ increased the number of colonies to 25.0 ± 4.5, 90.0 ± 22.6, 87.3 ± 15.5, 58.3 ± 17.6 and 54.0 ± 9.6 in MCF-7, clone s1, clone s3, clone as4 and clone as5, respectively. Unexpectedly, the number of colonies was increased in MCF-7/pCcmdm2as clones both in the absence and presence of E₂ compared with the parent MCF-7. However, their growth acceleration in response to E₂ was almost equivalent to that of MCF-7 (2.5-fold). A significantly high growth ratio (10-fold) in response to E₂ was observed in MCF-7/pCcmdm2 clones (*P* < 0.01 compared with MCF-7 and MCF-7/pCcmdm2as clones). These data indicate that overexpression of MDM2 results in a prominent growth advantage under E₂-supplemented conditions.

E₂ increased p53 expression, especially in MCF-7 cells overexpressing MDM2 The tumor suppressor p53 is a crucial regulator of cell growth through transactivation of p53-responsive genes such as *p21/waf-1*, an inhibitor of cyclin-dependent kinase.^{30–32} Recent evidence has suggested that E₂ treatment of MCF-7 induces the accumulation of p53.^{33,34} Moreover, it was also shown that p53 may function as a negative regulator of the ER signaling pathway.³⁵ The above results suggest the possibility that overexpression of MDM2 induced the rapid degradation of p53 in MCF-7/pCdm2, resulting in progression of ER-mediated transcription and a decrease of cell cycle inhibitor. Therefore, the levels of p53 protein in these cells were analyzed. Among four established clones, the results obtained from clone s1 (MCF-7/pCdm2) and clone as4 (MCF-7/pCdm2as) are shown in the following figures, since the data obtained from clone s3 and clone as5 were almost identical to those from clone s1 and clone as4, respectively. Fig. 3 shows p53 expression at 24 h after addition of various concentrations of E₂, as assessed by western blotting of cell lysates. Prior to addition of E₂, cells were maintained for 48 h in phenol red-free DMEM containing 10% FBS stripped of steroids. It should be noted that the level of p53 was higher in MCF-7/pCdm2 than in the parent MCF-7 in the absence of E₂ (indicated as 0 nM E₂ in Fig. 3). Also in MCF-7/pCdm2as, p53 expression paralleled the level of MDM2; both were lower than those in the parent MCF-7. In MCF-7, p53 accumulation was observed at 0.1 nM E₂ and its expression increased in a concentration-dependent manner. MCF-7/pCdm2 showed a remarkable elevation of p53 protein even at 0.1 nM E₂ ($P < 0.05$, compared with MCF-7). In contrast, only a modest increase in p53 was observed in MCF-7/pCdm2as.

When the cells were treated with 10 nM E₂ for 3–48 h, an increase in the p53 protein was detectable at 6 h, and its elevation was maintained for up to 48 h (Fig. 4A). p53 protein was expressed to a greater extent in MCF-7/pCdm2 than in the parent MCF-7 ($P < 0.01$). On the other hand, MCF-7/pCdm2as expressing the reduced level of MDM2 showed a lower expression of p53 than MCF-7 ($P < 0.01$). Under the same conditions, MDM2 protein in MCF-7 cells increased gradually and almost doubled at 48 h (Fig. 4B). In MCF-7/pCdm2, MDM2 increased rapidly within 6 h and maintained its elevated level for at least 48 h ($P < 0.01$, compared with MCF-7). In contrast, the induction of MDM2 was inhibited in MCF-7/pCdm2as ($P < 0.01$, compared with MCF-7).

These unexpected results imply that not only in the steady state, but also in response to E₂, the expression level of p53 correlates with that of MDM2, despite the well-known role of MDM as a promoter of p53 degradation^{7,8} and the results of growth assay. Therefore, in addition to protein level, the transcriptional activity of p53

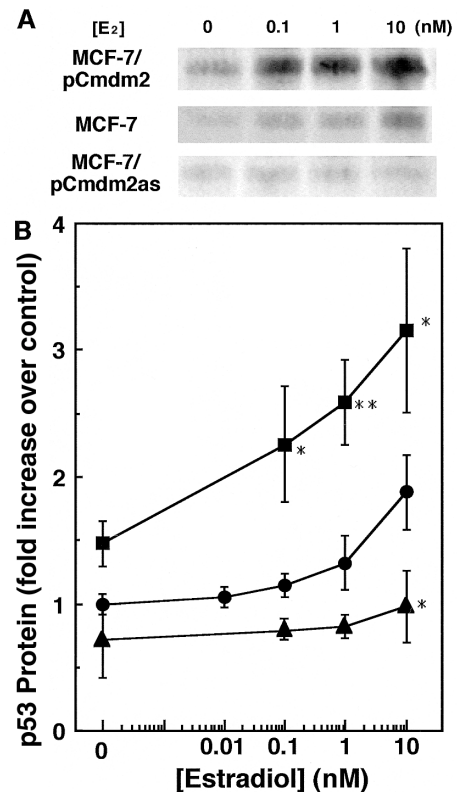


Fig. 3. Concentration-dependent effect of E₂ on p53 expression. Cells were maintained in medium without E₂ for 48 h and then incubated with various concentrations of E₂ for another 24 h. (A) Typical western blots. Cellular proteins were separated by 7.5% SDS-PAGE and transferred onto PVDF membranes. The membranes were probed with p53 monoclonal antibody (Ab-6). (B) Band density was quantified with a densitometer. The data (means \pm SD; $n = 3$) shown are expressed as the increase (fold) over the value obtained in parent MCF-7 in the absence of E₂ (control). ■ MCF-7/pCdm2, ● MCF-7, ▲ MCF-7/pCdm2as. * $P < 0.05$, ** $P < 0.01$ compared with MCF-7.

in response to E₂ was assessed by measuring the protein level of p21/waf-1, expression of which is dominantly controlled by the transcriptional function of p53.^{30–32} In MCF-7 and MCF-7/pCdm2as, p21 time-dependently increased and was almost doubled at 48 h (Fig. 4C). However, E₂-induced p21 expression was most prominent in MCF-7/pCdm2 ($P < 0.01$, compared with MCF-7). The increase was detectable as early as 6 h after E₂ treatment and reached more than 3-fold at 48 h.

UV-induced p53 expression was repressed in MCF-7 overexpressing MDM2 Since MCF-7/pCdm2 expressed full-length MDM2 derived from exogenously introduced cDNA, we must confirm the ability of this MDM2 to promote the degradation of p53 under other conditions. Fur-

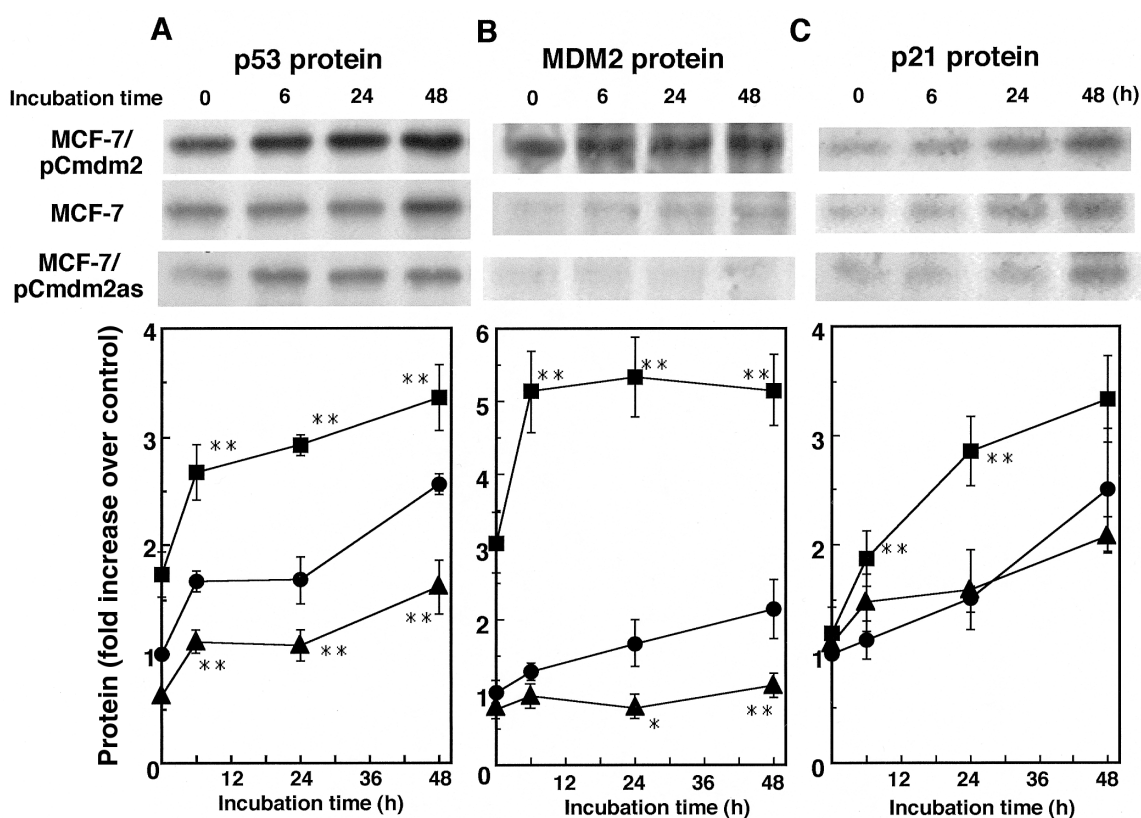


Fig. 4. Time-dependent effects of E₂ on p53, MDM2 and p21 expression. After preincubation for 48 h in medium without E₂, cells were stimulated with 10 nM E₂ for the indicated periods of time. p53 (A), MDM2 (B) and p21 (C) proteins were assessed by western blotting with p53 monoclonal antibody (Ab-6), MDM2 monoclonal antibody (Ab-1) and p21 polyclonal antibody (C-19), respectively. Upper panels are typical western blots from three separate experiments. Band density was quantified with a densitometer and data (means±SD; n=3) are expressed as increase (fold) over the value obtained in parent MCF-7 at 0 time (control). ■ MCF-7/pCcmdm2, ● MCF-7, ▲ MCF-7/pCcmdm2as. * P<0.05, ** P<0.01 compared with MCF-7.

thermore, to establish whether the cooperative increase in MDM2 and p53 was restricted to E₂ stimulation, the changes of p53 expression were examined in response to UV exposure, which is one of the most effective inducers of p53.³⁶⁻³⁸ Treatment of cells with UV resulted in a time-dependent increase in the level of p53 (Fig. 5). In MCF-7/pCcmdm2, p53 induction was inhibited by overexpressed MDM2 after 24 h. In contrast, MCF-7/pCcmdm2as showed the most prominent p53 induction, probably due to its reduced MDM2 expression.

E₂-induced p53 up-regulation was suppressed by transient reduction of MDM2 by antisense oligonucleotide The results obtained raised the possibility that the accumulation of p53 induced by E₂ was enhanced by MDM2 protein. To address this question, the production of MDM2 protein was transiently prevented by antisense oligonucleotide (AS oligo).²⁹ Treatment of MCF-7 cells with AS oligo resulted in a dose-dependent decrease in the level of MDM2 (Fig. 6B). The level of MDM2 was

slightly up-regulated by E₂ as compared with its absence. Fig. 6C showed the changes of p53 expression in response to various concentrations of MDM2 AS oligo. In the absence of E₂, p53 expression was proportional to the increase of AS oligo concentration. In sharp contrast, in the presence of 10 nM E₂, the level of p53 was reduced by MDM2 AS oligo, probably due to the inhibition of MDM2 expression. These results clearly indicate that E₂-induced accumulation of p53 correlated well with the level of MDM2 protein.

DISCUSSION

The results obtained in the present study imply a possible novel role of MDM2 in MCF-7 cells. MDM2 overexpression in MCF-7 cells afforded a growth advantage under E₂-supplemented conditions. In these cells, however, p53 accumulation induced by E₂ was remarkably higher than that in the parent MCF-7 cells. On the other

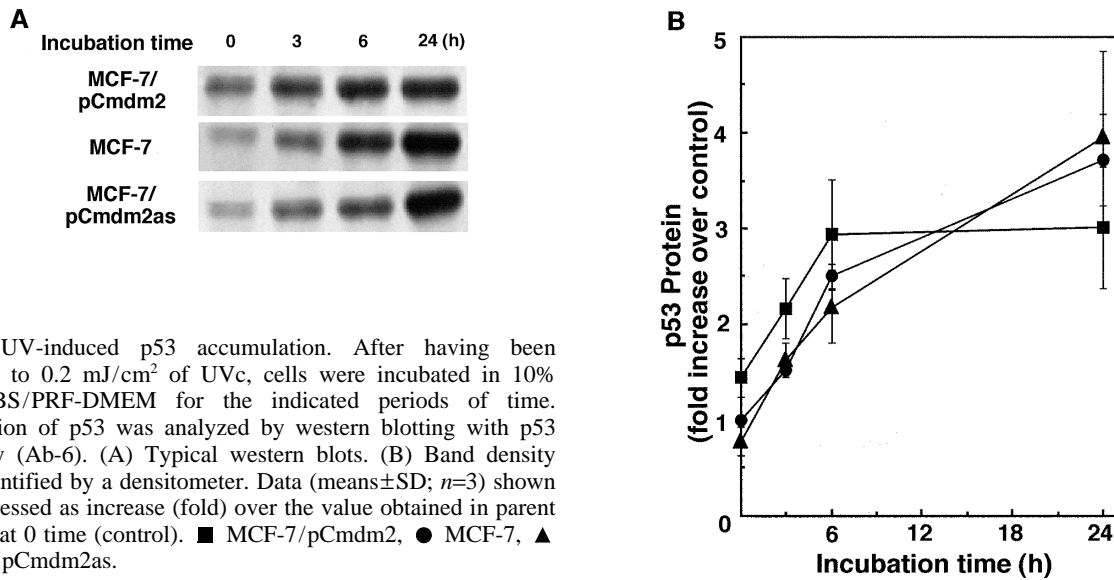


Fig. 5. UV-induced p53 accumulation. After having been exposed to 0.2 mJ/cm² of UVc, cells were incubated in 10% DCC-FBS/PRF-DMEM for the indicated periods of time. Expression of p53 was analyzed by western blotting with p53 antibody (Ab-6). (A) Typical western blots. (B) Band density was quantified by a densitometer. Data (means±SD; n=3) shown are expressed as increase (fold) over the value obtained in parent MCF-7 at 0 time (control). ■ MCF-7/pCdm2, ● MCF-7, ▲ MCF-7/pCdm2as.

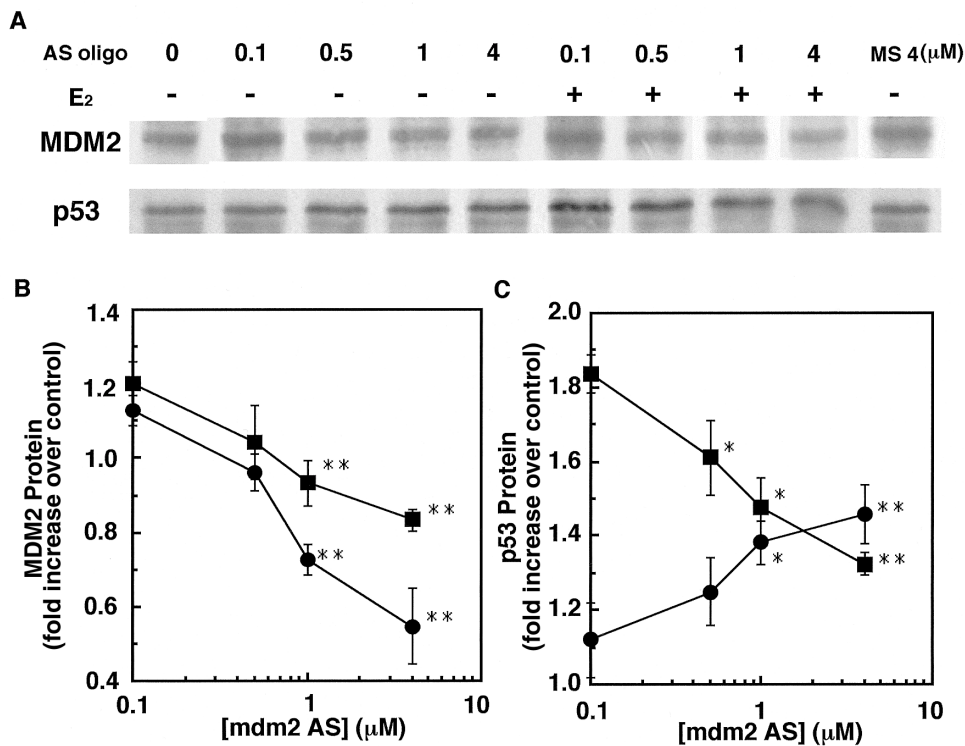


Fig. 6. The effects of *mdm2* antisense phosphorothioate oligonucleotide on the levels of MDM2 and p53 proteins. MCF-7 cells were treated with various concentrations of *mdm2* antisense oligonucleotide (AS oligo) or 4 μM mismatch oligonucleotide for 6 h and incubated for another 18 h in 10% DCC-FBS/DMEM with or without 10 nM E₂. Cell lysates were prepared and the levels of MDM2 and p53 proteins were evaluated by western blotting. (A) Western blots of MDM2 and p53. MS4 indicates the samples treated with 4 μM of mismatch oligonucleotide. (B, C) Band density was quantified by a densitometer. Data (means±SD; n=3) shown are expressed as increase (fold) over the value obtained in MCF-7 cells in the absence of AS oligo and E₂ (control). ■ E₂+, ● E₂-. * P<0.05, ** P<0.01 compared with the value of 0.1 μM AS oligo.

hand, reduction of MDM2 suppressed E₂-induced p53 accumulation. Also, changes of steady-state MDM2 expression caused a proportional increase or decrease in the level of p53, indicating that they might maintain a balance.

MDM2 is well known to act as a negative regulator of p53 by inhibiting its ability to activate transcription and by promoting its rapid degradation.^{3,4,7)} Kubbutat *et al.* reported that transient overexpression of MDM2 inhibited the accumulation of both endogenous and exogenous p53 in a dose-dependent manner.⁸⁾ However, there are several observations which are consistent with our findings showing a paradoxical relationship between MDM2 and p53. Many investigators reported high expression of both MDM2 and wild-type p53 in soft tissue sarcomas,¹⁹⁾ bladder carcinomas,³⁹⁾ testicular germ cell tumors,⁴⁰⁾ non-Hodgkin's lymphomas,⁴¹⁾ melanomas⁴²⁾ and breast cancers.⁴²⁾ Moreover, extended half-lives of wild-type p53 have been found in some choriocarcinoma cell lines which contain elevated levels of MDM2.⁴³⁾ These observations could be explained by the possibility that the elevated p53 induced MDM2 overproduction. However, Keleti *et al.* reported that in a subset of rhabdomyosarcoma cell line with MDM2 gene amplification, the wild-type p53 protein is also elevated and both p53 and MDM2 were co-localized in the same nuclei.⁴⁴⁾ In addition, both mutant-type p53 and MDM2 were elevated in some tumors.^{19,45,46)} These intricate interactions between MDM2 and p53 in the steady-state could not be explained by the mechanism previously reported. Therefore, it is reasonable to speculate that other mechanisms may regulate the expression levels of MDM2 and p53 via enhancement of transcription and/or protein stability.

In our preliminary investigations demonstrating E₂-induced accumulation of p53 and MDM2, we confirmed that MDM2 accumulation was dependent on enhanced transcription from the *mdm2*-P2 promoter, which possesses two p53 binding motifs.^{42,47)} In other words, the MDM2 level was under the control of p53 (data not shown). In the reverse transcriptase-polymerase chain reaction assay using promoter-specific primers, the *mdm2*-P2 transcript in MCF-7 was transiently observed at 6 h after addition of E₂. In contrast, it was maintained from 6 to 24 h in MCF-7/pCcmdm2. It is tempting to speculate

that accumulated MDM2, which was up-regulated by p53, may in turn enhance the p53 expression via direct or indirect transcriptional activation. This is compatible with the findings that MDM2 possesses zinc finger motifs of the type found in DNA-binding proteins and an acidic domain that has transcriptional activity.^{6,48,49)} Alternatively, E₂ may enhance the protein stability of MDM2 and p53. These possibilities are under investigation in our laboratory.

MDM2 is frequently overexpressed in human breast cancer cells,²²⁻²⁵⁾ and ER-positive breast cancer cell lines express relatively high levels of *mdm2* mRNA and protein compared to ER-negative cells.²⁶⁾ These findings strongly suggest the biological significance of MDM2 in ER-positive breast cancer. In fact, overexpression of MDM2 in MCF-7 cells affords a growth advantage under E₂-supplemented conditions, although the mechanism of this growth acceleration has not yet been established. The prominent accumulation of the cell cycle inhibitor p21/waf-1 and its inducer p53 in MCF-7/pCcmdm2 seems incompatible with the results obtained in growth assay. Further experiments are necessary to understand whether the intricate interactions between MDM2 and p53 in response to E₂ were specific in breast cancer cells and to gain further insight into the mechanism regulating this novel interaction.

In summary, our findings presented here indicate a novel function of MDM2 in regulating p53 expression in breast cancer. This may provide a clue for better understanding the relationship between the tumor suppressor and the oncogene product, and suggest a possible novel function of MDM2, which may promote cell growth in breast cancer cells independently of p53 inhibition.

ACKNOWLEDGMENTS

We are grateful to Dr. Klaus Roemer (University of California, San Diego) for pCcmdm2 and pCcmdm2as plasmids. We also thank Akio Suzuki for expert technical assistance and helpful advice. This work was supported in part by research grants from the Ministry of Education, Culture, Sports and Science of Japan.

(Received October 14, 1998/Revised November 30, 1998/
Accepted December 4, 1998)

REFERENCES

- 1) Cahilly-Snyder, L., Yang-Feng, T., Francke, U. and George, D. L. Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. *Somat. Cell Mol. Genet.*, **13**, 235-244 (1987).
- 2) Fakharzadeh, S. S., Trusko, S. P. and George, D. L. Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *EMBO J.*, **10**, 1565-1569 (1991).
- 3) Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L. and Vogelstein, B. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature*, **358**, 80-83 (1992).
- 4) Momand, J., Zambetti, G. P., Olson, D. C., George, D. and

- Levine, A. J. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*, **69**, 1237–1245 (1992).
- 5) Kussie, P. H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A. J. and Pavletich, N. P. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science*, **274**, 948–953 (1996).
 - 6) Boddy, M. N., Freemont, P. S. and Borden, K. L. The p53-associated protein MDM2 contains a newly characterized zinc-binding domain called the RING finger. *Trends Biochem. Sci.*, **19**, 198–199 (1994).
 - 7) Haupt, Y., Maya, R., Kazaz, A. and Oren, M. Mdm2 promotes the rapid degradation of p53. *Nature*, **387**, 296–299 (1997).
 - 8) Kubbutat, M. H., Jones, S. N. and Vousden, K. H. Regulation of p53 stability by Mdm2. *Nature*, **387**, 299–303 (1997).
 - 9) Barak, Y., Juven, T., Haffner, R. and Oren, M. mdm2 expression is induced by wild type p53 activity. *EMBO J.*, **12**, 461–468 (1993).
 - 10) Xiao, Z. X., Chen, J., Levine, A. J., Modjtahedi, N., Xing, J., Sellers, W. R. and Livingston, D. M. Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature*, **375**, 694–698 (1995).
 - 11) Martin, K., Trouche, D., Hagemeyer, C., Sorensen, T. S., LaThangue, N. B. and Kouzarides, T. Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature*, **375**, 691–694 (1995).
 - 12) Marechal, V., Elenbaas, B., Piette, J., Nicolas, J. C. and Levine, A. J. The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. *Mol. Cell. Biol.*, **14**, 7414–7420 (1994).
 - 13) Elenbaas, B., Dobbstein, M., Roth, J., Shenk, T. and Levine, A. J. The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. *Mol. Med.*, **2**, 439–451 (1996).
 - 14) Fiddler, T. A., Smith, L., Tapscott, S. J. and Thayer, M. J. Amplification of MDM2 inhibits MyoD-mediated myogenesis. *Mol. Cell. Biol.*, **16**, 5048–5057 (1996).
 - 15) Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., Cordon-Cardo, C. and DePinho, R. A. The Ink4a tumor suppressor gene product, p19^{Arf}, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell*, **92**, 713–723 (1998).
 - 16) Zhang, Y., Xiong, Y. and Yarbrough, W. G. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell*, **92**, 725–734 (1998).
 - 17) Sigalas, I., Calvert, A. H., Anderson, J. J., Neal, D. E. and Lunec, J. Alternatively spliced mdm2 transcripts with loss of p53 binding domain sequences: transforming ability and frequent detection in human cancer. *Nat. Med.*, **2**, 912–917 (1996).
 - 18) Matsumoto, R., Tada, M., Nozaki, M., Zhang, C. L., Sawamura, Y. and Abe, H. Short alternative splice transcripts of the mdm2 oncogene correlate to malignancy in human astrocytic neoplasms. *Cancer Res.*, **58**, 609–613 (1998).
 - 19) Cordon-Cardo, C., Latres, E., Drobnjak, M., Oliva, M. R., Pollack, D., Woodruff, J. M., Marechal, V., Chen, J., Brennan, M. F. and Levine, A. J. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res.*, **54**, 794–799 (1994).
 - 20) McCann, A. H., Kirley, A., Carney, D. N., Corbally, N., Magee, H. M., Keating, G. and Dervan, P. A. Amplification of the MDM2 gene in human breast cancer and its association with MDM2 and p53 protein status. *Br. J. Cancer*, **71**, 981–985 (1995).
 - 21) Quesnel, B., Preudhomme, C., Fournier, J., Fenaux, P. and Peyrat, J. P. MDM2 gene amplification in human breast cancer. *Eur. J. Cancer*, **30A**, 982–984 (1994).
 - 22) Inada, K., Toi, M., Yamamoto, Y., Suzuki, A., Kurisaki, T., Suzuki, H. and Tominaga, T. Immunocytochemical analysis of MDM2 protein expression and its relevance to tumor angiogenesis in primary breast cancer. *Oncol. Rep.*, **3**, 667–671 (1996).
 - 23) Hideshima, T., Shinohara, T., Baba, M. and Shirakusa, T. The expression of MDM2 and p53 protein in breast carcinoma. *Oncol. Rep.*, **4**, 297–300 (1997).
 - 24) Baunoch, D. A., Watkins, L. F., Tewari, A., Reece, M. T., Adams, L., Stack, R., Brown, A., Jones, L., Christian, D., Latif, N. A., Lawrence, W. D. and Lane, M. A. MDM2 overexpression in benign and malignant lesions of the human breast: association with ER expression. *Int. J. Oncol.*, **8**, 895–899 (1996).
 - 25) Marchetti, A., Buttitta, F., Girlando, S., Dalla Palma, P., Pellegrini, S., Fina, P., Doglioni, C., Bevilacqua, G. and Barbareschi, M. mdm2 gene alterations and mdm2 protein expression in breast carcinomas. *J. Pathol.*, **175**, 31–38 (1995).
 - 26) Gudas, J. M., Nguyen, H., Kiein, R. C., Katayose, D., Seth, P. and Cowan, K. H. Differential expression of multiple MDM2 messenger RNAs and proteins in normal and tumorigenic breast epithelial cells. *Clin. Cancer Res.*, **1**, 71–80 (1995).
 - 27) Roemer, K. and Friedmann, T. Modulation of cell proliferation and gene expression by a p53-estrogen receptor hybrid protein. *Proc. Natl. Acad. Sci. USA*, **90**, 9252–9256 (1993).
 - 28) Suzuki, A., Toi, M., Yamamoto, Y., Saji, S., Muta, M. and Tominaga, T. Role of MDM2 overexpression in doxorubicin resistance of breast carcinoma. *Jpn. J. Cancer Res.*, **89**, 221–227 (1998).
 - 29) Chen, L., Agrawal, S., Zhou, W., Zhang, R. and Chen, J. Synergistic activation of p53 by inhibition of MDM2 expression and DNA damage. *Proc. Natl. Acad. Sci. USA*, **95**, 195–200 (1998).
 - 30) el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825 (1993).

- 31) Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. and Elledge, S. J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816 (1993).
- 32) Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J. and Reed, S. I. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell*, **76**, 1013–1023 (1994).
- 33) Hurd, C., Khattree, N., Alban, P., Nag, K., Jhanwar, S. C., Dinda, S. and Moudgil, V. K. Hormonal regulation of the p53 tumor suppressor protein in T47D human breast carcinoma cell line. *J. Biol. Chem.*, **270**, 28507–28510 (1995).
- 34) Hurd, C., Khattree, N., Dinda, S., Alban, P. and Moudgil, V. K. Regulation of tumor suppressor proteins, p53 and retinoblastoma by estrogen and antiestrogens in breast cancer cells. *Oncogene*, **15**, 991–995 (1997).
- 35) Yu, C. L., Driggers, P., Barrera-Hernandez, G., Nunez, S. B., Segars, J. H. and Cheng, S. The tumor suppressor p53 is a negative regulator of estrogen receptor signaling pathways. *Biochem. Biophys. Res. Commun.*, **239**, 617–620 (1997).
- 36) Hall, P. A., McKee, P. H., Menage, H. D., Dover, R. and Lane, D. P. High levels of p53 protein in UV-irradiated normal human skin. *Oncogene*, **8**, 203–207 (1993).
- 37) Zhan, Q., Carrier, F. and Fornace, A. J., Jr. Induction of cellular p53 activity by DNA-damaging agents and growth arrest. *Mol. Cell. Biol.*, **13**, 4242–4250 (1993).
- 38) Perry, M. E., Piette, J., Zawadzki, J. A., Harvey, D. and Levine, A. J. The mdm-2 gene is induced in response to UV light in a p53-dependent manner. *Proc. Natl. Acad. Sci. USA*, **90**, 11623–11627 (1993).
- 39) Lianes, P., Orlow, I., Zhang, Z. F., Oliva, M. R., Sarkis, A. S., Reuter, V. E. and Cordon-Cardo, C. Altered patterns of MDM2 and TP53 expression in human bladder cancer. *J. Natl. Cancer Inst.*, **86**, 1325–1330 (1994).
- 40) Riou, G., Barrois, M., Prost, S., Terrier, M. J., Theodore, C. and Levine, A. J. The p53 and mdm-2 genes in human testicular germ-cell tumors. *Mol. Carcinog.*, **12**, 124–131 (1995).
- 41) Maestro, R., Gloghini, A., Doglioni, C., Gasparotto, D., Vukosavljevic, T., De Re, V., Laurino, L., Carbone, A. and Boiocchi, M. MDM2 overexpression does not account for stabilization of wild-type p53 protein in non-Hodgkin's lymphomas. *Blood*, **85**, 3239–3246 (1995).
- 42) Landers, J. E., Cassel, S. L. and George, D. L. Translational enhancement of mdm2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. *Cancer Res.*, **57**, 3562–3568 (1997).
- 43) Landers, J. E., Haines, D. S., Strauss, J. F., 3rd and George, D. L. Enhanced translation: a novel mechanism of mdm2 oncogene overexpression identified in human tumor cells. *Oncogene*, **9**, 2745–2750 (1994).
- 44) Keleti, J., Quezado, M. M., Abaza, M. M., Raffeld, M. and Tsokos, M. The MDM2 oncoprotein is overexpressed in rhabdomyosarcoma cell lines and stabilizes wild-type p53 protein. *Am. J. Pathol.*, **49**, 143–151 (1996).
- 45) Zou, M., Shi, Y., al-Sedairy, S., Hussain, S. S. and Farid, N. R. The expression of the MDM2 gene, a p53 binding protein, in thyroid carcinogenesis. *Cancer*, **76**, 314–318 (1995).
- 46) Gunther, T., Schneider-Stock, R., Rys, J., Niezabitowski, A. and Roessner, A. p53 gene mutations and expression of p53 and mdm2 proteins in invasive breast carcinoma. A comparative analysis with clinico-pathological factors. *J. Cancer Res. Clin. Oncol.*, **123**, 388–394 (1997).
- 47) Bull, E. K., Chakrabarty, S., Brodsky, I. and Haines, D. S. mdm2-P2 transcript levels predict the functional activity of the p53 tumor suppressor in primary leukemic cells. *Oncogene*, **16**, 2249–2257 (1998).
- 48) Borden, K. L. and Freemont, P. S. The RING finger domain: a recent example of a sequence-structure family. *Curr. Opin. Struct. Biol.*, **6**, 395–401 (1996).
- 49) Saurin, A. J., Hamlett, J., Clague, M. J. and Pennington, S. R. Inhibition of mitogen-induced DNA synthesis by bafilomycin A1 in Swiss 3T3 fibroblasts. *Biochem. J.*, **313**, 65–70 (1996).