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ARTICLES

# Increased Expression of MDM2, Cyclin D1, and p27$^{Kip1}$ in Carcinogen-Induced Rat Mammary Tumors

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**Abstract** It is thought that environmental pollutants, such as polycyclic aromatic hydrocarbons (PAH), contribute to human breast tumorigenesis, yet their roles remain incompletely elucidated. The prototypical PAH 7,12-dimethylbenz(α)anthracene (DMBA) specifically and effectively induces mammary tumor formation in rodent models. In an attempt to explore the molecular mechanisms by which PAH initiates and promotes mammary tumorigenesis, we examined the expression of several cell cycle regulators in rat mammary tumors induced by DMBA. Expression of cyclin D1, murine double minute-2 (MDM2), and Akt was up-regulated in tumors in comparison to normal mammary glands, as indicated by RT-PCR, Western blot analysis, and immunohistochemical staining. Expression of p27$^{Kip1}$ protein was also elevated in the tumors with increased cytoplasmic localization. However, RB protein remained hyperphosphorylated. To directly test the effects of DMBA, the MCF-7 human breast cancer cells were treated. DMBA induced MDM2 expression in a dose- and time-dependent fashion in the MCF-7 cells, and this activation appeared to be p53 dependent. These data suggest that activation of cyclin D1, MDM2, and AKT as well as increased expression and cytoplasmic localization of p27$^{Kip1}$ may play a role in this model of environmental pollutant-induced mammary tumorigenesis. J. Cell. Biochem. 95: 875–884, 2005.


**Key words:** carcinogen; DMBA; breast cancer; animal models; MDM2; p27$^{Kip1}$

It has been suggested that lipophilic environmental chemicals, such as polycyclic aromatic hydrocarbons (PAHs), contribute to human breast tumorigenesis [Rundle et al., 2000, 2002; Gammon et al., 2002; Li et al., 2002]. Laboratory animal models have been useful in understanding the initiation, promotion, and progression of mammary carcinogenesis. In particular, rodent mammary tumorigenesis induced by the model PAH carcinogen 7,12-dimethylbenz(α)anthracene (DMBA) provides an excellent system for elucidating the molecular mechanisms of mammary carcinogenesis.

DMBA selectively and effectively induces mammary tumor formation in female rats. A single dose of DMBA administrated intragastrically to 40–60 day-old female Sprague-Dawley (S-D) rats results in development of mammary tumors with an 8–21 week latency and incidence of 80%–100% [Russo and Russo, 1996]. These tumors range from benign tumors to papillomas and adenocarcinomas. The adenocarcinomas are histologically and cytologically malignant [Russo and Russo, 1987]. It is known that many factors such as estrogen exposure, prolactin blood levels [Russo and Russo, 1996], and diet [Rogers et al., 1998, 1999; Kavanagh et al., 2001] affect the progress of DMBA-induced carcinogenesis in rats,

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yet the molecular mechanisms remain largely unclear.

Central to the process of carcinogenesis are alterations in the expression and/or regulation of components of the cell cycle and checkpoint machinery, resulting in abnormal proliferation. Overexpression of cyclin D1 has been well documented in the pathogenesis of breast cancer [Buckley et al., 1993; Bartkova et al., 1994]. Increased cyclin D1 expression is found in a high percentage of primary human breast cancers [Bartkova et al., 1994], including those in early stages of the disease [Weinstat-Saslow et al., 1995]. While mice lacking cyclin D1 display profound defects in mammary cell proliferation and gland development [Sicinski et al., 1995], targeted overexpression of cyclin D1 in mammary epithelium of transgenic mice leads to mammary tumor formation [Wang et al., 1994].

Cell cycle progression is negatively regulated by members of the CIP/KIP family of cyclin-dependent kinase inhibitors (CKIs), including $p27^{Kip1}$ (hereafter referred to as p27). p27 plays an important role in coordinating the activation of cyclin E-cdk2 with the accumulation of cyclin D-cdk4, and in initiating a timely cell cycle exit in response to anti-mitogenic stimuli [Sherr, 1996]. In proliferating cells, p27 is primarily associated with cyclin D-cdk4/6 complexes, which remain catalytically active. In contrast, when p27 associates with cyclin E-cdk2, it inhibits cdk2 kinase activity, resulting in cell cycle arrest [Blain et al., 2003]. An inverse correlation between the p27 protein levels and poor prognosis was first noted in colon cancer and later in many human cancers, including breast, prostate, lung, and liver [Lloyd et al., 1999]. However, elevated p27 protein levels have also been found in some tumors and cancers, including breast cancers [Fredersdorf et al., 1997; Lloyd et al., 1997; Loda et al., 1997]. In this case, p27 protein is often found in an inactive form in the cytoplasm [Ciaparrone et al., 1998; Singh et al., 1998]. For example, approximately 40% of primary tumors display cytoplasmic p27 staining. It has been shown that Akt directly phosphorylates p27 resulting in its cytoplasmic localization [Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002].

Inactivation of the tumor suppressor protein p53 plays a central role in tumorigenesis. More than 50% of human cancers contain mutations in the p53 gene [Levine, 1997]. Indeed, inactivation of p53 by either mutations in the p53 gene, alterations of p53 modulators, or deregulation of components in the p53 pathway is believed to represent a ubiquitous neoplastic strategy [Vogelstein et al., 2000]. The primary negative regulator of p53 is murine double minute-2 (MDM2), a proto-oncoprotein that physically interacts with and inhibits p53. MDM2 functions as an ubiquitin E3 ligase for p53 to promote p53 degradation [Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997; Fang et al., 2000; Honda and Yasuda, 2000]. In addition, MDM2 possesses p53-independent oncogenic activities, exemplified by interaction with retinoblastoma protein (Rb) and E2F to promote cell cycle $G_1$–S transition [Martin et al., 1995; Xiao et al., 1995; Lundgren et al., 1997; Sun et al., 1998; Gu et al., 2003]. Importantly, MDM2 is a transcriptional target of p53 [Barak et al., 1993], creating a negative feedback loop in maintaining the low p53 protein levels in normal cells. The role of MDM2 in the development of human cancer is highlighted by the observation that overexpression of MDM2 is found in a variety of human tumors and cancers bearing wild-type (WT) p53 alleles [Momand et al., 1998], including human breast cancers [Quesnel et al., 1994; Marchetti et al., 1995; McCann et al., 1995].

In this study, we showed that the expression of cyclin D1, MDM2, and Akt was elevated in DMBA-induced rat mammary tumors. Interestingly, p27 protein was also elevated in the tumors and was often mislocalized to the cytoplasm. In addition, DMBA treatment of human breast cancer cells induced the expression of MDM2 in a p53-dependent manner. Taken together, these data suggest that activation of cyclin D1, MDM2, and Akt as well as deregulation of p27 expression and localization may contribute to carcinogen-induced mammary tumorigenesis.

## MATERIALS AND METHODS

### Animal Treatment and Tissue Processing

Animal treatment and tissue collection were performed as previously described [Kavanagh et al., 2001]. Briefly, to induce tumors, virgin female S-D rats, age 56 days, were administrated a single intragastric dose of 15 mg/kg of DMBA in 0.2 ml sesame oil or vehicle alone. Animals were sacrificed 16 weeks following treatment. Individual tumors and normal

mammary glands were removed, dissected, and processed for histology or snap frozen in liquid nitrogen. Snap-frozen tissues were pulverized on dry ice using a Bessman Tissue Pulverizer (Spectrum), catalogued, and stored at −80°C. Total RNA, DNA, and protein were isolated sequentially from pulverized frozen tissue samples using Trizol Reagent (Gibco) according to the manufacturer's instructions.

### Immunohistochemistry

Paraffin-embedded normal mammary gland and tumor tissue samples were sectioned at 5 μM and affixed to SuperFrost + microscope slides (Fisher Scientific). The sections were then deparaffinized, rehydrated, and subjected to antigen retrieval by incubation of the slides with Antigen Unmasking Working Solution (Vector Laboratories) at 90°C for 20 min. Following antigen retrieval, immunohistochemical staining was performed using the antibody specific for MDM2 (2A10) or p27 (C-19, Santa Cruz). The staining signals were detected using the Vectestain ABC kit (Vector Laboratories).

### Western Blot Analysis

Total proteins were extracted from rat mammary tissue samples and resuspended in a buffer containing 250 mM NaCl, 50 mM Tris, pH 7.4, and 1% SDS. For cell culture samples, cells were incubated in the lysis buffer (125 mM NaCl, 25 mM Tris pH7.4, 0.5% NP-40, 50 mM NaF, 0.5 mM $NaVO_4$, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 10 μg/ml leupeptin). Cytoplasmic and nuclear proteins were obtained using the procedure as described [Jiang et al., 2002]. Equal amounts of protein (20–60 μg per sample) were separated on a 10% polyacrylamide-SDS gel and subjected to Western blot analyses using antibodies purchased from Santa Cruz Biotechnology: p53 (DO-1 or FL-393), MDM2 (SMP-14), cyclin D1 (HD11), $p27^{Kip1}$ (C-19), IκB-α (C-21), and actin (C-11). Antibodies specific for phospho-Rb (Ser807/811) (Cell Signaling Technology), Akt (Cell Signaling Technology), or α-tubulin (T5168, Sigma) were also used.

### Northern Blot Analysis

Total RNA samples (15 μg) were resolved in a formaldehyde/agarose gel, and transferred to Hybond-XL Nylon membrane (Amersham). $^{32}P$-labeled probes for murine p27 and GAPDH were prepared with the Redi-Prime II random prime labeling kit (Amersham) and hybridized using Quick-Hyb (Stratagene). Films were scanned and densities determined using LabWorks Analysis Software v. 3.0.02 (Ultra-Violet Products).

### Cell Culture and Treatment

Human breast cancer cells (MCF-7, Hs578T, MDA-MB-231, T47D), and untransformed human mammary epithelial MCF-10F cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose supplemented with 10% fetal bovine serum (FBS; certified, Gibco), 100 units/ml penicillin and streptomycin (Gibco). WT and p53 null mouse embryonic fibroblast cells (MEFs, kindly provided by Dr. Tyler Jacks, Massachusetts Institute of Technology, Cambridge, MA) were maintained in DMEM supplemented with 12% FBS and used in early passage (5–10). Cells were treated DMBA (Sigma) or the vehicle (acetone) as indicated.

### RT-PCR Analysis

Two micrograms of total RNA isolated from mammary tissues was mixed with 20 pmol of oligo-dT primer (Pharmacia Biotech) and first strand synthesis performed using SuperScript II reverse transcriptase (Gibco). The single-stranded cDNA was used for PCR analysis. The primers for murine cyclin D1, 5′ (D103: CAGACGGCCGCGCCATGGAA) and 3′ (D105: AGGAAGTTGTTGGGGCTGCC) were used to generate a specific cyclin D1 PCR product of 681 base pairs. The PCR conditions for cyclin D1 were 40 s at 94°C, 1 min at 60°C, and 1 min at 72°C for 30 cycles. For β-actin, 5′ (GGGGAATTCATGCCATCCTGCGTCTGGA) and 3′ (GGGGAATTCCACATCTGCTGGAAGGTGG) primers were used to generate a specific PCR product of 480 base pairs. The conditions for β-actin were 40 s at 94°C, 1 min at 60°C, and 1 min at 72°C for 25 cycles. Both reactions were preceded by an initial 3-min melting phase at 94°C and followed by a 7-min final extension phase at 72°C.

## RESULTS

### Increased Expression of Cyclin D1 and MDM2 in DMBA-Induced Mammary Tumors

A single dose of 15 mg/kg of DMBA by gastric gavage in female S-D rats induced mammary tumors within 20 weeks. In two separate experiments, DMBA induced mammary tumors at incidences of 62% and 70% with 1.6 ± 0.9 and

**TABLE I. Histopathology of Mammary Tumors used in this Study**

| Tumor | Histopathology |
|---|---|
| 2-39 L3 | Non-invasive papillary and cribiform adenocarcinoma |
| 2-44 L5 | Invasive papillary and cribriform adenocarcinoma |
| 2-56 R1 | Non-invasive papillary and cribiform adenocarcinoma |
| 2-59 R1 | Invasive papillary and cribiform adenocarcinoma |
| 3-12 R3 | Invasive papillary and cribriform adenocarcinoma |
| 3-28 R4/5 | Invasive papillary and cribiform adenocarcinoma |
| 3-29 L3 | Invasive papillary and cribiform adenocarcinoma |

$2.0 \pm 1.2$ tumors per tumor bearing rat, respectively. The tumors ranged from papillomas to non-invasive and invasive papillary and cribiform adenocarcinomas [Rogers et al., 1998]. Seven tumors were selected randomly for this study (Table I). As overexpression of cyclin D1 is frequently found in human breast cancer, we analyzed the expression of cyclin D1 mRNA in the DMBA-induced rat mammary tumors and compared it to that of normal mammary glands. As shown in Figure 1, the relative levels of cyclin D1 over β-actin in tumors were $10.9 \pm 1.3$ compared to $2.6 \pm 1.0$ in normal samples ($P < 0.001$). These data suggest that cyclin D1 is induced in mammary tumors.

Since overproduction of MDM2 has been documented in human breast cancer [Quesnel et al., 1994; Marchetti et al., 1995; McCann et al., 1995], we examined the levels of MDM2 protein in four of the DMBA-induced rat tumors as compared to their histologically normal mammary gland counterparts. As shown in Figure 2A, the expression of MDM2 protein was evident in all of the mammary tumors whereas MDM2 protein was hardly detectable in histologically normal mammary glands from the corresponding rats. To assess the subcellular localization of MDM2, we performed immunohistochemical analysis for MDM2 expression in the tumor tissue and normal mammary gland samples. As shown in Figure 3, minor MDM2-specific staining was seen in normal mammary glands whereas high levels of nuclear MDM2 were observed in the DMBA-induced tumors. Thus, MDM2 is overexpressed in the DMBA-induced mammary tumors.

### Aberrant p27 Expression in DMBA-Induced Mammary Tumors

The cyclin-dependent kinase inhibitor p27 is a critical regulator of cell cycle progression. Alterations in p27 expression and activity are often associated with abnormal cell proliferation and tumor formation. We therefore examined the expression of p27 in the DMBA-induced rat mammary tumors as compared to that of normal mammary glands from the same animal. Surprisingly, p27 protein levels were significantly increased in the tumors (Fig. 2B). For instance, the tumor sample 2-39 L3 exhibited a two-fold increase in p27 protein expression compared to the normal gland (2-39) of the same rat, whereas a nearly ten-fold increase in tumor sample 2-59 R1 was noted when compared to its normal gland (2-59). The actin normalized p27 levels in tumor and normal glands were $10.8 \pm 1.7$ and $2.1 \pm 0.6$, respectively, ($P < 0.02$). These data suggest that the expression of p27 protein is increased in mammary tumors derived from DMBA-treated rats.

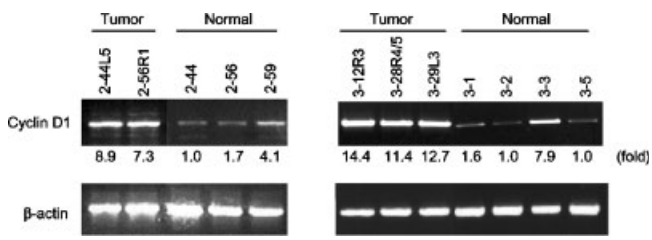


**Fig. 1.** Up-regulation of cyclin D1 mRNA expression in 7,12-dimethylbenz(α)anthracene (DMBA)-induced rat mammary tumors. Two micrograms of total RNA from DMBA-induced rat mammary tumors (2-44 L5, 2-56 R1, 3-12 R3, 3-28 R4/5, 3-29 L3) and the normal mammary glands from DMBA-treated animal (2-44, 2-56, 2-59) or from vehicle control (3-1, 3-2, 3-3, and 3-5) was used for first strand cDNA synthesis followed by PCR analysis using primers specific to murine cyclin D1 and β-actin. The PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. The intensity of the cyclin D1 band was normalized to the intensity of β-actin derived from the same RNA sample. The ratio of cyclin D1 over β-actin in the DMBA-treated normal mammary gland 2-44 or vehicle control 3-2 was arbitrarily set at 1.0. The relative expression of cyclin D1 was indicated as fold expression. Data were subjected to statistical analysis using Student's *t*-test.

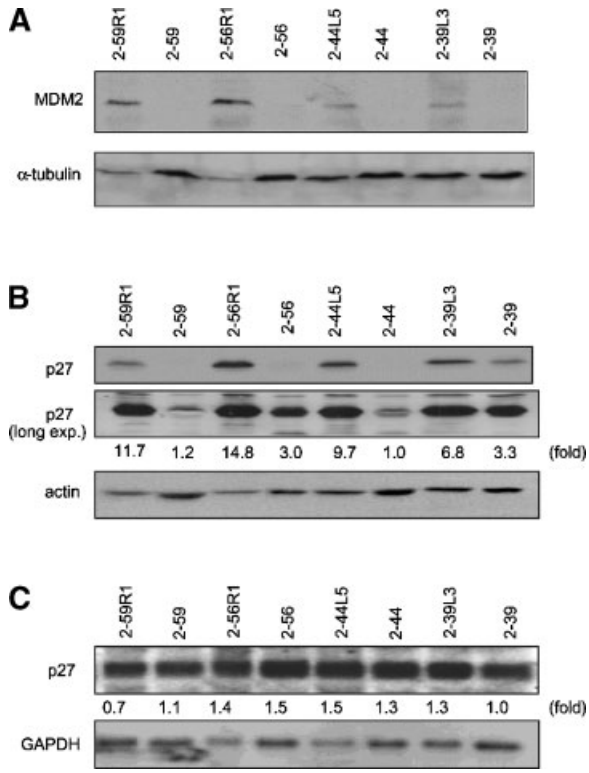


**Fig. 2.** Up-regulation of MDM2 and p27 in DMBA-induced rat mammary tumors. **A**: Fifty micrograms of total protein from DMBA-induced rat mammary tumors and the corresponding paired normal mammary glands from the same animal were separated by 10% PAGE–SDS and subjected to Western blot analyses using antibodies specific for MDM2 or α-tubulin. **B**: Samples were subjected to Western blot analysis, as above, using antibodies specific for p27 or actin. The level of p27 expression was normalized to actin in the same sample and the p27 expression level in normal mammary sample 2-44 was arbitrarily set at 1.0. **C**: Fifteen micrograms of total RNA was subjected to Northern blot analysis using $^{32}$P-labeled probes for murine p27 and GAPDH. Quantitative analysis was performed using densitometric scanning and the expression of p27 was normalized to GAPDH expression in the same sample. The p27 expression level in normal mammary sample 2-39 was arbitrarily set to 1.0.

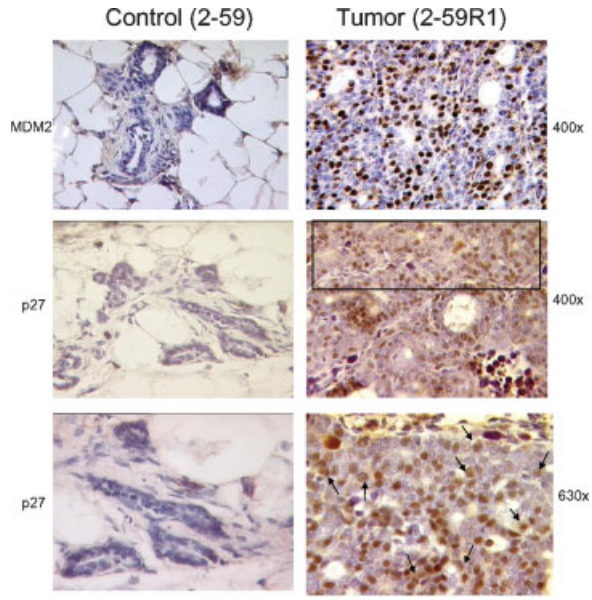


**Fig. 3.** Increased expression of MDM2 and p27 in rat mammary tumors. Paraformaldehyde-fixed, paraffin-embedded normal mammary gland from DMBA-treated rat (2-59), and mammary tumor from the same rat (2-59 R1) were subjected to immunohistochemical staining using antibody specific for MDM2 or p27. Cells were counterstained with hematoxylin, and pictures were taken using a Leitz microscope at 400× or 630× magnification, as indicated. The cytoplasmic p27 staining in tumor cells is indicated by a square in the middle right panel and in individual cells by arrows in the lower right panel.

Since p27 can be regulated at both the transcriptional [Yang et al., 2001] and post-transcriptional levels [Slingerland and Pagano, 2000], we performed Northern blot analysis to examine p27 mRNA expression in paired tumor and normal samples. As shown in Figure 2C, there were no significant differences in p27 mRNA levels between the tumor and normal samples when normalized to GAPDH loading control. These data suggest that alteration of p27 protein expression in the DMBA-induced rat mammary tumors occurs through a post-transcriptional mechanism.

To evaluate the cellular and subcellular distribution of p27 in normal and tumor tissues, we performed immunohistochemical staining of paraffin-embedded normal mammary glands and corresponding tumors. As shown in Figure 3, p27 was barely detectable in normal mammary gland. In tumor cells, the cytoplasmic localization of p27 was evident while the nuclear staining of p27 was also increased in scattered tumor cells (Fig. 3, the middle right panel and bottom right panel). Furthermore, when we examined the subcellular localization by fractionation, p27 was clearly accumulated in the cytoplasm as well as in nuclei (Fig. 4A). Interestingly, expression of Akt was evidently elevated in tumor samples. Analysis of the cytoplasmic IκB-α protein confirmed that the nuclear preparations were essentially free of cytoplasmic contamination (Fig. 4A). Thus, increased expression and cytoplasmic localization of p27 is associated with DMBA-induced mammary tumorigenesis, which correlated with elevated Akt expression.

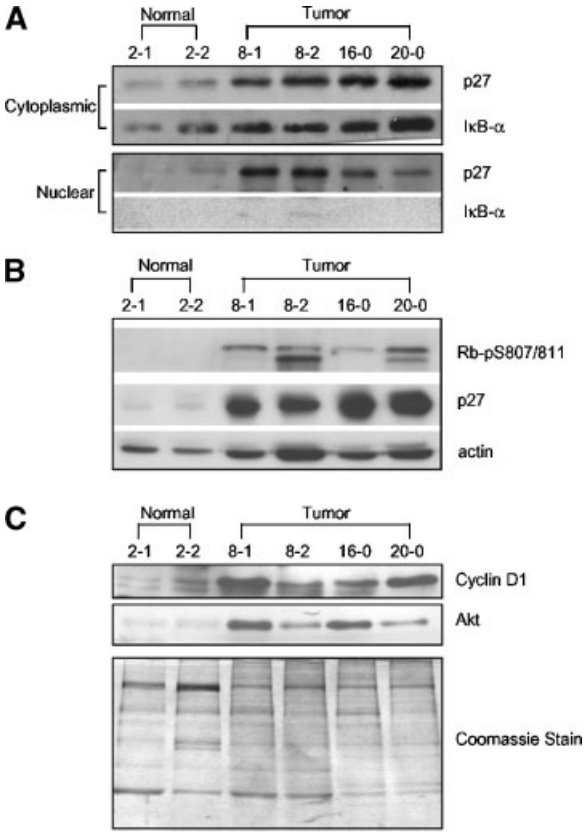


**Fig. 4.** Elevated expression of cyclin D1, Akt, p27, and phosphorylation of Rb in DBMA-induced rat mammary tumors. **A**: Lysates (40 μg of total proteins) of cytoplasmic or nuclear preparations derived from tumors or normal controls were subjected to Western blot analysis for p27. Cytoplasmic IκB-α protein was analyzed in parallel as a control for purity of preparation. **B**: Whole cell lysates (100 μg of total proteins) were subjected to Western blot analysis using antibodies specific for phospho-Rb (Rb-pS807/811), p27, or actin. **C**: Lysates of nuclear preparations (60 μg of total proteins) were evaluated by Western blot analysis using antibodies specific for cyclin D1 and Akt. Comparable protein loading was shown by staining the gel with Commassie brilliant blue R250 (lower panel).

We next examined the phosphorylation status of Rb. As shown in Figure 4B, phosphorylation of Rb at Ser807/811 was readily detected in tumors but not in the normal controls, and correlated with elevated expression of cyclin D1 protein (Fig. 4C). Since Ser807/811 of Rb are phosphorylated by cyclin D-dependent kinases [Adams, 2001], these data indicate that the cyclin D-associated kinases remain active despite of p27 protein accumulation.

### Elevated Expression of MDM2 and p27 in Human Breast Cancer Cells

Since the expression of MDM2 and p27 appear elevated in DMBA-induced mammary tumors as compared with normal control mammary glands, we investigated whether this phenomenon was recapitulated in human breast cancer cell lines. We examined the expression of MDM2 and p27 in four cell lines derived from human breast cancers (MCF-7, T47D, Hs578T, and MBA-MD-231) and untransformed human breast epithelial MCF-10F cells. As shown in Figure 5, MDM2 protein levels were clearly higher in cancer cell lines compared to the MCF-10F cells. Interestingly, p27 expression was also significantly higher in these breast cancer cells as compared with MCF-10F cells. These data are consistent with the notion that expression of MDM2 and p27 is elevated in human breast cancer cells.

### Activation of p53 by DMBA

Since MDM2 is up-regulated in the DMBA-induced mammary tumors and in human breast cancer cell lines, it is conceivable that up-regulation of MDM2 by DMBA might function to inhibit normal p53 activation during the course of tumorigenesis. Therefore, we examined the effect of DMBA treatment on the expression of p53 and MDM2 in MCF-7 breast cancer cells. As shown in Figure 6A, treatment with an increasing dose of DMBA for 24 h led to up-regulation of MDM2 in MCF-7 cells. Of note,

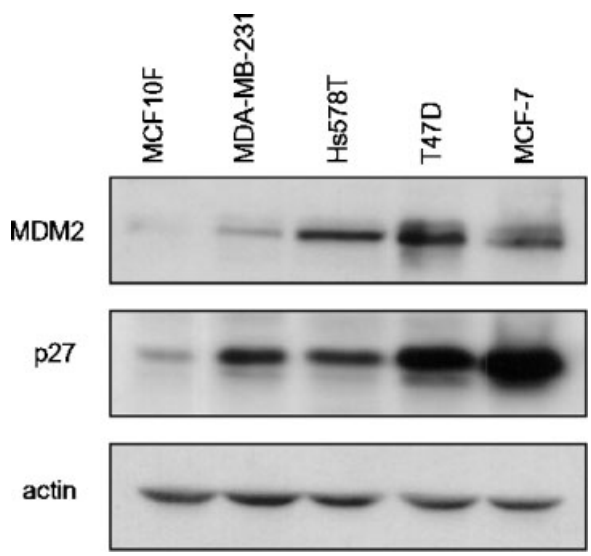


**Fig. 5.** Elevated expression of MDM2 and p27 in human breast cancer cell lines. Sub-confluent non-transformed human breast epithelial cells (MCF-10F) and human breast cancer cells (MDA-MB-231, Hs578T, T47D, MCF-7) were lysed and samples (50 μg) of total protein were subjected to Western blot analyses using antibodies specific for MDM2, p27, or actin.

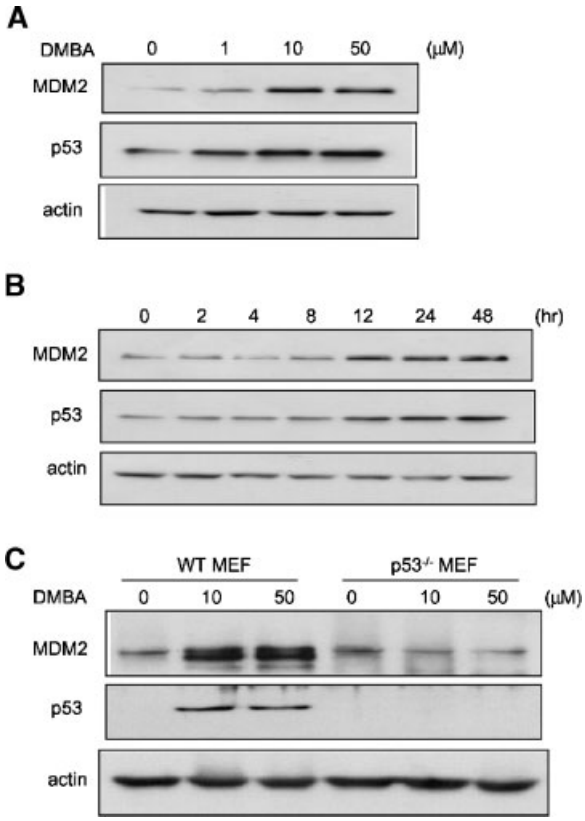


**Fig. 6.** DMBA treatment induces p53-dependent expression of MDM2 in MCF-7 cells. **A**: Sub-confluent MCF-7 cells were treated with vehicle control or increasing doses of DMBA. Twenty-four hours post-treatment, cells were lysed and equal amounts of total proteins were subjected to Western blot analyses using antibodies specific for MDM2, p53, or actin. **B**: Sub-confluent MCF-7 cells were treated with vehicle control or 10 μM DMBA for the times indicated. Whole cell lysates were obtained and Western blot analysis was performed. **C**: Sub-confluent wild type (WT) and p53 null ($p53^{-/-}$) MEFs were treated with vehicle control or the indicated dose of DMBA. Twenty-four hours post-treatment, cells were lysed and 35 μg of total protein subjected to Western blot analyses using antibodies specific for MDM2 (mixture of monoclonal antibodies 2A10, 4B2, and 3F3), p53 (FL-393), or actin.

the DMBA treatment also led to activation of p53. In addition, when a time course treatment with 10 μM DMBA was performed, a clear up-regulation of these proteins was observed after 12 h (Fig. 6B). Since MCF-7 cells contain WT p53 protein, these data suggest that DMBA-mediated acute activation of MDM2 may be correlated with activation of p53. To investigate whether p53 plays a role in DMBA-induced up-regulation of MDM2, we studied the effect of DMBA on MDM2 expression in WT and p53 null ($p53^{-/-}$) MEF cells. As shown in Figure 6C, treatment with DMBA for 24 h resulted in significant up-regulation of p53 and MDM2 in WT MEFs, but not in $p53^{-/-}$ MEFs. These data suggest that DMBA activates p53, which in turn stimulates expression of MDM2.

## DISCUSSION

In this study, we showed that increased expression of cyclin D1 is associated with DMBA-induced mammary tumors, in agreement with a previous report [Jang et al., 2000]. Indeed, overexpression of cyclin D1 is characteristic of human breast cancer [Gillett et al., 1994, 1996; Barnes and Gillett, 1998]. Interestingly, treatment of rats with the carcinogen *N*-methyl-*N*-nitrosourea (NMU) also results in up-regulation of cyclin D1 in mammary tumors [Sgambato et al., 1995]. Thus, deregulation of cyclin D1 appears a critical step in breast cancer development including carcinogen-induced breast tumorigenesis.

We also showed that expression of MDM2 is increased in DMBA-induced mammary tumors. It has been noted that MDM2 is overexpressed in many human cancers, often in the tumors containing WT p53. Because MDM2 is a critical negative regulator of p53, the enhanced MDM2 expression represents an alternate strategy for abrogation of p53 function. Interestingly, analysis of the p53 status of DMBA-induced rat mammary tumors reveals rare mutations of p53 [Kito et al., 1996], suggesting that the alternate mechanisms of p53 inactivation are critical in this carcinogenesis model. This notion is supported by our observation that MDM2 expression is up-regulated in DMBA-induced rat mammary tumors. The mechanism of how DMBA induces MDM2 expression in rat mammary tissues is not clear. Since p53 is a transcriptional activator for MDM2, it is conceivable that the initial treatment with DMBA can lead to acute activation of MDM2 through the action of p53. This hypothesis is supported by the observation that DMBA affects MDM2 expression only in the WT MEF but not in the p53-null MEF cells. However, chronic elevation of MDM2 clearly requires additional mechanisms. Nevertheless, up-regulation of MDM2 may represent a critical mechanism for abrogation of p53 tumor suppressor function in DMBA-induced tumorigenesis.

In this study, we found elevated p27 protein expression in DMBA-induced rat mammary tumors in contrast to a previous report [Jang

et al., 2000]. In addition, we also observed significant cytoplasmic p27. Elevated expression and mislocalization of p27 in human breast cancers has been reported [Fredersdorf et al., 1997; Lloyd et al., 1997; Loda et al., 1997]. It has been shown that Her-2/neu activates Akt, which directly phosphorylates p27 at theronine 157, leading to cytoplasmic localization of p27 [Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002]. Interestingly, we also observed elevated expression of Akt in DMBA-induced rat mammary tumors. Thus, it is possible that the Akt signaling pathway is activated, which plays a role in deregulation and mislocalization of p27 during DMBA-induced mammary tumorigenesis.

We also observed elevated nuclear p27 in the mammary tumors, the biological significance of which is not yet clear. It has been shown that p27 protein is an important factor for cdk4/6 activity since it functions as an assembly factor for cyclin D/cdk4 and cyclin D/cdk6 holoenzyme complexes [Blain et al., 1997; LaBaer et al., 1997]. Indeed, cells lacking p27 exhibit reduced cyclin D-associated kinase activity [Cheng et al., 1999]. On the other hand, sequestration of p27 by cyclin D-cdk4/6 complexes effectively frees cdk2 from p27-mediated inhibition allowing both cdk4/6 and cdk2 to remain active. Considering the critical role of cyclin D1 overproduction in breast cancer, it is conceivable that expansion of proliferating cells in mammary tissue requires an elevated expression of p27 for proper assembly of cyclin D/cdk complexes. Consistent with this notion is the observation that cyclin D1 protein expression is elevated and that the cyclin-associated kinases responsible for Rb phosphorylation remain active in the rat mammary tumors. Interestingly, a significantly increased p27 and cyclin D1 expression have been observed in several highly proliferative human breast cancer cell lines and in low-grade primary breast tumors [Fredersdorf et al., 1997]. Moreover, overexpression of cyclin D1 leads to up-regulation of p27 in mouse mammary epithelial cells [Han et al., 1996]. Thus, up-regulation of p27 in DMBA-induced rat tumors may be due to the increased expression of cyclin D1, allowing for proper activation of cdk complexes and promoting cell cycle progression of the developing tumor.

Furthermore, while low p27 levels have been shown to desensitize tumor cells to apoptotic signals thereby protecting these cells from undergoing apoptosis [Park et al., 1999; Carneiro et al., 2003], increased expression of p27 has been shown to promote survival of normal and neoplastic cells upon cellular stresses [Han et al., 1996]. For example, up-regulation of p27 by adhesion leads to protection of cells from drug-induced apoptosis [St Croix et al., 1996; St Croix and Kerbel, 1997]. Overexpression of p27 enhances survival of neuronal cells during inflammatory injury response [Park et al., 1997; Ophascharoensuk et al., 1998], and blocks cytochrome c release in preventing caspase activation in leukemia cells [Eymin et al., 1999]. Moreover, $p27^{-/-}$ MEF cells exhibit increased sensitivity to apoptosis upon growth factor withdrawal [Hiromura et al., 1999], which can be rescued by restoration of p27 expression. It is therefore plausible that up-regulation of p27 may be involved in abnormal survival of mammary cells that is important in DMBA-induced mammary tumorigenesis. Thus, deregulation of p27 may play a complex role in the control of apoptosis and proliferation of mammary tumor cells, and the ultimate outcome may depend upon physiological conditions and availability of critical growth factors.

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