Activation of Murine Double Minute 2 by Akt in Mammary Epithelium Delays Mammary Involvement and Accelerates Mammary Tumorigenesis

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Abstract

Amplification or overexpression of murine double minute 2 (MDM2) promotes a variety of human tumors by degrading tumor suppressor proteins such as p53. Phosphorylation of MDM2 on Ser166 and Ser186 by the survival kinase Akt inhibits p53-mediated apoptosis. However, it is unclear whether this pathway contributes to normal or malignant pathophysiology in vivo. To address these questions, we generated transgenic mice expressing the Akt-phosphorylated form of MDM2 (MDM2DD(S166D/S186D)) in the mammary epithelium. Activation of MDM2 delayed mammary gland involution and accelerated tumor progression in mouse mammary tumor virus/neu transgenic mice by inhibiting apoptosis in a manner associated with decreased p53 expression. Our findings offer in vivo evidence that activation of MDM2 by Akt contributes to mammary development and tumorigenesis. Cancer Res; 70(19); OF1–6. ©2010 AACR.

Introduction

Amplification or overexpression of murine double minute 2 (MDM2) is associated with a variety of human tumors. MDM2 was first identified as a negative regulator of p53, a well-known tumor suppressor which prevents tumorigenesis in response to stressors and oncogenic factors (1, 2). MDM2 is known to bind to the transactivation domain of p53 and inhibit its transactivity. As a ring finger–containing E3 ubiquitin ligease, MDM2 could also promote p53 degradation through a ubiquitin-mediated proteolytic process (3).

The serine/threonine protein kinase Akt, also known as protein kinase B, plays a critical role in tumorigenesis by regulating a variety of cellular processes, including preventing cells from undergoing apoptosis (4, 5). The importance of Akt in promoting cell survival was shown by a study that targeted the expression of constitutively activated Akt to the mammary epithelium of transgenic mice, significantly delaying mammary involution with impaired apoptosis (6). A number of Akt/protein kinase B downstream targets have been identified and were associated with apoptotic functions, such as NF-κB (7), Bad (8), Forkhead transcription factors (9, 10), and p21Cip1/WAF1 (11). In addition, activated Akt could inhibit p53-mediated apoptosis through phosphorylation of its downstream target MDM2 on Ser166 and Ser186 (12–14). The phosphorylation of MDM2 stimulates the translocation of MDM2 to the nucleus to bind to p53, and then targets p53 for degradation by the proteosome (13, 14). Activated Akt was also reported to phosphorylate MDM2 at Ser188, although its function is unclear (15).

However, the Akt/MDM2/p53 pathway was largely studied in the in vitro culture system. To directly study the role of Akt-activated MDM2 in mammary development and tumorigenesis, we generated transgenic mice expressing the Akt-phosphorylated form of MDM2 (MDM2DD(S166D/S186D)) in the mammary epithelium. Activation of MDM2 delayed mammary gland involution and accelerated tumor progression in mouse mammary tumor virus (MMTV)/neu transgenic mice by inhibiting apoptosis in a manner associated with decreased p53 expression. Our findings offer in vivo evidence that activation of MDM2 by Akt contributes to mammary development and tumorigenesis.

Materials and Methods

Generation of transgenics

The cDNA encoding MDM2 and MDM2DD were subcloned, respectively, from PCDNA3 vector into the p206 vector (6, 14). Generation of transgenic mice was described previously (6). The genotypes of transgenic mice were identified by transgene-specific PCR and further confirmed by Southern blot analysis described previously (6).

Primary antibodies

Primary antibodies used included rabbit polyclonal anti-MDM2 (N-20; Santa Cruz Biotechnology, Inc.), rabbit...
Polyclonal anti-p53 (FL-393; Santa Cruz Biotechnology), anti-p53 antibody (FL-393; Santa Cruz Biotechnology), anti-p21 antibody (ab7960; Abcam), and anti-FOXO3a antibody (H-144; Santa Cruz Biotechnology).

Isolation of primary mammary epithelial cells
Primary mammary epithelial cells (MEC) were isolated from FBV/n and MMTV/MDM2DD mammary glands by digestion with 2 mg/mL of collagenase and 100 units/mL of hyaluronidase (Sigma) for 3 hours. The cells were incubated with 2% fetal bovine serum DMEM in a Matrigel-coated plate.

Results and Discussion

Generation of transgenic mice
To study the role of Akt-activated MDM2 in mammary development and tumorigenesis, we generated transgenic mice expressing MDM2 or the Akt-phosphorylated form of MDM2 (MDM2DD) in the mammary epithelium. MDM2DD mimics the active phosphorylated state of MDM2 by Akt, which was generated by substituting both the serine residues at 166 and 186 with aspartic acid. Full-length cDNA of MDM2 or MDM2DD were placed under the MMTV promoter (Fig. 1A). Genotypes of transgenic mice were identified by transgene-specific PCR (data not shown). Transgene expressions were examined at the RNA level by quantitative reverse transcription-PCR (Fig. 1B and C) and at the protein level by immunohistochemical staining (Fig. 1D–I). The transgenes were uniformly expressed in the mammary epithelium, with the expression of MDM2DD predominantly in the nucleus and the expression of MDM2 in both the cytoplasm and the nucleus, although very little MDM2 was expressed in FVB/n control mice. The results were consistent with previous studies which indicated that AKT-mediated MDM2 Ser\textsuperscript{166} and Ser\textsuperscript{168}. 

![Figure 1. Generation of transgenic mice. A, structure of the transgene. B and C, quantitative reverse transcription-PCR analysis of MDM2DD transcript expressions in MMTV/MDM2DD (B) and MMTV/MDM2 (C) strains. D–I, immunohistochemical analysis of MDM2 expressions on pregnant 7 d mammary glands from MMTV/MDM2DD (D and E), MMTV/MDM2 (F and G), and FVB/n (H and I) mice.](image)
phosphorylations were critical for the translocation of MDM2 to the nucleus (14).

**MMTV/MDM2DD mice exhibited delayed mammary involution**

To determine the contribution of MDM2 and MDM2DD to mammary gland development, we observed the morphologic changes by whole mount and histologic analysis, and checked the functional differentiations by β-casein RNA expressions on virgin, pregnant, and lactating mammary glands. The results suggested that overexpression of MDM2 or MDM2DD had no effect on puberty, pregnancy, and lactation of mammary glands (Supplementary Figs. S1 and S2). Interestingly, the involution process was affected. One day postweaning, mammary glands from both MMTV/MDM2 and MMTV/MDM2DD mice showed no difference compared with those from FVB/n mice (Fig. 2A, B, and C). The alveoli were expanded and surrounded with single-layered epithelial cells. Three days postweaning, the FVB/n mice showed that the majority of lobuloalveolar structures were collapsed. In addition, ducts began to appear and fat cells were obvious (Fig. 2D). Mammary glands from MMTV/MDM2 mice

![Figure 2. MMTV/MDM2DD mice showed delayed mammary involution. A–F, histologic patterns on involution 1 d and 3 d mammary glands from FVB/n (A and D), MMTV/MDM2DD (B and E), and MMTV/MDM2 (C and F) mice. G and H, TUNEL analysis on involution 3 d mammary glands from FVB/n (E) and MMTV/MDM2DD (G) mice. Arrows, apoptotic cells. I and J, immunohistochemical analysis of p53 expression on mammary glands from FVB/n (I) and MMTV/MDM2DD (J) mice. K, apoptotic indices of FVB/n and MDM2DD mice at involution 3 d. L, immunoblot analysis of p53 expression on mammary glands from FVB/n and MDM2DD mice. M, quantitative reverse transcription-PCR analysis of Tweak transcript expression in mammary glands from MMTV/MDM2DD and FVB/n mice. O, cell number counting on MECs from MMTV/MDM2DD and FVB/n mammary glands with etoposide (25 μg/mL) treatment for 3 d. Re-expressing of p53 was achieved by p53-containing lentivirus infection for 2 d.](https://cancerres.aacrjournals.org/content/70/19/6831/F1.large.jpg)
showed similar morphologies as the control mice (Fig. 2F), whereas MMTV/MDM2DD mice showed delayed involution progression with alveoli still expanding and surrounded with single-layered epithelial cells (Fig. 2E). These observations indicated that the expression of MDM2DD delayed mammary gland involution in transgenic mice.

MMTV/MDM2DD mice showed decreased epithelial apoptosis and p53 expression

Because apoptosis is the major characteristic during mammary involution, TUNEL assay was performed to assess whether the delayed mammary involution in MMTV/MDM2DD mice was associated with defective apoptosis. Significantly less apoptosis was found in MMTV/MDM2DD glands (10.7%) as compared with FVB/n glands (26%; Fig. 2G, H, and K). Previous studies showed that Tweak, LIF, and TGFβ3 expression was increased during mammary gland involution, which could induce apoptosis (16), we therefore compared their RNA expression in MDM2DD mice with FVB/n mice and found that the increased RNA expression of these genes in FVB/n mice during involution were inhibited in MDM2DD mice (Fig. 2M; Supplementary Fig. S3A and B). These observations suggested that MDM2DD slows mammary gland involution by inhibiting apoptosis, which corresponded with the decreased expressions of genes known to be involved in involution.

An earlier study showed that Akt-activated MDM2 has an increased ability to degrade p53, we then examined whether defective apoptosis in mammary glands from MMTV/MDM2DD mice was dependent on the change of p53 expression. We first examined the expression of p53 on mammary glands by immunohistochemical staining and immunoblotting and found that there was less p53 expression in MMTV/MDM2DD mice at involution 3 days compared with that in the FVB/n mice (Fig. 2l, J, and L). Next, we treated primary MECs isolated from MMTV/MDM2DD and FVB/n mice with etoposide and found that there was significantly less cell death in MDM2DD-expressing MECs compared with those from FVB/n mice (P < 0.01). Re-expressing p53 in MDM2DD-expressing MECs increased the cell death to similar levels of those from p53-expressing wild-type MECs (Fig. 2O). These results suggested that the impaired apoptosis during mammary gland involution in MDM2DD mice was dependent on the decreased expression of p53.

Although the functionality of MDM2 is not only dependent on its ability to degrade p53, MDM2 becomes more closely related to p53 function after it is activated by Akt (14). This close regulation was proved in the current study in which the expression of MDM2DD in transgenic mice delayed mammary gland involution, which was related to decreased expression of p53 (Fig. 2J, L, and O). It was also reported that transgenic mice expressing myr-Akt1 in the salivary glands showed a significant increase in MDM2 phosphorylation and a reduction in apoptosis and p53 expression after gamma irradiation (17). These results indicate that Akt-activated MDM2 is critical in suppressing p53-dependent apoptosis in vivo.

A previous study showed that MDM2 transgenic mice driven by β-lactoglobulin promoter (BLG/MDM2) displayed lactation defects during mammary gland development which was independent of p53 expression (18). There are two reasons accounting for the differences with regard to the effect of MDM2 to the mammary gland development shown in these two studies. First, the hormone regulations on these two promoters are different, the MDM2 expression level and expression duration are different in these two transgenic mice. Second, these two transgenic mice were generated in two different genomic backgrounds (BLG/MDM2 in C57B1/6J and MMTV/MDM2 in FVB/n), which has been shown to have an effect on the phenotype of mice.

Coexpression of MDM2DD and neu results in accelerated tumor formation

To explore the roles of MDM2 and MDM2DD in mammary tumorigenesis, cohorts of virgin female MMTV/MDM2 and
MMTV/MDM2DD mice were monitored for tumor formation. None of these two groups developed mammary tumors after a year of observation.

We next examined whether MDM2DD expression could collaborate with other oncogenes in promoting mammary tumorigenesis. We generated bitransgenic mice, coexpressing either MDM2 or MDM2DD, with activated neu in the mammary epithelium by interbreeding MMTV/neu mice with MMTV/MDM2 and MMTV/MDM2DD mice, respectively. Cohorts of virgin female bitransgenics and MMTV/neu mice were monitored for tumor formation. The results revealed that expression of MDM2DD accelerated tumor formation in MMTV/neu mice (Fig. 3A). Fifty percent of the bitransgenic mice showed tumor formation at 165 days (n = 27) as compared with 179 days (n = 32) from MMTV/neu mice (P < 0.01). However, expression of MDM2 did not accelerate tumor formation in MMTV/neu mice (Fig. 3B).

To confirm that the accelerated tumor formation in MMTV/neu/MDM2DD bitransgenic mice was due to transgene expression, immunohistochemical staining was performed on mammary tumors. The results revealed that expressions of MDM2 were detected in bitransgenic mammary tumors (Fig. 4D, E, and F). Nuclear localization of MDM2 is important for its function in accelerating tumorigenesis because many MDM2-targeted tumor suppressor genes are predominantly localized in the nucleus. Our data showed that although MMTV/neu could promote nuclear localization of MDM2 to some extent, the amount of nuclear localized MDM2 was still lower in MDM2DD-expressing tumors than in MDM2DD-expressing tumors (Fig. 4E and F), which might explain why MDM2 did not have a significant effect on tumorigenesis in MMTV/neu mice compared with MDM2DD. This raises the interesting question of whether nuclear localized MDM2 (MDM2DD) might be more potent in inducing tumor formation than wild-type MDM2, which is distributed in both the cytoplasm and the nucleus.

As the overexpression of MDM2DD in mammary glands was associated with an inhibition of apoptosis during involution, we therefore examined whether the accelerated tumor formation in bitransgenics was related to the survival..
advantage provided by MDM2DD. The degrees of apoptosis in mammary tumors from MMTV/neu/MDM2DD and MMTV/neu mice were measured. The results revealed that MDM2DD mammary tumors had a decrease in apoptosis (7.25%) compared with neu tumors (32.5%; Fig. 4G, H, and K). Because MDM2DD has an increased ability to degrade p53, we next examined whether expression changes of p53 were related to MDM2DD in mammary tumors and showed that the expression of p53 and its target p21 were decreased in bitransgenic mammary tumors (Fig. 4I, J, and L). Interestingly, when another MDM2 target, FOXO3a, was examined, FOXO3a expression levels were indeed lower in the MDM2/MDM2DD mammary tumors than that of the neu-expressing tumors (Fig. 4L). However, we did not observe a significant difference between MDM2- and MDM2DD-expressing mammary tumors, suggesting that there may be a threshold level for the reduction of FOXO3a that was already reached with wild-type MDM2 and MDM2DD is not able to reduce it further. Thus, the MDM2-mediated downregulation of FOXO3a might not significantly contribute to the differential response between MDM2 and MDM2DD mice. These observations suggested that MDM2DD accelerates mammary tumor formation in MMTV/neu mice with an impaired apoptosis, which correlates with decreased p53 expression.

Taken together, these observations suggest that expression of Akt-activated MDM2 in the mammary epithelium inhibits apoptosis during mammary gland involution and accelerates tumor formation in MMTV/neu mice. The expression of activated MDM2 corresponded with the decreased expression of p53 in vivo.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
In memory of Serena Lin-Guo for her courageous fight against breast cancer.

Grant Support

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Received 09/01/2009; revised 07/01/2010; accepted 07/18/2010; published OnlineFirst 09/14/2010.

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Cancer Res  Published OnlineFirst September 14, 2010.