Differential Expression of Multiple MDM2 Messenger RNAs and Proteins in Normal and Tumorigenic Breast Epithelial Cells

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ABSTRACT
The MDM2 gene is a nuclear phosphoprotein that is regulated by p53 and functions, in one capacity, to inhibit the transcriptional activity of the wild-type p53 protein. Multiple MDM2 transcripts were detected in human breast epithelial cells. In estrogen receptor-negative normal, immortal, and tumorigenic breast epithelial cells, we found a good correlation between MDM2 mRNA levels and expression of wild-type p53. When wild-type p53 was overexpressed in estrogen receptor-negative tumor cells containing a mutant or no endogenous p53, MDM2 mRNA levels increased significantly, indicating that wild-type p53 positively influences MDM2 mRNA levels in these tumor cells. Because all estrogen receptor-positive breast tumor cells containing high MDM2 mRNA levels regardless of the status of their endogenous p53 protein, other factors likely influence MDM2 expression in these cells. Distinct MDM2 proteins (range, Mr = 54,000-68,000 and 90,000-100,000, respectively) were differentially expressed in human breast epithelial cells. The lower molecular weight MDM2 proteins were most abundant in the normal mammary cells but present at varying levels in many of the tumor cells examined. MDM2 was a nuclear protein; however, nuclear staining intensity did not always correlate with the amount of MDM2-immunoreactive protein as determined by Western blot analysis. This discrepancy suggests that MDM2 interacts with novel cellular proteins in different kinds of breast epithelial cells.

INTRODUCTION
The human homologue of the mouse MDM2 gene encodes a zinc finger-containing protein that is a putative transcription factor (1, 2). Overexpression of this gene in BALB/c 3T3 cells increases their tumorigenic potential, suggesting that the MDM2 protein possesses oncogenic activity. Further evidence that MDM2 plays a role in the process of cell transformation derives from experiments where this protein was shown to complex with and inhibit the transcriptional activity of wild-type p53 (3, 4). MDM2 interacts with the amino terminal acidic activation domain of the p53 protein, raising the possibility that MDM2 inhibits p53 function by disrupting its interaction with the general transcription machinery (5).

Recent evidence indicates that MDM2 is one of the downstream effector genes in cells that express a wild-type p53 protein (6, 7). The first intron of the MDM2 gene contains a p53 DNA binding site. This element confers wild-type p53 responsiveness to the endogenous MDM2 gene and to reporter genes when placed upstream in a heterologous promoter (7). More recent data suggest that this p53 response element may actually be part of an internal, cryptic p53-dependent promoter (8). When considered together, these observations suggest that regulation of p53 and MDM2 are intimately linked and that these two proteins may act in concert to control cell proliferation.

Our laboratory has been interested in regulatory mechanisms that play a role in the genesis of breast cancer. The importance of p53 in this process is underscored by the finding that ~40% of primary human breast tumors harbor mutations in the coding sequences of the p53 gene (9, 10). Moreover, recent studies indicate that expression of mutant p53 is an important prognostic marker for node-negative breast cancers (11, 12). Presumably, the remaining tumors utilize other mechanisms to bypass the negative regulatory effects of the wild-type p53 gene. One possibility is that these tumor cells can up-regulate the expression of MDM2 to a level sufficient for binding and titrating the effects of wild-type p53. Such a mechanism has been proposed to explain the overexpression of MDM2 in soft tissue sarcomas, osteosarcomas, and a subset of malignant gliomas (13-17). Recently, Chen et al. (18) reported that the amplification of MDM2 in osteosarcoma cells resulted in inhibition of wild-type p53-induced growth arrest following treatment with ionizing radiation. These results have provided important evidence demonstrating that MDM2 overexpression can affect p53 function in a known physiological pathway. To date, amplification and overexpression of MDM2 has not been shown to play a significant role in the genesis of human epithelial tumors (19-21).

To begin to understand the complex regulation between these two cellular proteins, we examined levels of p53 and MDM2 mRNA and protein in human breast epithelial cells. We determined the sizes of the mRNAs and proteins for MDM2 present in mammary epithelial cells at different stages of cell transformation. One goal of our study was to determine whether a relationship existed between p53 levels and corresponding levels of MDM2 in mammary epithelial cells that expressed wild-type versus mutant p53 proteins. Because MDM2 levels were high in ER⁺ breast cancer cells regardless of their p53
status, we examined the possible influence of estrogens on MDM2 gene expression. Finally, to determine the intracellular localization of these proteins, we performed immunocytochemical analyses of MDM2 and p53 proteins in representative breast epithelial cell lines.

MATERIALS AND METHODS

Cells and Cell Culture. NMECs derived from reduction mammaplasties were purchased from Clonetics Corp. (San Diego, CA) and the Corriel Institute (Camden, NJ). The immortalized breast epithelial cell line MCF-10 was generously provided by Dr. Sam Brooks, Michigan Cancer Foundation (22). Immortalized 184B5 and HBL100 cells were obtained from the ATCC. The ER+ cell line MCF-7 was obtained from Dr. R. Buick, University of Toronto (23) and the ER+ BT20T cells were provided by Dr. K. Keyomarsi, New York State Department of Health. Other ER+ cells including T47-D, ZR75-1, and BT474 and the ER+ tumor cell lines MDA-MB-157, MDA-MB-231, MD-MBA-453, MD-MBA-468, HS578T, and SKBr3 were all obtained from the ATCC.

NMECs and 184B5 cells were cultured in a mammary epithelial basal medium (Clonetics Corp.) supplemented with vitamins, 0.5% fetal bovine serum, 20 ng/ml EGF, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, and 52 µg/ml bovine pituitary extract (24). MCF-10 cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (GIBCO) supplemented with 2.5% horse serum, 10 mM HEPES, 2 mM glutamine, 0.1 mM nonessential amino acids, 20 ng/ml EGF, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, and 10 µg/ml transferrin. The ER+ and ER+ breast cancer cells were cultured in α-MEM (GIBCO) supplemented with 10 mM HEPES, 2 mM glutamine, 0.1 mM nonessential amino acids, 10% fetal bovine serum, 1 ng/ml EGF, and 2 µg/ml insulin with the exception of MDA-MB-468, SKBr3, and BT474 cells. These cells were grown in improved MEM without EGF and insulin was added to a final concentration of 5 µg/ml. All cells were routinely screened for contamination and maintained at 37°C in an atmosphere of 6.5% CO2.

Adenovirus Infection of Breast Tumor Cells. An Adenovirus vector containing a cDNA encoding the wild-type p53 gene (AdWTP53) was constructed according to established procedures.4 MDA-MB-157 and MDA-MB-231 cells were plated and allowed to grow for 1 or 2 days before infection with 10 plaque-forming units of AdWTP53 or the same virus without the p53 gene (AdCon). Cells were harvested for protein and RNA analyses 24 h after infection.

RNA Isolation and Northern Blot Analysis. Total RNA was prepared by lysing cell monolayers in guanidinium isothiocyanate and centrifuging over a 5.7 M CsCl cushion as described previously (25). RNA (20 µg) was electrophoresed on denaturing formaldehyde gels, transferred to MagnaNT membranes, and cross-linked with UV. The probes used for Northern blot analyses were obtained as follows. A 2.1-kilobase BamHI-XhoI fragment, a 750-bp BamHI-Xba I, and an 800-bp HindIII fragment were excised from different regions of the MDM2 cDNA (a generous gift from Dr. Bert Vogelstein; Ref 13). A 2.0-kilobase BamHI fragment from the human p53 cDNA was purchased from ATCC and an 800-bp PstI fragment was excised from p36B4 (26). All probes were labeled with [α-32P]dCTP or dATP to a specific activity of ~1 × 106 cpm/µg DNA using a random-primed labeling kit (Boehringer Mannheim).

Immunoprecipitation and Western Blot Analyses. For immunoprecipitation studies, cells were washed 3 times in ice-cold PBS and harvested in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.75% NP40, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 50 mM sodium fluoride, 10 mM sodium o-vanadate). Equal amounts of protein were subjected to immunoprecipitation by incubating with 1 µg IF2 monoclonal antibody (MDM2 antibody 1; Oncogene Science) or 3 µl culture supernatant from SB10-producing hybridoma cells (5). Specific complexes were collected following incubation with Protein A + G agarose (Oncogene Science). After washing three times in SNBTE buffer (5% sucrose; 0.5 mM NaCl; 1% NP-40; 50 mM Tris, pH 7.4; 5 mM EDTA) and twice with radioummunoprecipitation assay buffer, the precipitates were electrophoresed in 8% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed for MDM2 as described below.

For Western blot analyses cells were washed three times with ice-cold PBS, scraped into hot (80–90°C) Laemmli lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2 mM EDTA, 15% sucrose, 10% glycerol, 3% SDS, 0.7 M 2-mercaptoethanol) and the proteins denatured by boiling for 10 min. After centrifugation, protein lysate (50 µg/lane) was electrophoresed on 8 or 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The filters were blocked with Tris-buffered saline containing 8% dried milk and 0.1% Tween 20 and then probed with the respective antibody. MDM2 was detected using 2.5 µg/ml IF2 antibody (Oncogene Science), p53 was detected using 4 µg/ml PAbs 1801 and DO-1 antibodies (Oncogene Science) and actin was detected with 3 µg/ml actin antibody-1 (Oncogene Science). After washing in Tris-buffered saline containing 0.1% Tween 20, and subsequent incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (Bio-Rad), specific complexes were detected using a chemiluminescent technique according to the manufacturer’s recommendations (New England Nuclear).

Immunocytochemistry and Autoradiography. Cells were grown in tissue culture chamber slides, washed three times in ice-cold PBS, and immediately fixed in methanol:acetone (1:1) for 2 min. After allowing the slides to air dry, they were stained for MDM2 using the IF2 monoclonal antibody (MDM2 Ab-1; Oncogene Science) and PAb 1801 (Ab-2) (Oncogene Science) for detection of p53. The MDM2 and p53 antibodies were diluted to a final concentration of 4.5 µg/ml or 1 µg/ml, respectively. After washing in Tris-buffered saline containing 0.05% Tween 20, the specific complexes were visualized using the avidin-biotin complex immunoperoxidase system (Vector Laboratories, Burlingame, CA) according to the manufacturer’s recommendations.

RESULTS

MDM2 mRNA Expression in Human Mammary Epithelial Cells. In our initial experiments we sought to determine whether there were any differences in the expression of

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**Fig. 1** Northern blot analysis of MDM2 and p53 RNA in human breast epithelial cells. The p53 status of the cells was obtained from several sources and, if known, is indicated in parentheses (30, 31, 33, 36). RNA was isolated from three independently derived normal human mammary epithelial cells HMEC 1–3 (WT), immortalized, nontumorigenic HBL100, MCF-10 (WT) and 184B5 (WT) cells, estrogen receptor-positive MCF-7 (WT), ZR75–1 (WT), T47-D (codon 194 mutation) and BT-474 (codon 285 mutation) tumor cells and estrogen receptor-negative MDA-MB-157 (null), MDA-MB-231 (codon 280 mutation), MDA-MB-468 (codon 273 mutation), SKBr-3 (codon 175 mutation), HSS78T (codon 157 mutation) breast cancer cells, electrophoresed, transferred, and hybridized with probes for the MDM2, p53, and 36B4 genes, respectively. The lane designated SJSA-1 contains RNA from an osteosarcoma line that has an amplification of the MDM2 gene. Arrows to the left of the top panel, sizes of the MDM2 transcripts present in these cells. Single panel to the right, a shorter exposure of the lane containing RNA from the SJSA-1 cell line.

MDM2 in normal and immortal versus transformed human mammary epithelial cells. For these experiments total RNA was isolated from exponentially growing populations of NMECs derived from reduction mammoplasties, immortalized but nontumorigenic mammary epithelial cells and estrogen receptor-positive and -negative breast cancer cell lines. Normal and immortalized, nontumorigenic breast cells grown in culture do not express the estrogen receptor. This observation likely reflects the biology of mammary epithelial cells as very few cells are ER⁺ in normal nonlactating mammary glands (27, 28). To ascertain the estrogen receptor status of the various cells, we probed the filter for ER mRNA (Fig. 1, Panel 2). As expected, only the ER⁺ breast tumor cell lines expressed detectable levels of estrogen receptor mRNA.

Relatively high levels of MDM2 mRNA were present in all three independently derived ER⁺, NMEC strains (Lanes 1–3). The amount of MDM2 mRNA detected in spontaneously arising MCF10 (Lane 5) and carcinogen-induced 184B5 (Lane 6) ER⁻, immortalized, nontumorigenic cells was similar to the amount present in NMECs. In contrast, HBL100 mammary epithelial cells, that were immortalized by SV40 large T antigen, expressed a much lower level of MDM2 mRNA (Lane 4). This is an important observation as SV40 T antigen binds to and inactivates the function of wild-type p53. The four estrogen receptor positive breast cancer cell lines MCF-7, ZR75-1, T47-D and BT-474 (Lanes 7–10) expressed similar MDM2 mRNA levels to those found in normal mammary epithelial cells. In contrast, the five estrogen receptor negative breast cancer cell lines examined, MDA-MB-157, MDA-MB-231, MDA-MB-468, SKBr-3, and HSS78T, all expressed MDM2 RNA levels that were 5 to 10 fold lower than in any of the other cells analyzed (Lanes 11–15). Although all human mammary epithelial cells expressed readily detectable MDM2 transcripts, the abundance of this mRNA was well below the amount present in the overexpressing human osteosarcoma cell line, SJSA-1 (16).

MDM2 mRNA having several different molecular weights were detected in our panel of mammary epithelial cells. To determine the sizes of the various transcripts, identical
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Fig. 2  Immunoprecipitation of MDM2 protein from human breast epithelial cells. Protein extracts were prepared from representative breast epithelial cells and immunoprecipitation was performed by incubating 2 mg protein extract with the IF2 (Oncogene Science) or 5B10 antibody at 4°C. Specific protein complexes were separated in an 8% SDS-polyacrylamide gel, electrophoretically transferred to a nitrocellulose membrane, and subsequently probed for MDM2 using the IF2 antibody. Numbers, migration of prestained molecular weight markers that were coelectrophoresed and transferred with the samples. The lane labeled Control contains 2 mg protein extract from MCF-7 cells precipitated with an isotypic nonspecific antibody. Arrows, positions and intensities of the MDM2 proteins.

amounts of RNA standards were coelectrophoresed and their migration was compared with MDM2 mRNAs from mammary epithelial and SJSA-1 osteosarcoma cells. The major form of MDM2 mRNA in human mammary epithelial cells migrated as a 6.7-kilobase mRNA. We often saw higher molecular weight transcripts of ~8.0 and 12.5 kilobase in normal and immortal mammary epithelial cells but rarely in tumorigenic mammary epithelial cells. In the MDM2 overexpressing SJSA-1 cells, another band of ~4.5 kilobase could be readily detected. This band was detected occasionally in RNA samples from all mammary epithelial cells.

Since MDM2 is known to lie downstream of p53 in a nuclear signal transduction pathway (6, 7), we examined the relationship between p53 and MDM2 mRNA levels in the same mammary epithelial cells. When the filter shown in the upper panel was stripped and hybridized with a probe for p53, most of the cell lines showed readily detectable levels of p53 mRNA regardless of their state of transformation. The two exceptions were ZR75-1 cells which expressed significantly lower levels of p53 mRNA and MDA-MB-157 cells which had no detectable p53 mRNA at all. The different patterns of p53 and MDM2 mRNA expression observed among the mammary epithelial cells could not be attributed to unequal loading of the lanes as all cells expressed similar levels of the control p36B4 mRNA.

MDM2 Protein Levels in Mammary Epithelial Cells. MDM2 is expressed as a series of phosphoproteins ranging in molecular weight from 55,000 to 90,000 in murine 3T3DM cells that contain multiple copies of the MDM2 gene (1, 29). To determine the molecular weight(s) of the MDM2 proteins expressed in human mammary epithelial cells, immunoprecipitation studies were performed using the IF2 and 5B10 antibodies which recognize epitopes in the amino and carboxy terminal regions of MDM2, respectively (5, 14). The results presented in Fig. 2 demonstrate that the IF2 antibody recognized at least four distinct forms of MDM2 in breast epithelial cells. The M, 90,000 form of MDM2 was the predominant protein present in most of the cell lines examined. Two additional MDM2 proteins with molecular weights of around 75,000 and 85,000 were detected at lower levels from all mammary epithelial and SJSA-1 osteosarcoma cells. In the normal and immortalized 184B5 cells, a higher M, 95,000 MDM2 protein was also detected.

Immunoprecipitations performed with the 5B10 antibody, which recognizes an epitope in the carboxy terminus of MDM2, yielded slightly different results. This antibody efficiently precipitated the $M_1$, 90,000 and 95,000, and, to a lesser extent, the $M_2$, 75,000 forms of MDM2. The relative differences in intensities of the various bands likely reflect bona fide differences in protein levels as equivalent amounts of actin were detected from each sample (data not presented). As a control for these experiments we demonstrate that numerous protein bands with molecular weights ranging from 75,000 to 90,000 are also precipitated from SJSA-1 cells that contain an amplified MDM2 gene.

The level of many cell cycle-regulated proteins does not always correlate with the abundance of their mRNAs. To determine quantitative differences in the levels of MDM2 protein in our panel of mammary epithelial cells, we prepared total cell protein extracts and performed Western blot analyses. The predominant form of MDM2 detectable by Western blot was the M, 90,000 protein (Fig. 3). We found a good correlation between the level of MDM2 mRNA and the amount of the M, 90,000 MDM2 protein in the different cells. The normal (Fig. 3, Lanes 1 and 2), immortal, noninvasive (Fig. 3, Lanes 3 and 4) and the ER+ breast cancer cells (Fig. 3, Lanes 5–8) all had higher levels of the M, 90,000 MDM2 protein than the ER- breast cancer cells (Fig. 3, Lanes 9–13). An additional MDM2 protein having a $M_3$, ~57,000 was present in the SJSA-1 osteosarcoma cells and many of the breast epithelial cells examined. This band was not detectable in the immunoblotting experiments because it comigrates on gels with the mouse monoclonal antibodies used for immunoprecipitation. The bands corresponding to the major MDM2 proteins detected in the SJSA-1 osteosarcoma line are indicated (Fig. 3, arrows on the right). To ensure that the differences in protein levels detected in the various mammary epithelial cells were not due to unequal loading and transfer of proteins, the lower portion of the same membrane was subsequently probed for actin (Fig. 3, bottom).

Several groups have described a binding site for wild-type p53 within the first intron of the MDM2 gene (7, 8). This DNA sequence is apparently involved in the regulation of MDM2 gene transcription by wild-type p53. Thus, in the presence of wild-type p53, MDM2 mRNA and protein levels were concomitantly increased presumably due to the influence of wild-type
A more critical consideration in these comparisons is whether the p53 expressed in the various cells is mutant or wild type. Normal and immortalized, nontumorigenic breast cells grown in culture do not express the estrogen receptor (Fig. 1). This finding likely reflects the biology of mammary epithelial cells since very few cells are ER in normal nonlactating mammary glands (27, 28). The normal and immortal 184B5 mammary epithelial cells used in these studies express a wild-type p53 protein that has increased stability (30–32). The p53 gene in the immortalized MCF10 cells has been sequenced along its entirety and shown to be wild type. All of these cells expressed relatively high levels of MDM2 protein. In contrast, the ER− tumor cells (Lanes 10–14) expressed either a mutant or no p53 and had relatively low levels of MDM2 mRNA and protein (33). Thus, when examining ER− mammary epithelial cells, we found a good correlation between wild-type p53 expression and corresponding levels of MDM2 mRNA and the M, 90,000 MDM2 protein.

The same positive correlation between wild-type p53 and MDM2 protein expression did not hold true for the ER+ breast cancer cells. All of the ER+ breast cancer cells expressed relatively high levels of full length MDM2 protein when compared to the ER− cells. MCF-7 and ZR75−1 cells express a wild-type p53 protein (34, 35); however, T47-D and BT-474 cells both express mutant p53 (33, 36). Thus, factors other than p53 are likely responsible for the high levels of MDM2 mRNA and protein in ER+ breast tumor cells. Among the various cell types examined, MDM2 mRNA levels showed proportionally greater variations than did MDM2 protein levels. These findings suggest additional regulation of MDM2 protein accumulation at some posttranscriptional level in human breast epithelial cells.

Wild-Type p53 Induces MDM2 Expression in ER− Tumor Cells. To obtain independent evidence that wild-type p53 influences MDM2 expression in human breast tumor cells, two ER+ tumor cell lines, MDA-MB-157 and MDA-231, were infected with an adenovirus vector carrying a wild-type p53 gene at 10 plaque-forming units/cell. The relative levels of wild-type p53 protein present in the cells 24 h after infection are shown in Fig. 4A. We observed no difference in p53 expression between uninfected cells (Fig. 4A, Lanes 1 and 4) and cells

F. Diella, personal communication.
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MDM2 mRNA levels were examined from cells independently infected with the same viral constructs (Fig. 4B). MDM2 mRNA levels were relatively low in the uninfected cells (Fig. 4B, Lanes 1 and 4), and in the cells infected with a control virus (Fig. 4B, Lanes 2 and 5). MDA-MB-157 and MDA-MB-231 cells expressing a wild-type p53 protein showed 6- and 10-fold increases in MDM2 mRNA levels (Fig. 4B, Lanes 3 and 6). These increases in MDM2 mRNA levels are in accordance with the finding of slightly higher levels of wild-type p53 protein in the MDA-MB-231 cells. When considered together, these data support the idea that wild-type p53 positively influences MDM2 mRNA expression in ER" breast tumor cells that express mutant or no endogenous p53 protein.

Effect of Estrogen on MDM2 Expression. Because all of the ER" tumor lines contained high levels of MDM2 protein, regardless of the status of the p53 gene, we examined whether estrogen could influence MDM2 expression in these cells. MCF-7 and ZR75-1 cells were grown in phenol red-free medium containing charcoal-stripped serum for 5 days. These conditions have been shown previously to slow the growth of the cells and down-modulate the expression of estrogen-dependent genes (37, 38). After scanning the autoradiograms and correcting for slight loading differences, a 2-fold increase in MDM2 mRNA levels was observed in MCF-7 cells after refedding the cells with estrogen-containing medium for 4 to 8 h (Fig. 5A). The slight increase in MDM2 mRNA detected in ZR75-1 cells at 4 and 8 h after estrogen repletion could be attributed to minor loading differences as the level of the control mRNA, 36B4, was also increased slightly in these samples (bottom, Fig. 5B). We examined MDM2 protein levels in the same samples and found that they were not affected by estrogen levels in the culture medium. In contrast to the minor changes observed in MDM2 mRNA levels, the estrogen inducible gene, p52, was down-regulated in estrogen-depleted cells and increased ~20-fold 4 to 8 h after the addition of 3H-estriadiol to the culture medium (middle). When considered together, these data suggest that estrogen levels may not directly influence MDM2 mRNA expression in cultured breast epithelial cells.

Immunocytochemical Localization of MDM2 and p53 in Human Mammary Epithelial Cells. To determine the intracellular location of the MDM2 protein, we stained representative breast cell lines using the IF2 antibody. After evaluating different fixatives, methanol:acetone (1:1) was found to yield the most reproducible staining results. All of the mammary epithelial cells studied contained clearly detectable MDM2 protein using Western blot analysis (Fig. 3); however, considerable variation in immunocytochemical staining patterns was observed. The NMECs and immortalized 184B5 mammary epithelial cells showed distinct nuclear staining for MDM2 with some variation in intensity from one cell to another (Fig. 6A). When the same cells were stained for p53 using the PAb 1801 antibody, a similar pattern of heterogenous nuclear staining was observed. In contrast another immortalized, mammary epithelial cell line MCF-10 showed less intense and more variable staining for both MDM2 and p53 proteins. The decreased staining intensity observed in these cells may be due to the slightly lower levels of protein accumulation as determined by Western blotting (Fig. 3).

The estrogen receptor-positive breast epithelial lines all contained relatively high levels of MDM2 protein when examined using Western blots (Fig. 3). However, when analyzed immunocytochemically, the ER" tumor cells stained poorly, if at all, for MDM2. In MCF-7 cells the small amount of perinuclear staining obtained with the MDM2 antibody is likely nonspecific because we see similar results using isotypic monoclonal antibodies for other cellular proteins that are not expressed specified.

J. M. Gudas, unpublished data.

Fig. 4 A, infection of ER" tumor cells with an adenovirus vector containing a wild-type gene. Protein lysate prepared from exponentially growing MDA-MB-157 and MDA-MB-231 ER" tumor cells (Lanes 1 and 4), cells infected with a control adenovirus vector (Lanes 2 and 5), and cells infected with AdWTpS3 virus (Lanes 3 and 6) was examined for p53 expression using Western blot analysis. B, RNA was isolated from cells treated as described in the legend to A and examined for MDM2 expression using Northern blot analysis. Lower panel, 36B4 expression from the same filter.
in these cells. Very few nuclei from ER+ ZR75–1 cells stained for MDM2 protein and some cells may show some cytoplasmic localization. Interestingly, staining of the ZR75–1 cells for p53 revealed a distinct cytoplasmic staining that was highly localized within discrete foci. To our knowledge, a similar staining pattern for p53 has not been described in other cell types.

The ER−, MDA-MB-231 breast cancer cells had very low levels of MDM2 when examined using Western blot analysis (Fig. 3) and did not stain at all for MDM2. Two other ER− breast cancer cell lines that expressed very low levels of MDM2 protein by immunoprecipitation and Western blotting, MDA-MB-157 and MDA-MB-453, were also negative for MDM2 when examined immunocytochemically (data not shown). We believe that the level of MDM2 protein may be below the detection limit by immunocytochemistry in the ER− breast cancer cells. As expected for cells that express a mutant p53, all nuclei in MDA-MB-231 cells stained intensely for p53. As controls for the immunocytochemistry, we show that SJSA-1 cells, which overexpress MDM2, stained intensely for this antigen while very few nuclei from these cells were positive for p53 (Fig. 6).

Discussion

In this study we report two independent observations concerning MDM2 expression in human breast epithelial cells. The first finding is that a good correlation exists between expression of wild-type p53 in normal and immortalized mammary epithelial cells with accumulation of relatively high levels of MDM2 mRNA and protein in the same cells. Because the magnitude of the difference in relative MDM2 mRNA levels was greater than that observed for corresponding MDM2 protein levels, other factors, including protein stability and translational efficiency, likely play a role in determining the total amount of MDM2 protein in a cell. The normal and immortalized human mammary epithelial cells used for these studies are estrogen receptor negative and express a wild-type p53 protein. The wild-type p53 is unusual in these cells because it has a half-life of ~3 h (30) compared to a half-life of ~20 min in many other cell types (30, 39, 40).

In contrast, all of the estrogen receptor-negative breast tumor cell lines studied had either a mutant or no p53 protein and expressed significantly less MDM2 mRNA and protein. Because the ER− cell lines we examined all had mutations in the conserved DNA binding domain of the protein (41), they are all likely defective in their ability to transactivate cellular target genes. Previous studies from other laboratories have demonstrated a consensus p53 DNA binding site in the MDM2 gene which is necessary for regulation by wild-type p53 (6, 7). To determine whether wild-type p53 could, indeed, affect MDM2 expression, we introduced a wild-type p53 gene into ER− breast tumor cells that contained a mutant or no p53 protein. In both cell types wild-type p53 expression resulted in an increase in MDM2 mRNA levels. These data suggest that wild-type p53 functions to increase the expression of MDM2 mRNA and protein in estrogen receptor-negative breast epithelial cells and this regulatory circuit becomes disrupted when the cells progress and gain mutations in the p53 gene. ER− breast tumors are usually more differentiated than ER− tumor cells (42, 43). Because MDM2 mRNA and protein levels are lower in ER− breast cancer cells, our data suggest a diminution rather than an increase in MDM2 expression during the progression of mammary tumors.

The second major finding from this study is that MDM2 mRNA and protein levels are significantly higher in ER− breast...
Fig. 6  A, immunocytochemical localization of MDM2 and p53 proteins in normal and immortal human breast epithelial cells. Cells were grown on glass tissue culture chamber slides, fixed in methanol-acetone (1:1), and subsequently stained for MDM2 and p53 using the 1F2 or PAb 1801 antibodies, respectively. After washing, specific complexes were detected using the avidin-biotin complex immunoperoxidase detection system as recommended by the manufacturer (Vectastain). B, MDM2 and p53 immunostaining in tumorigenic breast epithelial cells.

Because this phenotype was only observed in the ER+ breast cancer cells, we asked whether some function or genes regulated by the estrogen receptor could be involved in the increased expression of MDM2 in these cells. When ER+ cells were cultured in estrogen-depleted medium, the mRNA for the estrogen-regulated gene, pS2, was decreased and increased significantly on refeeding the cells with medium containing β-estradiol. Under the same conditions only a 2-fold change in MDM2 mRNA levels in MCF-7 cells and no change in ZR75–1 cells was detected. Therefore, the elevated levels of MDM2 mRNA and protein in these cells may not involve a direct effect of the estrogen receptor itself. Many other genes including glutathione-S-transferase π and glutathione peroxidase are differentially expressed in ER+ versus ER- breast cancer cells (44–46). Presumably, differences in the posttranscriptional processing of these mRNAs differences and/or differences in their chromatin structure accounts for preferential mRNA expression in one cell type versus another. These results are similar but not identical to those reported by Saeed et al. (47) who found that estrogen increased MDM2 expression in ER- cells transfected with a gene encoding the estrogen receptor. Because the estrogen receptor does not always induce estrogen-dependent promoters when introduced into ER- cells (48), the two results are not directly comparable.
Several different MDM2 transcripts, ranging in size from 4.5 to 12.5 kilobases, were detected in mammary epithelial cells. At present we do not know if these various mRNA species have functional significance or if some of the higher molecular weight transcripts represent partially or alternatively processed MDM2 mRNAs. Interestingly, evidence from DNA sequencing studies indicates that both the human and mouse MDM2 mRNAs can be alternatively spliced (1, 49). Because all mRNA bands hybridized with probes obtained from both the 5’ and 3’ end of the coding region of the human MDM2 cDNA, we believe that they are all bona fide MDM2 transcripts.

Results from immunoprecipitation and Western blot analyses showed a complex pattern of many different MDM2 proteins in human breast epithelial cells. The predominant protein detected from all mammary epithelial cells migrated as a Mr 90,000 band. This form likely represents the full-length MDM2 protein while the smaller, ~57,000 form, may be an alternatively spliced variant as described in mouse and rat cells (1, 29). Haines et al. (29) have recently reported that the different molecular weights of the MDM2 proteins in mouse cells represent alternatively spliced variants. Moreover, they have demonstrated that these proteins differ in their ability to physically associate with, and thus inhibit, the function of wild-type p53 (29). When considered along with the finding of multiple mRNA species, we believe that the different forms of MDM2 present in breast epithelial cells likely represent distinct MDM2 proteins that may have both overlapping and unique biological functions.

Immunocytochemical staining for MDM2 showed that the protein was nuclear in breast epithelial cells; however, the intensity and number of positive cells did not always correlate with the level of protein detectable by biochemical methods. Some of the variation in nuclear MDM2 staining seen in the NMECs and 184BS cells may reflect differences in cell cycle distribution as we have previously shown that these cells stain variably for p53, depending upon their position in the cell cycle (32). ER” MCF-7, ZR75–1, and BT474 (data not shown) cells showed little or no staining for MDM2 although they had comparable amounts of the Mr 90,000 MDM2 when assayed using Western blot analysis (Fig. 3). One possible explanation for the anomalous immunocytochemical results in ER” cells is that MDM2 is somehow sequestered in a larger complex that is only dissociated on lysis of the cells. We are presently performing more detailed experiments to test this hypothesis. With the exception of a unique cytoplasmic focal staining pattern for p53 observed in ZR75–1 cells, our results for p53 staining in breast epithelial cells are similar to those reported by other investigators (30, 33, 50).

Increasing evidence suggests that p53 and its downstream effector proteins MDM2 and CIP/Waf-1 play a role in determining cellular responses to endogenous and exogenous signals. The differential expression of multiple MDM2 mRNAs and proteins raises the possibility that these products may perform distinct functions in mammary epithelial cells at different stages of tumor development. We believe that further study of p53 and its downstream effector proteins MDM2 and CIP-1 (51, 52) should enhance our understanding of the processes of cell transformation and the means by which cells modulate their response to DNA damage and environmental stress. A better understanding of these cellular processes should in turn translate into more specific and, hopefully, more effective therapeutic approaches for the treatment of breast cancers.

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Differential expression of multiple MDM2 messenger RNAs and proteins in normal and tumorigenic breast epithelial cells.


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