

Activation of Murine Double Minute 2 by Akt in Mammary Epithelium Delays Mammary Involution 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 (MDM2DDS166D/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/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 ligase, 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 which 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- κ B (7), Bad (8), Forkhead transcription factors

(9, 10), and p21^{Cip1/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 proteasome (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 (MDM2DDS166D/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 (S166D/S186D) 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

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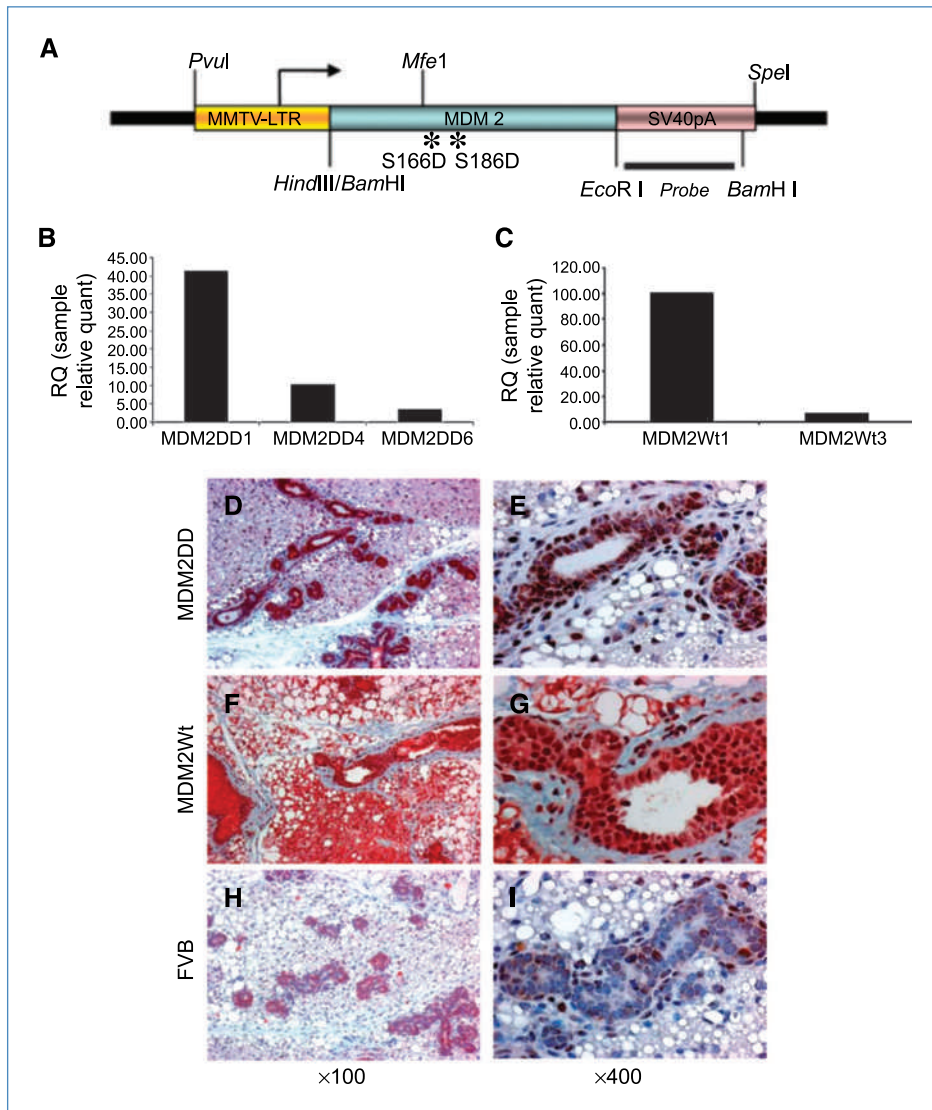


Figure 1. Generation of transgenic mice. A, structure of the transgene. B and C, quantitative reverse transcription-PCR analysis of MDM2DD transcript expressions in MMTV/MDM2DD (B) and MMTV/MDM2 (C) strains. D–I, immunohistochemical analysis of MDM2 expressions on pregnant 7 d mammary glands from MMTV/MDM2DD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

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 FVB/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 Ser¹⁶⁶ and Ser¹⁶⁸

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

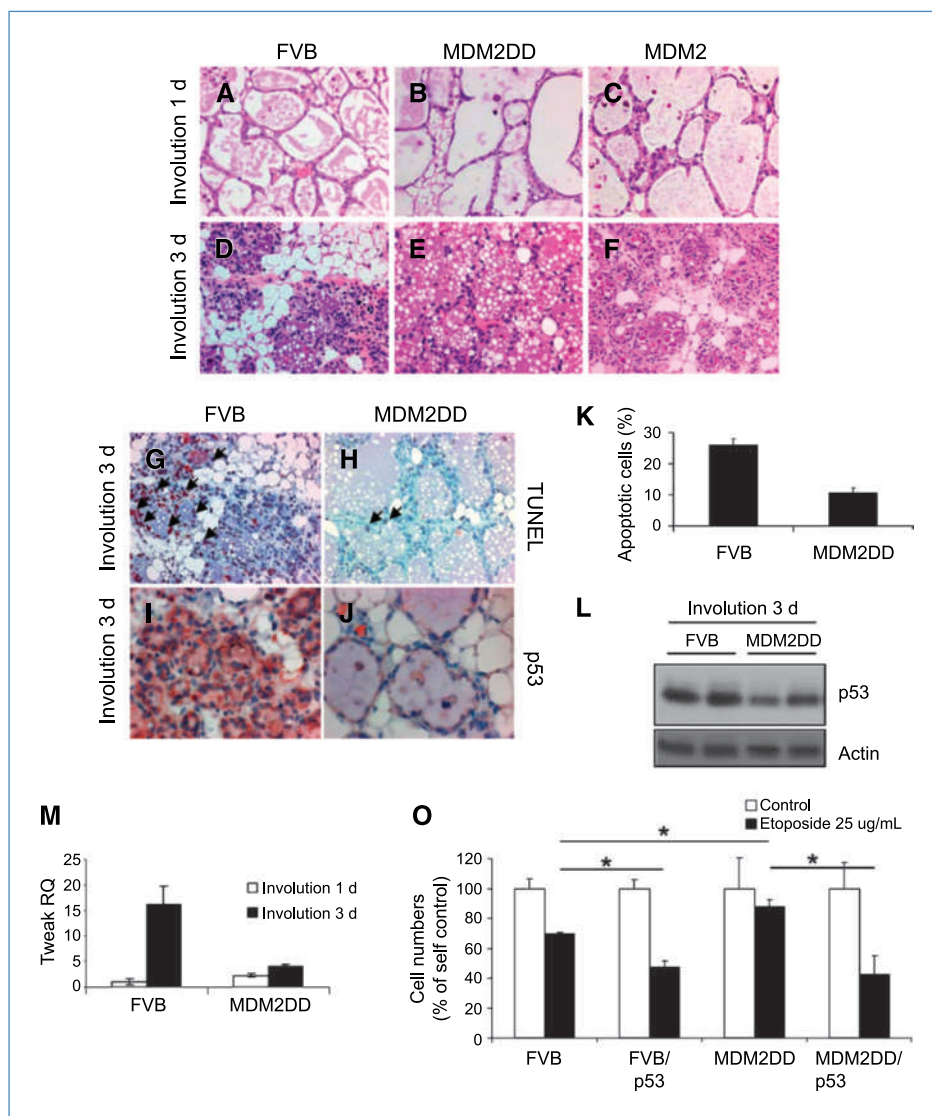


Figure 2. MMTV/MDM2DD mice showed delayed mammary involution. A–F, histologic patterns on involution 1 d and 3 d mammary glands from FVB/n (A and D), MMTV/MDM2DD (B and E), and MMTV/MDM2 (C and F) mice. G and H, TUNEL analysis on involution 3 d mammary glands from FVB/n (E) and MMTV/MDM2DD (G) mice. 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 MMTV/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 μ g/mL) treatment for 3 d. Re-expressing of p53 was achieved by p53-containing lentivirus infection for 2 d.

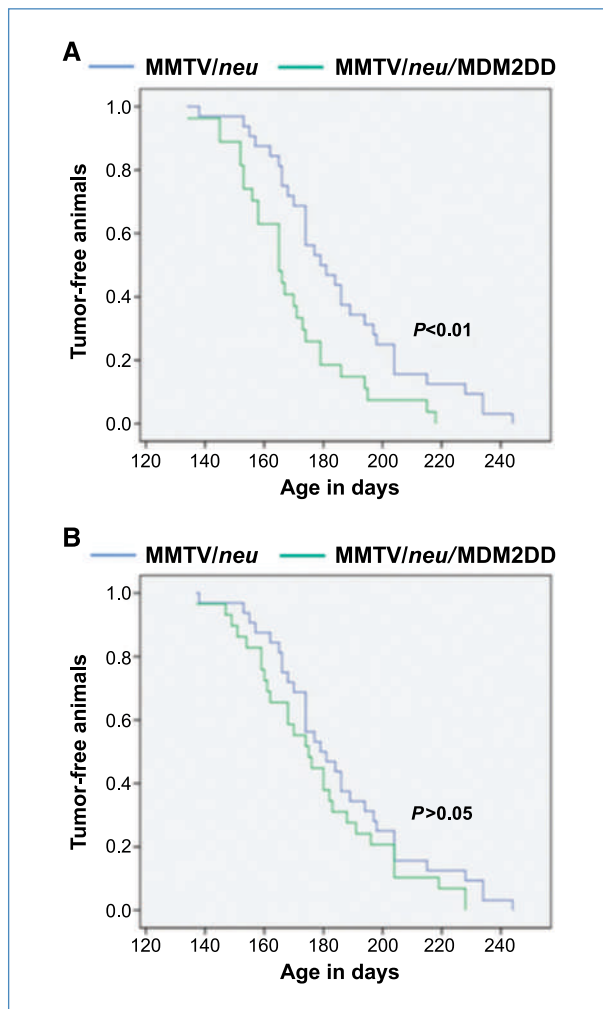


Figure 3. MDM2DD in affecting tumor onset. A, MDM2DD accelerated tumor onset in MMTV/*neu* mice ($P < 0.01$ by Mantel-Cox test). B, MDM2 did not accelerate tumor onset in MMTV/*neu* mice ($P > 0.01$ by Mantel-Cox test).

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 *TGF β 3* 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. 2I, 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 per-

formed 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 MDM2-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

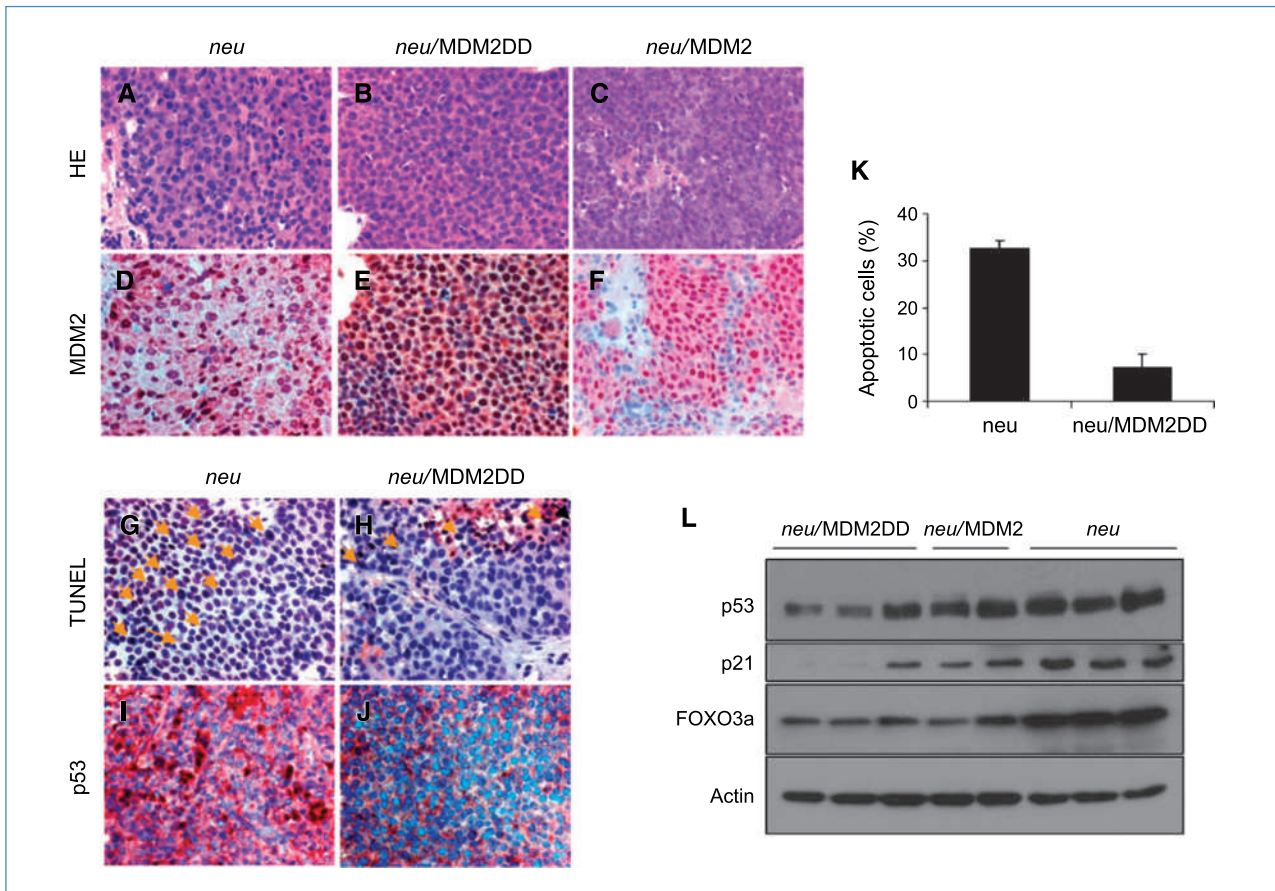


Figure 4. MDM2DD inhibited apoptosis and decreased p53 expression in MMTV/*neu* tumor. A–C, histologic patterns of MMTV/*neu* (A), MMTV/*neu*/MDM2DD (B), and MMTV/*neu*/MDM2 (C) tumors. D–F, immunohistochemical analysis of MDM2 expressions in MMTV/*neu* (D), MMTV/*neu*/MDM2DD (E), and MMTV/*neu*/MDM2 (F) tumors. G and H, TUNEL analysis in MMTV/*neu* (G) and MMTV/*neu*/MDM2DD (H) tumors. Arrows, apoptotic cells. I and J, immunohistochemical analysis of p53 expressions in MMTV/*neu* (I) and MMTV/*neu*/MDM2DD (J) tumors. K, apoptotic indices of MMTV/*neu* and MMTV/*neu*/MDM2DD mammary tumors. L, immunoblot analysis of p53, p21, and FOXO3a expressions in mammary tumors from MMTV/*neu*, MMTV/*neu*/MDM2DD, and MMTV/*neu*/MDM2 mice.

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.

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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.

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