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To examine *mdm2* function *in vivo*, we have performed experiments in two mouse models. The *mdm2* null mouse is an embryo lethal whose phenotype is completely rescued in the absence of *p53*. Analysis if the embryos indicate that they are dying by apoptosis. Analysis of mice null for *p53* and the presence or absence of the *mdm2* gene indicate a longer tumor latency in *p53-/-mdm2+/-* mice. In addition, these mice exhibit an increase in sarcomas with a concomitant decrease in lymphomas. We have also analyzed transgenic mice that overexpress *mdm2* in the breast epithelium and find that the mammary epithelial cells undergo multiple rounds of DNA synthesis without cell division. Importantly, this phenomenon is independent of p53 suggesting novel functions for MDM2. Th mammary epithelial cells are overexpressing cyclin A, but not cyclin B suggesting that the cells never exit S-phase. The S-phase transcription factor that binds MDM2, E2F1, appears to have no role in this defect.

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INTRODUCTION:

The development of human tumors is the result of multiple events that undermine the signals involved in normal growth control. The *p53* tumor suppressor gene is often mutated in the development of human cancers. An important negative regulator of p53 function is the product of the *mdm2* oncogene. MDM2 binds and inactivates p53 function. Amplification of *mdm2* occurs in many sarcomas and breast carcinomas suggesting an alternative mechanism of inactivating p53. This proposal was designed to examine the *in vivo* function of the *mdm2* oncogene in concert with the tumor suppressor *p53*. In addition, the interaction of MDM2 with other proteins and their effect on MDM2 function and tumor development will be examined.

The *mdm2* gene was originally cloned from a transformed murine cell line (3T3DM) as one of three amplified genes stably maintained in the form of double minutes (1). The overexpression of *mdm2* alone in primary rat embryo fibroblasts (REFs) resulted in immortalization or in transformation when transfected with the activated *ras* gene leading to the hypothesis that *mdm2* functioned as an oncogene in the process of cell transformation (2).

Subsequently, MDM2 was discovered as a protein that bound the p53 tumor suppressor (3). Moreover, the binding of MDM2 to p53 inactivated p53 function as a transcriptional activator. The interaction of MDM2 with p53 created much excitement in the field since the *p53* tumor suppressor gene is mutated in greater than 50% of human cancers (4). Cloning and localization of the human *mdm2* gene indicated that it resides on human chromosome 12q13-14, a region often altered in sarcomas (5). Analysis of both osteosarcomas and soft tissue sarcomas revealed amplification of the *mdm2* gene in approximately one third of tumors (5, 6). Additional experiments showed amplification or overexpression of *mdm2* in glioblastomas and breast carcinomas (7-9). These data thus led to the hypothesis that overexpression of MDM2 by gene amplification represents an alternate mechanism of inactivating p53 function.

To examine the interaction of p53 and MDM2 *in vivo*, we deleted the *mdm2* gene in mice using homologous recombination in ES cells (10). *mdm2* null mice died during embryogenesis 5.5 days of gestation. Since one of the functions of MDM2 is the negative regulation of p53, we hypothesized that the embryonic lethality seen in *mdm2* homozygous mutants was due to an inability to down-regulate p53 function. *p53* null mice are viable (11, 12) and we therefore tested our hypothesis by interbreeding mice heterozygous for both *mdm2* and *p53* genes. Strikingly, we found viable mice that were homozygous null for both *mdm2* and *p53*. Rescue of the *mdm2 -/-* lethality in a *p53* null background suggests that a critical *in vivo* function of MDM2 is the negative regulation of p53 activity.

However, evidence has accumulated indicating that MDM2 has separate functions in addition to its interaction and inhibition of p53 function. In some sarcomas, both amplification of *mdm2* and mutations in *p53* were found (13). Patients with these tumors had significantly reduced survival as compared to those with only one of these alterations. These data are indicative of independent functions. Indeed, recent developments suggest that MDM2 binds other factors implicated in growth control. MDM2 binds another tumor suppressor, the

retinoblastoma (Rb) gene product (14). This interaction also disrupts Rb function as a growth suppressor and inhibitor of transcription. In addition, a complex was detected between MDM2 and E2F1, a transcription factor important for the G1/S transition (15). In this case, however, the interaction further stimulated the activity of E2F1. Thus, MDM2 appears to inhibit Rb and p53 function, but its interaction with E2F1 suggests that in addition to inactivation of tumor suppressors, MDM2 can augment cell proliferation by activating genes involved in S-phase progression.

Our hypothesis is that MDM2 is an important component of the p53 pathway, but it has additional independent functions that affect tumorigenesis.

The specific aims of this proposal are:

1) to determine the mechanism of death of the *mdm2-/-* embryo by investigating growth arrest and apoptosis
2) to examine *mdm2-/- p53-/-* mice for timing and spectrum of tumor development
3) to assay for other *in vivo* interactions by mating *mdm2+/-* mice with *p21-/-* mice and with *Rb+/-* mice
4) to analyze MDM2 function using cell lines developed from the *mdm2/p53* double null mice.

BODY:

Experimental methods, assumptions, and procedures:

Mouse Breeding and Genotyping.

Mice null for *p53* (12) and were either wild-type, heterozygous, or homozygous for the *mdm2* null allele (10) were used to study the timing and spectrum of tumor development. To generate these genotypes, mice heterozygous for the *mdm2* null mutation were crossed to normal C57BL/6J females to produce two generations of mice of mixed 129/Sv:C57BL/6 background. These mice were crossed to 129/Sv *p53* null mice (obtained from Jackson Laboratory) to generate heterozygous mice for both mutant alleles. These double heterozygote mice were crossed to each other to obtain the mice used in this tumorigenic study. Genotyping for each of the null alleles was performed by PCR as described (10, 12).

BLG*mdm2* transgenic mice (line TG3640) (29) were maintained as hemizygotes by crossing to C57Bl/6J wild type mice. 129/Sv-C57BL/6 mice heterozygous for a mutant E2F1 allele (*E2F1+/-*) were obtained from L. Yamasaki (42) and crossed with mice carrying the BLG*mdm2* transgene. BLG*mdm2/E2F1+/-* progeny were backcrossed with *E2F+/-* mice to generate offspring that were BLG*mdm2/E2F1-/-*. BLG*E2F1* transgenic mice (line TG3604) were crossed with BLG*mdm2* mice (line TG3640) to create mice that carried both transgenes in a hemizygous state.

The presence of the BLG*mdm2* transgene was determined by a dominant coat color marker that had been coinjected with the transgene and by PCR with transgene-specific

primers BLG and MDM2 as described previously (29). *E2F1* heterozygous and homozygous mutant animals were identified by PCR as described previously (42), with the following exceptions. PCR was performed for both wild-type and mutant alleles together using 16 pmol each of L26 and L28 primers and 32 pmol of L31 primer per reaction. The PCR reactions (25 μl) were amplified using AmpliTaq (Perkin-Elmer) for 1 cycle (94°C 5 min), 35 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 1 min) and 1 cycle (72°C for 7 min).

Nulliparous females of the appropriate genotypes were mated and sacrificed at day 14 or 18 of gestation or at day 10 of lactation. If necessary, pups born to BLG*mdm2* females were removed to a foster mother and replaced with slightly older pups to allow for continued nursing. Most animals were given an intraperitoneal injection of 100 μg of BrdU in PBS/g body weight approximately 2 hours before sacrifice. The first abdominal (#4) mammary glands on both sides of the animal were dissected for analysis.

Tumor analysis

Mice that developed visible tumors approximately 1 cm in diameter were sacrificed and subjected to necropsy. Mice that did not develop visible tumors but became moribund were also sacrificed and subjected to necropsy. In addition to tumor samples, tissues from heart, lung, kidney, spleen, liver, and testis were recovered. The mice were carefully examined for the presence of any other abnormalities. Brain samples were taken in those few cases from moribund mice with no apparent pathology or tumor. All the tissues were fixed in 10% buffered formalin, processed for histology, and paraffin embedded. Four-micrometer-thick paraffin sections were stained with hematoxylin and eosin. The histopathologic analysis was performed without knowledge of the genotypes of the mice.

Statistical analysis

Comparison of the tumor latency for the three strains of mice was performed using Kaplan-Meier analysis. Significant differences in the type and dissemination of tumors between mice of the three genotypes were established using the chi-square test.

TUNEL

Tdt-mediated dUTP Nick End Labeling (TUNEL) was carried out using the In Situ Cell Death POD kit (Boehringer Mannheim, Indianapolis, IN). Blastocysts were flushed from the uterus using M15 medium and the zona pellucida removed by Acid Tyrode's solution (137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl \cdot 6H_2O$, 0.4 mM $Na_2HPO_4 \cdot H_2O$, 6 mM glucose, 1 X 10^{-5} M polyvinylpyrollidone, and 0.66% (v/v) HCl). They were fixed in 4% (w/v) paraformaldehyde by a 15 minute incubation in 0.3% (v/v) Triton X-100 in 0.1% (w/v) H2O2/methanol. Cells were permeabilized suing 0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate, then treated with terminal deoxytransferase and fluorescein conjugated dUTP. Labeling was detected using a peroxidase conjugated anti-fluorescein antibody and 3,3' diaminobenzidine (DAB) (Vector Labs, Burlingame, CA).

Immunohistochemistry.

Mammary gland tissues were fixed in 0.4% paraformaldehyde in PBS at 4°C overnight, washed twice in PBS, and dehydrated through a graded series of ethanols, from 70% to

100%, according to standard procedures. The tissue was then incubated in xylene for 30 min. before it was embedded in paraffin. Sections were cut at 7-μm and placed on lysine-coated slides. After rehydration, the slides were incubated in 0.01 M citrate buffer pH 7.0 in a steamer for 25 min. for antigen retrieval and then soaked in 0.3% hydrogen peroxide in methanol for 15 min. After rinsing in PBS, the slides were blocked with serum from the Vectastain kit (Vector Labs, Burlingame, CA) and then incubated with the appropriate antibody for 1 hour at 37°C in a humidified chamber. MDM2 (9312) and cyclin E (1014) antibodies were rabbit polyclonal antisera raised by our laboratory and used at a 1:250 dilution. Cyclin A (C-19; polyclonal; 1:250) and cyclin D1 (72-13G; monoclonal; 1:100) were obtained from Santa Cruz Biotechnology. All immunohistochemistry was performed with the Elite Vectastain Kit for mouse or rabbit (Vector Labs) according to the manufacturer's instructions. Staining was detected with the substrate DAB (Vector Labs). All immunostained slides were counterstained with Nuclear Fast Red (Vector Labs) before dehydrating and mounting with Permount. BrdU immunostaining was performed using the BrdU Staining kit (Zymed, San Francisco, CA) according to the manufacturer's instructions.

Results and discussion:

Our work during the last two years of funding have led to the development of two mouse models. In one model, the loss of *mdm2* results in embryonic lethality due to the inability to down modulate p53 function. Using this model, we have undertaken an examination of the critical components of the p53 pathway. In another model, the overexpression of *mdm2* in mouse mammary epithelial cells led to disruption of the cell cycle and tumor development. We have used both models to further our understanding of MDM2 function in tumorigenesis.

In the first model, the early embryonic lethality seen in *mdm2* null embryos is due to an inability to inactivate p53, and is completely rescued by the absence of p53 (10, 16). Because the MDM2/p53 interaction is critical to the survival of this *in vivo* model, it is ideal for studying the p53 pathway, specifically the contribution of downstream effectors of p53. In the previous year of funding, we have clearly shown the deletion of the p53 downstream target p21 had no effect on embryo lethality (28).

While the lethality of *mdm2* null embryos is rescued in the absence of p53, surprisingly, the loss of *p21* could not substitute, even partially, for loss of *p53.* p53 is a regulator of multiple pathways and the possibility exists that no single target can effect the functions of p53, a fact that is supported by the lack of mutations in *p21* in human tumors (24-26) and the lack of susceptibility of *p21* null mice to tumorigenesis (20, 27). However, the lack of *p21* induction by p53 in wild type embryos at 5.5 days of development indicates that p21 is not part of this pathway. Thus, we have unveiled an *in vivo* situation in which the ability of p53 to exert its effects is not dependent on activation of *p21.*

While an important observation, the inability of loss of *p21* to rescue the *mdm2* null phenotype did not yield any information as to the mechanism of embryonic cell death. Since the process was p53 dependent, we began to examine *mdm2* null embryos for apoptosis or

cell cycle arrest, the two biological outcomes of activating p53. Since at 5.5 days of gestation, few if any cells are visible in the decidua, we first examined earlier staged embryos for gross abnormalities. The analysis of blastocysts (3.5 days post coitum) revealed differences in their abilities to hatch depending of their genotype (Fig. 1). *Mdm2* null blastocysts were delayed in hatching indicating that the effects of loss of *mdm2* were detectable as early as 3.5 dpc. Using TUNEL assay to detect apoptosis, we showed that 25% of the embryos from a heterozygous cross were undergoing massive apoptosis while normal embryos have a few apoptotic cells (Fig. 2). Thus, these experiments establish that p53-dependent apoptosis is leading to death of the *mdm2* null cells.

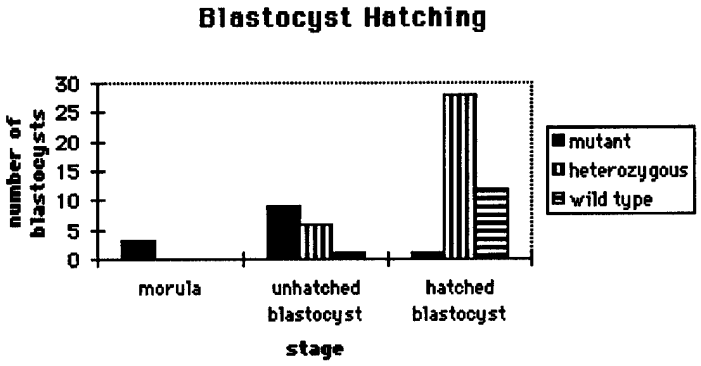


Fig. 1. Blastocysts from wild type or *mdm2* heterozygous crosses were isolated at 3.5 days post coitum, placed in culture for 24 hours and staged. After staging, they were genotyped.

Since a critical downstream target of p53, bax, is an inducer of apoptosis, we have begun to genetically determine if loss of *bax* rescued the phenotype by crossing *bax* null mice with the *mdm2* null heterozygous mice. The analysis of these mice should yield insight into the p53 pathway that functions during embryogenesis.

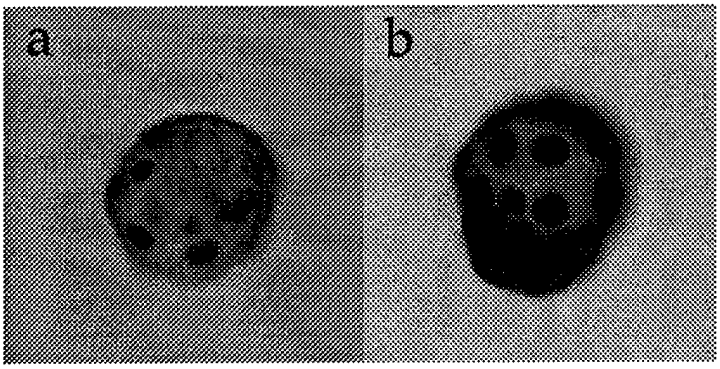


Fig 2. Blastocysts stained by TUNEL for apoptosis. a, normal embryo; b, *mdm2* null.

Aim 2 of our proposal was to examine the mdm2-/-p53-/- mice for tumor development. To assess the influence of the absence of one or two *mdm2* alleles on the latency and pathogenesis of neoplastic disease in *p53* null mice, crosses were performed using mice with different combinations of *p53* and *mdm2* null alleles as described in materials and methods to generate the following cohort: *p53* null mice (34); *p53* null/*mdm2* heterozygous mice (33); and *p53*/*mdm2* double null mice (58). The mice used in this study were in a mixed C57/129 background.

To measure tumor incidence, we plotted the number of animals that remained tumor free against time (Figure 3A). The mean latency for *p53-/-mdm2+/+*, *p53-/-mdm2+/-*, and *p53-/-mdm2-/-* mice was 150.5, 156.5, and 140.5, respectively. Latency in the *p53-/-mdm2 +/-* mice was significantly longer than in *p53 -/-mdm2-/-* mice (p=0.024) (Figure 3B).

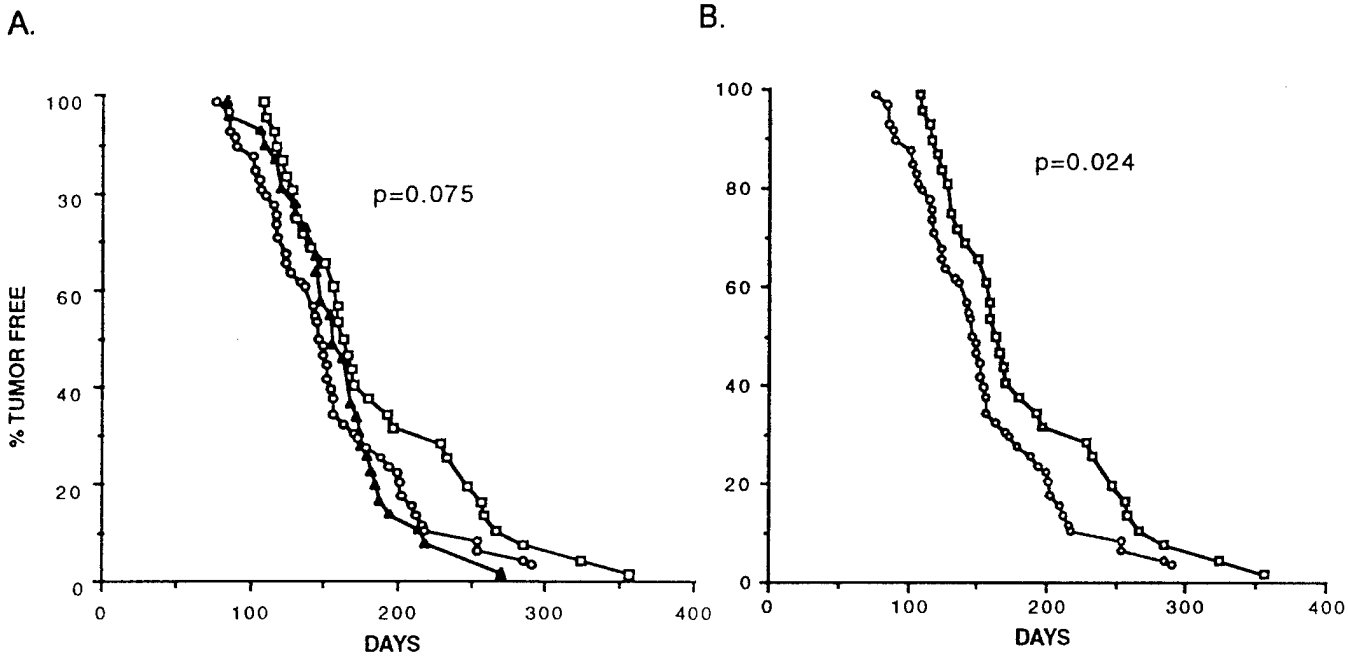


Fig. 3. Effects of a *mdm2* deletion on tumor latency in *p53* null mice. Thirty-four *mdm2 +/+* (solid triangles), 33 *mdm2 +/-* (squares), and 58 *mdm2 -/-* mice (circles), all null for *p53* and with tumors were used to calculate tumor latency. The percentage of mice remaining tumor free is plotted against age. The P value determined using Kaplan-Meier analysis is shown for all three genotypes in A and for *p53-/-mdm2+/-* (squares) and *p53-/-mdm2-/-* (circles) mice in B.

We also performed a pathological examination of the tumors that arose in the three genotypic strains of mice. Most tumors arising in mice of each genotype were lymphomas, constituting approximately 85% of the tumors in the *p53-/-* mice and 90% in the *p53 -/- mdm2-/-* mice. Although lymphomas were the most common malignancy in the *p53-/-mdm2+/-* mice, 64%, they were significantly less common than in mice of the other two genotypes (p=0.007)(Figure 4). The majority of lymphomas were thymic in origin; this type constituted 73% of the lymphomas in the *p53-/-* mice, 83% of the lymphomas in the *p53-/-mdm2-/-* mice, and 90% of the lymphomas in the *p53-/-mdm2+/-* mice. The thymic lymphomas in all three genotypes were high-grade large cell malignancies with numerous mitotic figures and evidence of apoptotic cell death.

Sarcomas constituted the next most common malignancy, accounting for approximately 21% of the neoplasms arising in *p53-/-* mice and 17% of the neoplasms arising in *p53-/-mdm2-/-* mice. Sarcomas were significantly more common in the *p53-/-mdm2+/-* mice,

comprising 42% of the neoplasms (p= 0.022)(Figure 4). The mean latency period for sarcomas ranged from 183-195 days and was not significantly different in the three genotypic strains of mice.

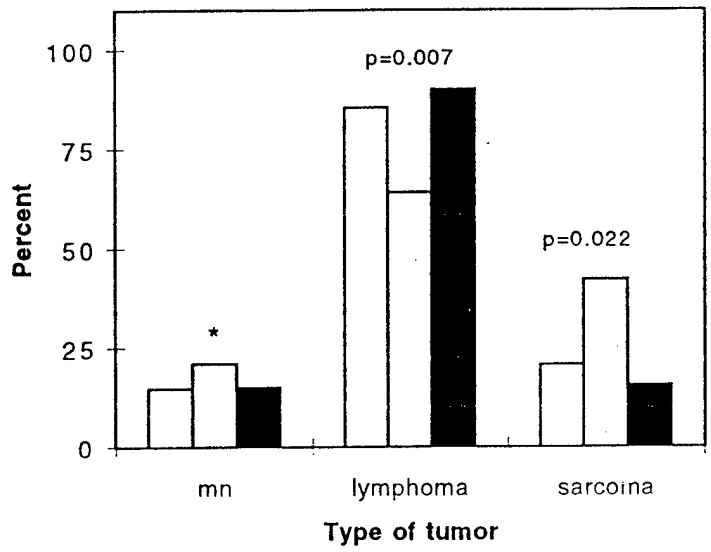


Figure 4. Effects of *mdm2* mutation on tumor development in *p53* null mice. The frequency of lymphomas, sarcomas and mice with multiple neoplasms (mn) were compared between the different genetic backgrounds. The frequency of each type of tumor is relative to the total number of tumors observed in each genotype. Comparison of the frequencies between tumor type and genetic background by chi square analysis indicates a statistically significant difference in the incidence of lymphomas and sarcomas, p=0.007 and p=0.022, respectively, in *p53-/-mdm2+/-* mice as compared to the other two genotypes. White, gray and black bars represent *p53-/-mdm2+/+*, *p53-/-mdm2+/-*, and *p53-/-mdm2-/-* mice respectively.

The fact that the *p53-/-mdm2+/-* mice survive longer than *p53-/-mdm2+/+* mice suggests that the level of MDM2 is a critical determinant of tumor development. In *p53-/-mdm2+/+* mice, the absence of p53, hence loss of interaction with MDM2, would by default result in more MDM2 protein in the cell. Increased levels of MDM2 due to gene amplification have been observed in sarcomas and other tumors (5, 6). Moreover, the overexpression of *mdm2* in the breast epithelium of transgenic mice led to tumor development (29). Thus, increased MDM2 protein in the cell can contribute to tumor development. The loss of one *mdm2* allele in *p53-/-mdm2+/-* mice might then be expected to result in decreased levels of MDM2 and a reduction in the rate of tumorigenesis.

Another important observation is the increased number of sarcomas in *p53* null/*mdm2* heterozygous mice as compared to *p53* null and *p53/mdm2* double null mice. This observation further substantiates the critical nature of MDM2 levels in mesenchymal tissue. The amplification of the *mdm2* gene is seen more often in sarcomas than in any other tumor type (5, 6). The increased incidence of spontaneous sarcomas in the *p53-/-mdm2+/-* mice suggests that an insufficient level, as well as an excess, of MDM2 could contribute to multistep carcinogenesis in a cell type-specific manner.

This is an example of a phenotype present in heterozygous mice that is absent in null or normal mice and is probably due to the complex interactions between MDM2 and several proteins that are critical for the regulation of cell proliferation. Competition between these

proteins and limiting amounts of MDM2 result in the observed phenotype. The generation of mice with a targeted deletion of *mdm2* that would bypass the problem of embryo lethality should yield insight into this problem.

The second model we are studying is one in which an *mdm2* minigene was expressed during gestation and lactation in the mammary gland of both wild-type p53 (p53+/+) and p53 knockout (p53-/-) mice. The deregulated expression of MDM2 inhibited normal development and morphogenesis of the mammary gland, and caused hypertrophy and nuclear abnormalities. These abnormalities included both multinucleated cells and enlarged cells with giant nuclei. Analysis of DNA content showed that 30-45% of the cells were polyploid, with DNA contents up to 16N, indicating that overexpression of *mdm2* caused mammary epithelial cells to undergo multiple rounds of S-phase without cell division. This phenotype was similar in the p53+/+ and p53-/- background, demonstrating a role for MDM2 in the regulation of DNA synthesis that is independent of the ability of MDM2 to inhibit p53 activity. Additionally, multiple lines of BLGMDM2 transgenic mice developed mammary tumors, confirming that overproduction of MDM2 contributes to tumorigenesis in epithelial cells *in vivo*. This study was published during the first year of funding (29).

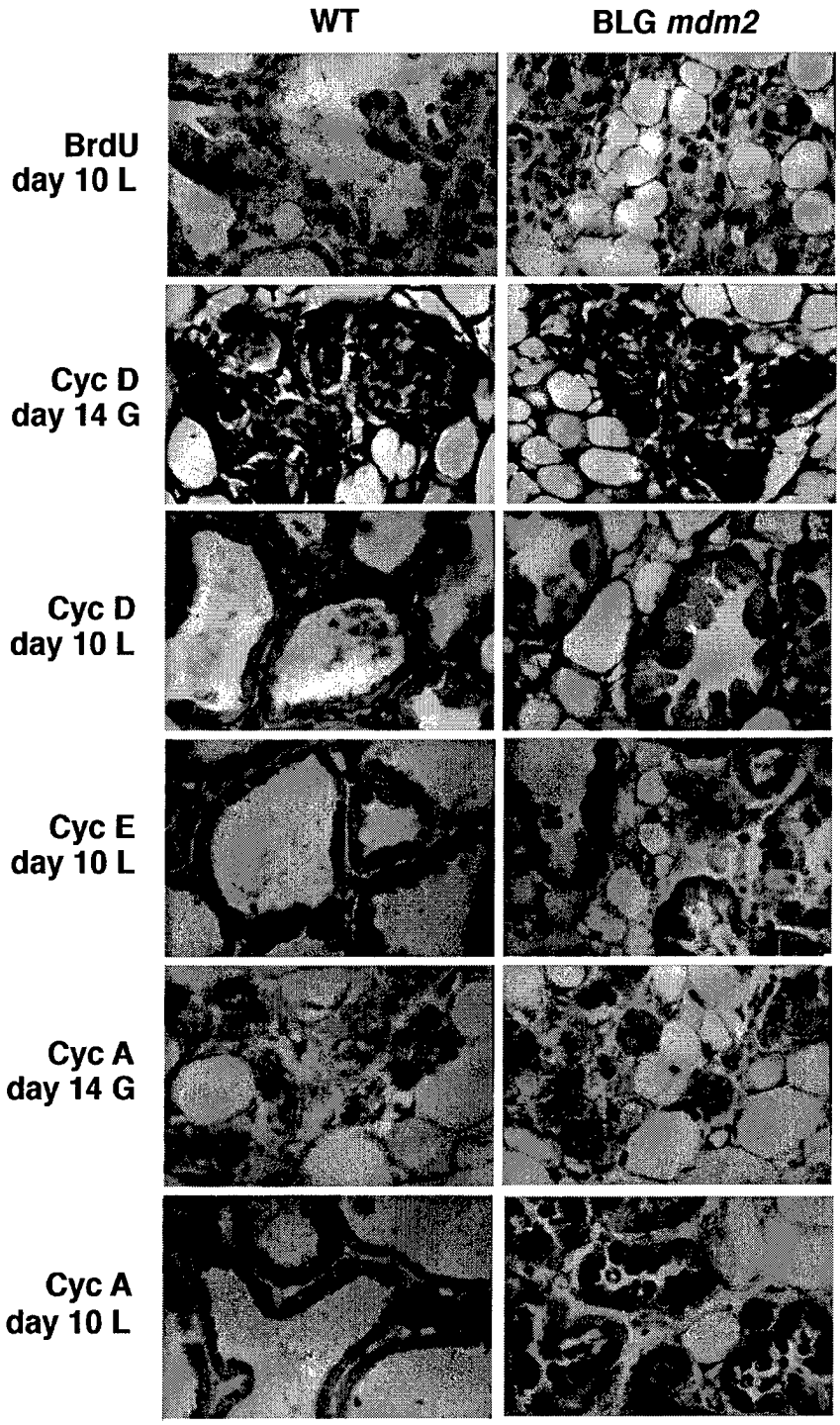
To further elucidate the molecular nature of the transgenic phenotype, we asked whether there were any alterations in the production of various cell cycle-regulated proteins at different stages of mammary gland development. Because cyclin expression is tightly regulated in various phases of the cell cycle, we focused on cyclin D1 (G1), cyclin E (G1/S), cyclin A1 (S/G2), and cyclin B1 (G2/M). To that end, we performed immunohistochemistry on wild type and BLG*mdm2* transgenic mammary glands at mid-pregnancy and mid-lactation, using antibodies raised against the various cyclins.

Two G1 cyclins tested, cyclin D1 and cyclin E, showed similar production patterns in both wild type and transgenic mammary glands. Specifically at day 14 of pregnancy, when growth factors are stimulating cyclin D1 production (41), mammary epithelial cells from both wild type and transgenic mice were positive for cyclin D1 (Fig. 5). This expression decreased somewhat in animals of either genotype by day 10 of lactation, when the cells are no longer receiving proliferation signals from the extracellular matrix (Fig. 5). Cyclin E, a G1/S phase cyclin, was produced in most cells of the mammary gland of either genotype at day 14 of gestation (data not shown), or at day 10 of lactation (Fig. 5).

Immunohistochemistry performed with an antibody to the S/G2-specific cyclin A revealed an interesting difference between the wild-type and the BLG*mdm2* transgenic mammary gland. Cyclin A was detectable in both tissues during pregnancy, but during lactation only the BLG*mdm2* transgenic gland continued to be positive for cyclin A (Fig. 5).

We also tested for cyclin B production using two different cyclin B antibodies, but were unable to detect a noticeable level of expression (data not shown). The lack of cyclin B expression in the cells of the BLG*mdm2* mammary gland suggests that the cells do not receive signals for M phase.

Fig. 5. Analysis of cell cycle gene expression in wild type or BLG*mdm2* transgenic mammary glands at day 10 lactation (L) or day 14 gestation (G).



The mechanism by which MDM2 overproduction disrupts the coordination of DNA synthesis in S phase and cytokinesis is unknown. However, one possible model involves the ability of MDM2 to bind and stimulate the activity of the S-phase transcription factor E2F1/DP1 (15). During the G1 phase of the cell cycle, the tumor suppressor Rb binds and inhibits E2F1 activity (30-32). E2F1 is released as a function of Rb phosphorylation by cyclin-dependent kinases late in G1, and becomes transcriptionally active. E2F1/DP1 then activates the expression of a number of genes involved in S phase, such as *cyclin E* (33), *dihydrofolate reductase* (34,35), *thymidine kinase* (36), and *DNA Pol* α (33, 37). E2F1 is a potent facilitator of DNA synthesis, as quiescent cells in tissue culture can be driven into S phase by overexpression of E2F1 alone (38-40).

We therefore generated BLG*mdm2* mice that were null for *E2F1*. We observed no notable differences in histology or cyclin gene expression between BLG*mdm2* and BLG*mdm2/E2F1-/-* mice, indicating that endogenous E2F1 activity was not required for the BLG*mdm2* phenotype. Since, depending on the experimental system, either loss of E2F1 function or overexpression of E2F1 results in transformation, we also tested whether overexpression of E2F1 augmented the severity of the BLG*mdm2* phenotype by generating mice bitransgenic for BLG*mdm2* and BLG*E2F1*. We observed a unique mixture of the two single transgenic phenotypes histologically, and found no significant changes in cyclin levels, indicating that overexpression of E2F1 had no effect on the BLG*mdm2* transgenic phenotype. Thus, increased expression or absence of E2F1 does not affect the ability of MDM2 to disrupt the cell cycle. This study has been submitted for publication.

Recommendations:

A manuscript describing the results of aim 1 is in preparation. The results from aim 2 have been submitted for publication. Part of aim 3 has already been published. The crosses with Rb+/- mice appear unlikely to yield important information in light of our other observations and will not be performed. Instead, we will continue working on the *mdm2* transgenic mouse model since *mdm2* overexpression leads to the development of breast tumors. This is a beautiful model to determine critical MDM2 interactions in tumor development. A manuscript describing the inability of E2F1 to affect the phenotype has also been submitted. During the last year of funding we will concentrate on aim 4. Several constructs have already been made. In addition, studies using a cell line containing a temperature sensitive p53 mutant that therefore is null only for *mdm2* are in progress.

CONCLUSIONS:

The work presented in this progress report makes major contributions to our understanding of p53 and MDM2. The loss of p21 cannot substitute, even partially, for loss of p53 in this the *mdm2-/-* model. We have determined that embryonic lethality in *mdm2* null mice is due to apoptosis. We have begun crosses to determine the need for bax, an inducer of apoptosis and a target of p53.

An *in vivo* transgenic model was used to study the functions of MDM2. The overexpression of *mdm2* in the breast epithelium led to multiple rounds of S-phase without cytokinesis. This phenotype was independent of p53. The mammary epithelial cells of the *mdm2* transgenic express high levels of cyclin A, but not cyclin B. These data suggest the cells do not exit S-phase. Crosses to determine if the S-phase transcription factor, E2F1, which binds MDM2, was important indicated that the absence or overexpression of E2F1 did not affect the abnormalities seen in the mammary gland.

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Appendix 1

Loss of one but not two *mdm2* null alleles alters tumor spectrum in *p53* null mice

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Summary

The transcriptional activity of the p53 tumor suppressor is inhibited by binding to MDM2. The *in vivo* significance of this interaction was established in *mdm2*-null mice. Embryonic lethality due to loss of *mdm2* is completely rescued by deletion of *p53*, indicating that the lethality is due to inability to down-modulate p53 function. The production of mice null for both *p53* and *mdm2* led us to assess the role of MDM2 in tumor development. We monitored tumor latency and spectrum in *p53*-null mice in the presence or absence of *mdm2*. Two unusual findings resulted: Tumor latency in *p53* null/*mdm2* heterozygous mice was longer than in *p53/mdm2* double-null mice. The incidence of sarcomas was higher in *p53* null/*mdm2* heterozygous mice than in *p53* null or *p53/mdm2* double-null mice. These data raise the possibility that heterozygosity at the *mdm2* locus in the absence of p53 affects the development of tumors of mesenchymal origin.

Key words: tumor suppressor genes, transgenic mice, genetics

Introduction

Mutation of the tumor suppressor *p53* negates its ability to arrest the cell cycle and to induce apoptosis, and is the most common alteration observed in tumors.[1-4] p53 function can also be altered by amplification of the *mdm2* gene, which has been observed in approximately one third of sarcomas and to a lesser extent in glioblastomas and breast carcinomas.[5-7] Since MDM2 binds p53 and inhibits p53 function,[8] these data have led to the hypothesis that amplification of *mdm2* is an alternate mechanism of inactivating p53.

MDM2 binds the amino terminal transactivation domain of p53, inhibiting its interaction with the transcriptional apparatus.[9-11] Mutation of p53 amino acids leu22 and trp23 or MDM2 amino acids gly58, glu68, val75, or cys77 results in lack of binding, limiting the interaction motif to the amino terminus of both proteins.[12] The crystal structure of the p53/MDM2 complex indicated an unusual interaction motif that is primarily dependent on van der Waals forces with only two hydrogen bonds.[13] The binding of MDM2 to p53 also promotes the rapid degradation of p53.[14-15]

The importance of the inhibition of p53 transcriptional activity by MDM2 is most clearly exemplified in the analysis of *mdm2* function *in vivo*.[16-17] The deletion of *mdm2* in mice leads to early embryonic lethality, at 5.5 days of development. This phenotype is completely rescued in the absence of *p53*, suggesting that lethality is due to the inability to down-modulate p53 activity during embryo development.

These data, however, do not preclude alternative functions for MDM2 and several observations suggest that MDM2 has functions independent of p53. For example, some tumors contain both a mutation of the *p53* gene and overproduction of MDM2 suggesting that in cells, these two genetic changes do not have identical effects.[18] In addition, overexpression of *mdm2* leads to cell cycle defects in breast epithelial cells.[19] The enlarged and multinucleated *mdm2*-

expressing epithelial cells undergo multiple rounds of DNA synthesis without cytokinesis. More important, the absence of p53 has no effect on this phenotype. The p53-independent functions of MDM2 may be attributed to the fact that MDM2 binds other proteins such as Rb and E2F1.[20,21] The binding of MDM2 to Rb prevents its function as an inhibitor of the cell cycle, and the binding to E2F1 augments E2F1 transcriptional activity during S-phase progression. In addition, MDM2 binds L5, a member of the ribosome complex, and its associated 5S ribosomal RNA.[22-23] Together these observations indicate additional functions for MDM2 other than solely inactivation of p53.

These findings led us to analyze mice null for *p53* and *mdm2* for tumorigenesis. Tumor latency was prolonged in *p53* null-*mdm2* heterozygous mice as compared to *p53* null mice. In addition, the tumor spectrum also changed with an increased number of sarcomas in *p53* null-*mdm2* heterozygous mice as compared to *p53* null and *p53/mdm2* double null mice. To our knowledge, this is the first example of a phenotype that results from having only one functional allele which disappears upon deletion of the other normal allele. This may be attributed to MDM2's unique ability to inhibit p53 function, and to interact with other proteins. These data further support an additional role for MDM2 other than inhibition of p53 function.

Materials and Methods

Mice

Mice with three different genotypes generated by gene targeting were used in this study. All of the mice were null for *p53*[24] and were either wild-type, heterozygous, or homozygous for the *mdm2* null allele.[16] To generate these genotypes, mice heterozygous for the *mdm2* null mutation were crossed to normal C57BL/6J females to produce two generations of mice of mixed 129/Sv:C57BL/6 background. These mice were crossed to 129/Sv *p53* null mice[24] (obtained from

Jackson Laboratory) to generate heterozygous mice for both mutant alleles. These double heterozygote mice were crossed to each other to obtain the mice used in this tumorigenic study. Genotyping for each of the null alleles was performed by PCR as described.[16,24]

Tumor analysis

Mice that developed visible tumors approximately 1 cm in diameter were sacrificed and subjected to necropsy. Mice that did not develop visible tumors but became moribund were also sacrificed and subjected to necropsy. In addition to tumor samples, tissues from heart, lung, kidney, spleen, liver, and testis were recovered. The mice were carefully examined for the presence of any other abnormalities. Brain samples were taken in those few cases from moribund mice with no apparent pathology or tumor. All the tissues were fixed in 10% buffered formalin, processed for histology, and paraffin embedded. Four-micrometer-thick paraffin sections were stained with hematoxylin and eosin. The histopathologic analysis was performed without knowledge of the genotypes of the mice.

Statistical analysis

Comparison of the tumor latency for the three strains of mice was performed using Kaplan-Meier analysis. Significant differences in the type and dissemination of tumors between mice of the three genotypes were established using the chi-square test.

Results

p53 null mice are prone to the development of multiple types of tumors early in their lives.[24,25] Our ability to rescue mice null for *mdm2* in a *p53*-null background prompted us to examine the contribution of *mdm2* to tumorigenesis in *p53*-null mice. We wished to assess the influence of the absence of one or two *mdm2* alleles on the latency and pathogenesis of neoplastic disease in *p53* null mice. Crosses were performed using mice with different combinations of *p53*

and *mdm2* null alleles as described in materials and methods to generate the following cohort: *p53* null mice (34); *p53* null/*mdm2* heterozygous mice (33); and *p53/mdm2* double null mice (58). The mice used in this study were in a mixed C57/129 background.

To measure tumor incidence, we plotted the number of animals that remained tumor free against time (Figure 1A). The mean latency for *p53-/-mdm2+/+*, *p53-/-mdm2+/-*, and *p53-/-mdm2-/-* mice was 150.5, 156.5, and 140.5, respectively. Latency in the *p53-/-mdm2 +/-* mice was significantly longer than in *p53 -/-mdm2-/-* mice (p=0.024) (Figure 1B).

Lymphomas

We also performed a pathological examination of the tumors that arose in the three genotypic strains of mice. Most tumors arising in mice of each genotype were lymphomas, constituting approximately 85% of the tumors in the *p53 -/-* mice and 90% in the *p53 -/-mdm2-/-* mice (Table 1). Although lymphomas were the most common malignancy in the *p53-/-mdm2+/-* mice, 64%, they were significantly less common than in mice of the other two genotypes (p=0.007)(Figure 2). The majority of lymphomas were thymic in origin; this type constituted 73% of the lymphomas in the *p53-/-* mice, 83% of the lymphomas in the *p53-/-mdm2-/-* mice, and 90% of the lymphomas in the *p53-/-mdm2+/-* mice (Table 2). There was no significant difference in latency to detection of lymphomas arising in the three genetic backgrounds. The thymic lymphomas in all three genotypes were high-grade large cell malignancies with numerous mitotic figures and evidence of apoptotic cell death (Figure 3A).

The thymic lymphomas exhibited a characteristic pattern of dissemination and organ involvement that, in general, was similar in each genotypic background. In every case examined, there was histologic evidence of dissemination most commonly to sites above the diaphragm (Table 2). Most also involved the heart (Table 2); the incidence of cardiac involvement was significantly lower in the *p53-/-mdm2+/-* mice than in mice of the other genotypes

(p=0.039)(Figure 4). Characteristically, cardiac involvement was limited to an expansion of the epicardial space (Figure 3B). Appreciable infiltration of the myocardium or endocardial involvement was not observed. The significance of the lower incidence of cardiac involvement in thymic lymphomas arising in *p53-/-mdm2+/-* mice is speculative although it may indicate that these tumors are somewhat less aggressive locally than their counterparts arising in other genetic backgrounds.

The lung was the most common site of extrathymic lymphoma involvement representing 84% of the cases in the *p53-/-* mice, 80% in *p53-/-mdm2+/-* mice, and 95% in *p53-/-mdm2-/-* mice (Table 2). Pulmonary involvement characteristically involved multifocal expansion of the bronchovascular bundles by lymphoma (Figure 3C). More extensive involvement was characterized by the progressive expansion of the alveolar septa and eventual filling of the alveolar spaces.

The most common site of extrathymic lymphoma involvement below the diaphragm was the liver (Figure 3D). Hepatic involvement was observed in 50% of the cases in *p53-/-* mice, 53% in *p53-/-mdm2+/-* mice, and 69% in *p53-/-mdm2-/-* mice (Table 2). Typically, multifocal expansion of the portal tracts was observed. Expansion of the hepatic sinusoids by lymphoma was characteristic of more extensive involvement. Approximately one-third of the cases of thymic lymphomas exhibited renal involvement (Table 2). In most cases renal involvement centered around the interlobular vessels. Occasionally, lymphomatous expansion of the renal hilum, centered around the hilar vasculature was observed. Approximately 20-30% of the thymic lymphomas exhibited splenic involvement (Table 2). Characteristically, this resulted in splenomegaly due to extensive lymphomatous infiltration of the red pulp (Figure 3E). The splenic architecture was occasionally disrupted.

Splenic lymphomas constituted 23% of the lymphomas arising in the *p53-/-* mice, 5% of the lymphomas in the *p53-/- mdm2+/-* mice, and 11% of lymphomas in the *p53-/-mdm2-/-* mice (Table 2). In contrast to secondary involvement of the spleen by thymic lymphoma, these malignancies were centered in the white pulp (Figure 3F). The histopathologic features were consistent with high grade large cell lymphoma and in all cases there was histologically confirmed evidence of dissemination. Dissemination most commonly involved sites below the diaphragm , most often the liver and kidney. Splenic lymphomas involved the lung 50% of the time in *p53-/-* mice, 40% in *p53-/-mdm2-/-* mice, and 100% (1 case) in *p53-/-mdm2+/-* mice. Dissemination of splenic lymphoma to the heart was not observed. The histologic pattern of organ infiltration was similar to that of thymic lymphomas except that capsular infiltration by retroperitoneal disease was a common feature of renal involvement by splenic lymphoma (Figure 3G).

The remainder of the lymphomas were nodal lymphomas which exhibited the histologic features of high grade large cell malignancies (Table 2). These malignancies invariably demonstrated extranodal extension.

Sarcomas

Sarcomas constituted the next most common malignancy, accounting for approximately 21% of the neoplasms arising in *p53-/-* mice and 17% of the neoplasms arising in *p53-/-mdm2-/-* mice (Table 1). Sarcomas were significantly more common in the *p53-/-mdm2+/-* mice, comprising 42% of the neoplasms ($p= 0.022$)(Figure 2). The mean latency period for sarcomas ranged from 183-195 days and was not significantly different in the three genotypic strains of mice.

Fibrosarcomas represented the most common histologic variant (Figure 5A). These tumors were characterized by spindle cells frequently arranged in a fasciculated pattern. Mitotic figures were frequent. Cellular pleomorphism was minimal, but occasional tumors exhibited foci

of tumor giant cells (Figure 5B). These tumors most commonly arose in subcutaneous connective tissue and attained diameters of 1-3 centimeters. They were locally aggressive and invaded underlying skeletal muscle; however, metastases were not observed.

Angiosarcomas were the next most common sarcoma. Interestingly, 12 of the 13 total cases of angiosarcoma developed in female mice. These tumors were relatively small (0.2-0.5cm) and grossly hemorrhagic. Most were intraabdominal and fixed to the peritoneal membrane. Like fibrosarcomas, these tumors were locally invasive but not metastatic. Histologically, they were characterized by irregular anastomotic vascular channels (Figure 5C). The endothelial lining of these vascular channels was markedly pleomorphic and numerous mitotic figures were observed.

Three cases of osteosarcoma were observed (Table 1). Two developed in *p53-/-mdm2+/-* mice and one in a *p53-/-mdm2-/-* mouse. Two were grossly consistent with extraskeletal osteosarcomas and one arose from a rib. These tumors were characterized by considerable cellular pleomorphism and haphazardly arrayed foci of osteoid and bone (Figure 5D). All three osteosarcomas displayed locally invasive growth. Two of the three tumors were associated with metastatic lesions involving the liver and spleen.

Teratoma and teratocarcinoma

Five cases of teratoma and two cases of teratocarcinoma were diagnosed (Table 1). Both teratocarcinomas were testicular in origin and occurred in *p53-/-mdm2-/-* mice. Four of the five teratomas were testicular and one was ovarian in origin. Three of the five teratomas arose in *p53-/-mdm2-/-* mice and one in each of the other two genotypes. The teratomas possessed solid and cystic elements and exhibited structures derived from ectoderm, mesoderm, and endoderm (Figure 5E). Histologically, these structures included keratinizing epithelium, glandular epithelium, respiratory tissue, neuronal tissue, and bone. In both teratocarcinomas the malignant component consisted of undifferentiated cells.

The remainder of the neoplasms consisted of unclassified malignant neoplasms (2 cases) and myelogenous leukemia (1 case). A total of three carcinomas were observed including 2 of adenocarcinomas (submandibular gland and pancreas) and one of endometrial carcinoma.

Discussion

In the experiments described in this report, we compared the tumor incidence in mice lacking *p53* that were also either wild type, heterozygous, or null for *mdm2*. These experiments were aimed at determining the influence of *mdm2* on survival and tumor spectrum of *p53* null mice. Several important observations were made: the latency to tumor development was longer for the *p53-/-mdm2+/-* mice than the *p53-/-* mice; significantly fewer lymphomas arose in *p53-/-mdm2+/-* mice than in *p53-/-* or *p53-/-mdm2-/-*; significantly fewer of the thymic lymphomas involved the heart in *p53-/-mdm2+/-* mice than in *p53-/-*; and sarcomas were significantly more common in *p53-/-mdm2+/-* mice. The possibility that his difference is due to genetic background is unlikely due to the way we backcrossed these mice. Thus, mice null for *p53* and heterozygous for *mdm2* differed significantly from double null mice, suggesting that heterozygosity at the *mdm2* locus altered not only the timing of tumor development, but also the tumor type.

The fact that the *p53-/-mdm2+/-* mice survive longer than *p53-/-mdm2+/+* mice suggests that the level of MDM2 is a critical determinant of tumor development. In *p53-/-mdm2+/+* mice, the absence of p53, hence loss of interaction with MDM2, would by default result in more MDM2 protein in the cell. Increased levels of MDM2 due to gene amplification have been observed in sarcomas and other tumors.[5-7] Moreover, the overexpression of *mdm2* in the breast epithelium of transgenic mice led to tumor development.[19] Thus, increased MDM2 protein in the cell can contribute to tumor development. The loss of one *mdm2* allele in *p53-/-mdm2+/-* mice would

then be expected to result in decreased levels of MDM2 and a reduction in the rate of tumorigenesis.

Tumor incidence in mice null for both *p53* and *mdm2* was not significantly different from that of *p53* null mice and is more difficult to explain. Perhaps the absence of interactions of MDM2 with other proteins augmented cell proliferation. MDM2 has been shown to bind the E2F1 transcription factor, and loss of E2F1 in mice leads to tumorigenesis.[21] If MDM2 is important for E2F1 function as a tumor suppressor[26,27], its loss could result in loss of a component of E2F1 function and tumorigenesis.

Another important observation is the increased number of sarcomas in *p53* null/*mdm2* heterozygous mice as compared to *p53* null and *p53/mdm2* double null mice. This observation further substantiates the critical nature of MDM2 levels in mesenchymal tissue. The amplification of the *mdm2* gene is seen more often in sarcomas than in any other tumor type.[5-7] The increased incidence of spontaneous sarcomas in the *p53-/-mdm2+/-* mice suggests that an insufficient level, as well as an excess, of MDM2 could contribute to multistep carcinogenesis in a cell type-specific manner. An independent study using a different deletion of *mdm2* yielded no significant difference in sarcoma incidence between mice of these three genotypes.[28] A tendency toward increased sarcomas in *p53-/-mdm2+/-* as compared to *p53-/-* mice, was observed in that study, but was not statistically significant. The small number of mice used in that study could account for lack of statistical significance.

This is an example of a phenotype present in heterozygous mice that is absent in null or normal mice and is probably due to the complex interactions between MDM2 and several proteins that are critical for the regulation of cell proliferation. Competition between these proteins and limiting amounts of MDM2 result in the observed phenotype. The generation of mice with a

targeted deletion of *mdm2* that would bypass the problem of embryo lethality should yield insight into this problem.

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Figure Legends.

Figure 1. Effects of a *mdm2* deletion on tumor latency in *p53* null mice. Thirty-four *mdm2* +/+ (solid triangles), 33 *mdm2* +/- (squares), and 58 *mdm2* -/- mice (circles), all null for *p53* and with tumors were used to calculate tumor latency. The percentage of mice remaining tumor free is plotted against age. The P value determined using Kaplan-Meier analysis is shown for all three genotypes in A and for *p53-/-mdm2+/-* (squares) and *p53-/-mdm2-/-* (circles) mice in B.

Figure 2. Effects of a *mdm2* mutation on tumor development in *p53* null mice. The frequency of lymphomas, sarcomas and mice with multiple neoplasms (mn) were compared between the different genetic backgrounds. The frequency of each type of tumor is relative to the total number of tumors observed in each genotype. Comparison of the frequencies between tumor type and genetic background by chi square analysis indicates a statistically significant difference in the incidence of lymphomas and sarcomas, p=0.007 and p=0.022, respectively, in *p53-/-mdm2+/-* mice as compared to the other two genotypes. White, gray and black bars represent *p53-/-mdm2+/+*, *p53-/-mdm2+/-*, and *p53-/-mdm2-/-* mice respectively.

Figure 3. Histopathologic features of malignant lymphomas arising in *p53-/-mdm2-/-* and *p53-/-mdm2+/-* mice. A) Thymic lymphoma arising in a *p53-/-mdm2-/-* mouse showing the characteristically high rates of apoptosis and mitosis. B) Section of heart showing epicardial involvement by thymic lymphoma with minimal involvement of the myocardium. C) Pulmonary involvement by thymic lymphoma showing expansion of the bronchovascular bundle with proximal extension into the alveolar septa. D) Hepatic involvement by lymphoma showing expansion of the portal tract and sinusoidal infiltration. E) Disseminated thymic lymphoma involving preferential infiltration of the splenic red pulp. F) Primary splenic lymphoma showing

replacement of splenic white pulp by high-grade large cell lymphoma. G) Renal dissemination of splenic lymphoma showing the characteristic capsular involvement. A focus of lymphoma showing expansion of interlobular vascular spaces is also present in the renal cortex.

Figure 4. Effects of a *mdm2* mutation on lymphoma dissemination in *p53* null mice. Twenty-two *mdm2*+/+, 19 *mdm2*+/- and 44 *mdm2*-/- mice, all of them null for *p53*, that had developed lymphoma were used to determine the tumor dissemination. Heart, lung, liver, spleen, kidney, and testis were fixed and examined by histopathology as described. Comparison of the frequencies between organ involvement and genetic background was performed using chi square analysis and is depicted above the appropriate bars. *, not significant. White, gray and black bars represent *p53-/-mdm2*+/+, *p53-/-mdm2*+/-, and *p53-/-mdm2-/-* mice respectively.

Figure 5. Histopathologic features of sarcomas. A) Characteristic histologic appearance of soft tissue fibrosarcoma showing interlacing fascicles of spindle cells. B) Occasional foci of tumor giant cells are observed in soft tissue sarcomas. C) Angiosarcoma demonstrating anastomosing pattern of vascular channels and considerable cellular pleomorphism. D) Osteosarcoma arising in a *p53-/-mdm2*+/- mouse showing nascent bone formation. E) Testicular teratoma arising in a *p53-/-mdm2-/-* mouse showing ectodermal and endodermal derived structures.

Table 1. Tumor spectrum in *mdm2* null, heterozygous, and wild type mice in a *p53*-null background

	mdm2+/+	*mdm2*+/-	*mdm2*-/-
Lymphomas	29[1] (85%)[2]	21 (64%)	52 (90%)
Sarcomas			
spindle cell carcinoma	6 (18%)	6 (18%)	5 (9%)
angiosarcomas	2 (6%)	7 (21%)	4 (7%)
osteosarcomas	-	2 (6%)	1 (2%)
Multiple neoplasms	5 (15%)	8 (24%)	8 (14%)
Teratoma	1 (3%)	1 (3%)	2 (3%)
Carcinoma	1 (3%)	2 (6%)	2 (3%)
Other neoplasms	1 (3%)	1 (3%)	1 (2%)

Thirty-four *mdm2*+/+, 33 *mdm2*+/- and 58 *mdm2*-/- mice, all of them null for *p53* that had developed a tumor were subjected to histopathologic analysis to identify the type of tumor.

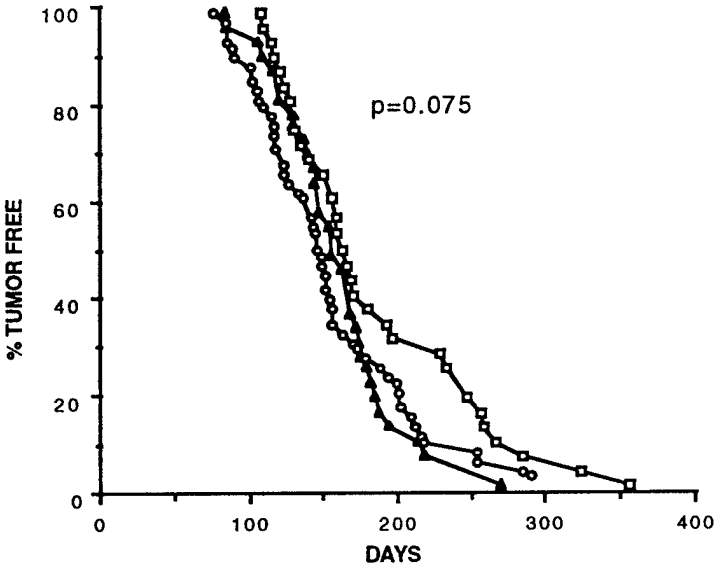
[1] One mouse had two lymphomas

[2] The percentages for each genotype will not equal 100% since multiple tumors arose in some mice.

Table 2. Incidence of lymphoma and its dissemination

	Heart	Lung	Liver	Spleen	LN	Kidney
p53-/-mdm2+/+						
Thymic (22 cases)	18/20(90%)[1]	16/19(84%)	10/20(50%)	4/20 (20%)	2/2(100%)	6/20(30%)
Splenic (7 cases)	0/5	3/6 (50%)	5/5(100%)	7/7(100%)	1/1(100%)	3/6(50%)
Peripheral (1 case)	-	-	+	-	+	-
p53-/-mdm2+/-						
Thymic (19 cases)	10/18(56%)	12/15(80%)	9/17(53%)	4/18(22%)	0/1	5/18(28%)
Splenic (1 case)	-	+	+	+	+ (MLN)	+
Peripheral (1 case)	+	+	+	+	+	+
p53-/-mdm2-/-						
Thymic (44 cases)	33/42(79%)	35/37(95%)	24/35(69%)	10/31(32%)	1/1(100%)	12/37 (32%)
Splenic (6 cases)	0/6	2/5(40%)	5/5(100%)	6/6(100%)	4/5(80%)	4/5(80%)
Peripheral (3 cases)	2/3(67%)	2/3(67%)	2/3(67%)	3/3(100%)	3/3(100%)	3/3(100%)

[1] only a fraction of the total number of samples were analyzed for dissemination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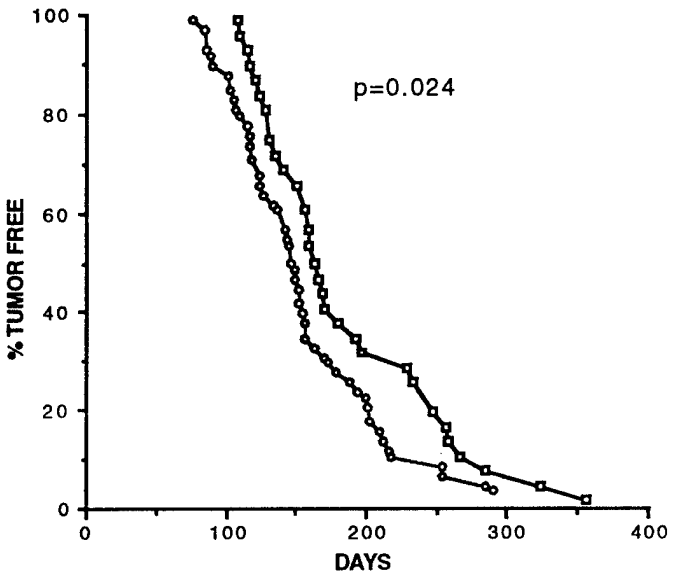
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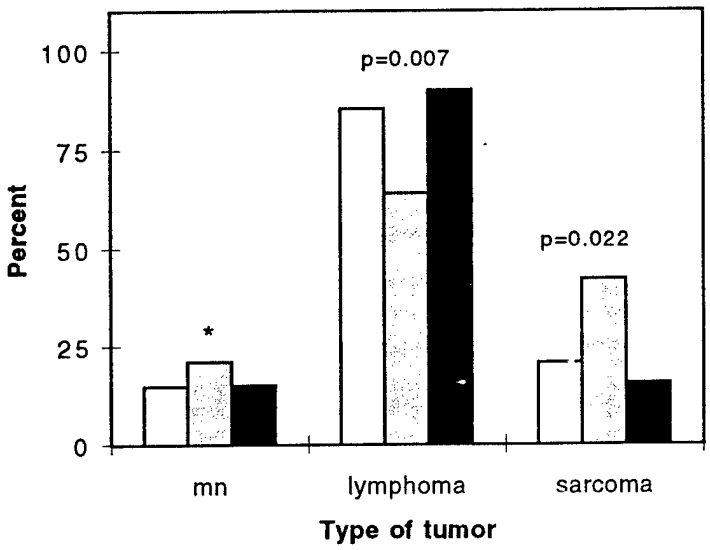
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lymphoma
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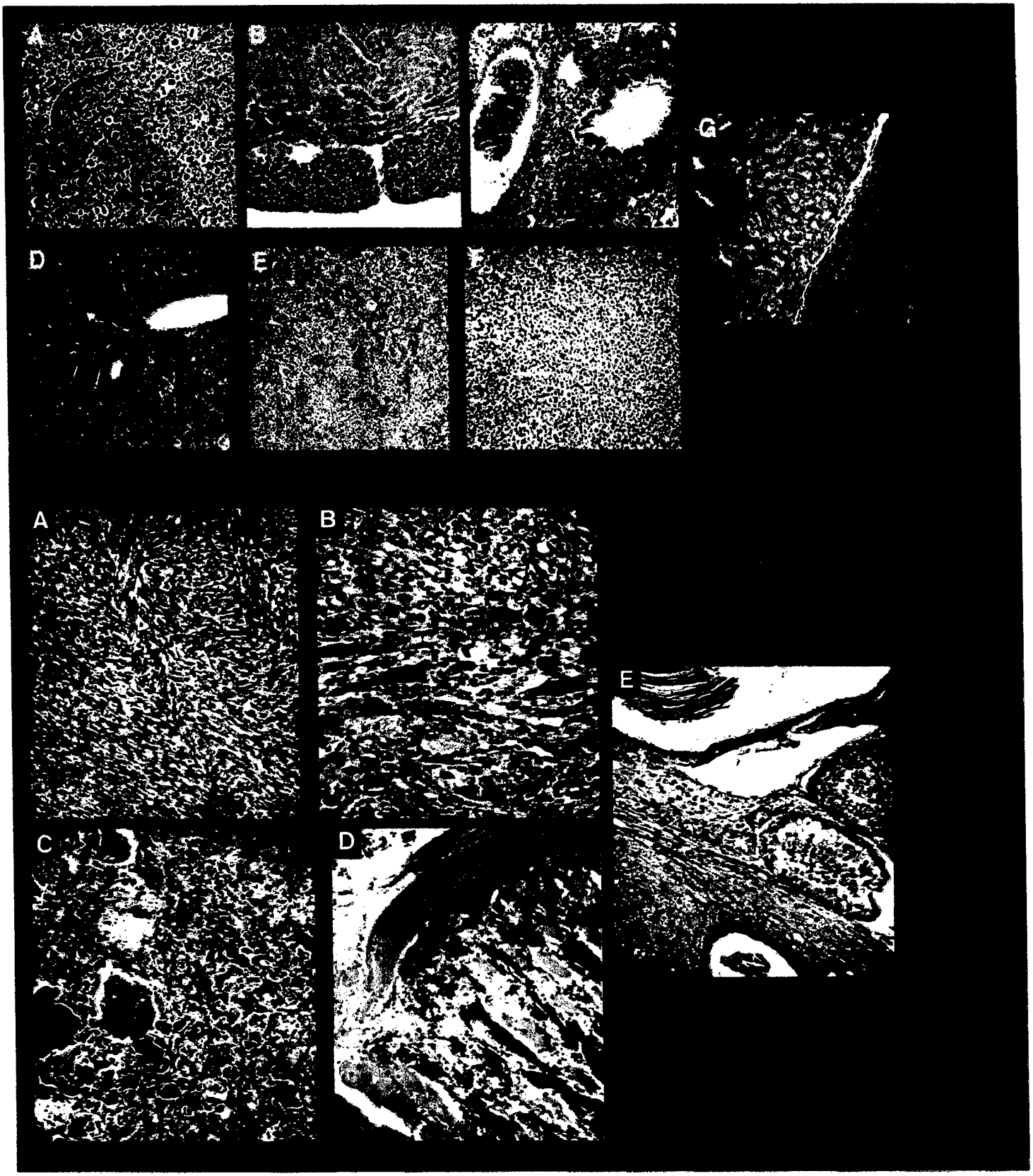
Type of tumor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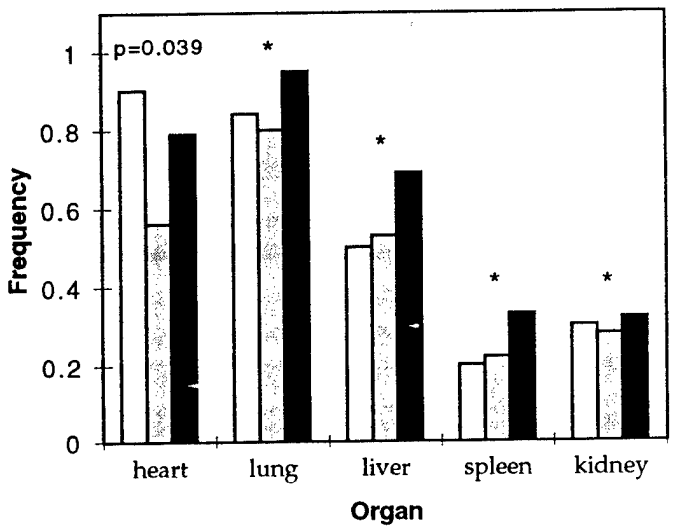
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Frequency
heart
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spleen
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Organ

Appendix 2

8/24/98

Overproduction of MDM2 *in vivo* Disrupts S-phase Independent of E2F1[1]

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[4]The abbreviations listed are: BLG, β-lactoglobulin

Running Title: MDM2 does not act through E2F1 *in vivo*

Abstract

Expression of an *mdm2* transgene (BLG*mdm2*) in the epithelial cells of the mouse mammary gland causes an uncoupling of S phase from M phase, resulting in polyploidy and tumor formation. The cell cycle defects are independent of interactions with p53. As MDM2 also binds and activates the S-phase-specific transcription factor E2F1, we hypothesized that increased E2F1 activity causes the development of the BLG*mdm2* phenotype. We therefore generated BLG*mdm2* mice that were null for *E2F1*. We observed no notable differences in histology or cyclin gene expression between BLG*mdm2* and BLG*mdm2/E2F1-/-* mice, indicating that endogenous E2F1 activity was not required for the BLG*mdm2* phenotype. Since, depending on the experimental system, either loss of E2F1 function or overexpression of E2F1 results in transformation, we also tested whether overexpression of E2F1 augmented the severity of the BLG*mdm2* phenotype by generating mice bitransgenic for BLG*mdm2* and BLG*E2F1*. We observed a unique mixture of the two single transgenic phenotypes histologically, and found no significant changes in cyclin levels, indicating that overexpression of E2F1 had no effect on the BLG*mdm2* transgenic phenotype. Thus, increased expression or absence of E2F1 does not affect the ability of MDM2 to disrupt the cell cycle.

Introduction

The most well understood function of the protein encoded by the *mdm2* oncogene is its ability to bind and inactivate the p53 tumor suppressor (1). Because *mdm2* is also a transcriptional target of p53 (2, 3), it thereby completes a feedback loop for the regulation of p53 activity. Genetic experiments in the mouse have illustrated the functionally significant relationship of these two molecules. The early embryonic lethality of mice lacking *mdm2* is completely rescued by deletion of *p53* (4, 5). These data demonstrate that MDM2 is both necessary and sufficient to regulate p53 activity in early mouse development. However, these experiments cannot address possible additional functions of MDM2 because the *mdm2*-deficient mouse dies at 5.5 days of embryogenesis.

Other lines of evidence indicate that MDM2 does indeed have a function in addition to regulation of p53. Most human tumors with an amplified *mdm2* gene retain a wild type *p53*, indicating that overexpression of MDM2 is sufficient to abrogate p53 activity (6). However, tumors that contain both a *p53* mutation and *mdm2* amplification have been identified and are associated with a worse prognosis than are tumors with a single alteration, indicating that a dual mutation can provide an additional growth advantage (7). Additionally, overexpression of MDM2 in tissue culture cells that lack p53 confers a transformation phenotype on those cells (8).

Finally, and most convincingly, overexpression of *mdm2 in vivo* leads to disruption of the normal cell cycle, independent of p53 (9). In these experiments, the β-lactoglobulin (BLG) promoter, which is active only in the pregnant and lactating mammary gland (10), was used to drive expression of an *mdm2* transgene (BLG*mdm2*) in the mouse. Mammary

glands from lactating female transgenic mice displayed abnormal development, marked histologically by the presence of abnormally large, polyploid epithelial cells. Additionally, these epithelial cells still synthesized DNA at a time when the cells of a normal gland would have ceased to proliferate and been fully differentiated. Most remarkably, this phenotype occurred even in a *p53-/-* background, indicating that increased MDM2 levels can affect cell proliferation and differentiation in this tissue independently of p53.

The mechanism by which MDM2 overproduction disrupts the coordination of DNA synthesis in S phase and cytokinesis is unknown. However, one possible model involves the ability of MDM2 to bind and stimulate the activity of the S-phase transcription factor E2F1/DP1 (Fig. 1) (11). During the G1 phase of the cell cycle, the tumor suppressor Rb binds and inhibits E2F1 activity (12-14). E2F1 is released as a function of Rb phosphorylation by cyclin-dependent kinases late in G1, and becomes transcriptionally active (15, 16). E2F1/DP1 then activates the expression of a number of genes involved in S phase, such as *cyclin E* (17), *dihydrofolate reductase* (18, 19), *thymidine kinase* (20), and *DNA Pol* α (17, 21). E2F1 is a potent facilitator of DNA synthesis, as quiescent cells in tissue culture can be driven into S phase by overexpression of E2F1 alone (22-24).

The creation of mice deficient for *E2F1* provides support for an opposing function of E2F1. Mice lacking *E2F1* are predisposed to hyperplasia and tumor development in certain tissues (25, 26). These data indicate that E2F1 can actually act as a negative growth regulator *in vivo*. The molecular mechanism for this activity is unknown, but has been attributed to the fact that E2F1 can promote p53-dependent apoptosis (23, 24, 27). Lack of p53 dependent apoptosis in *E2F1*-deficient mice (26) might accelerate tumor

formation. Additionally, E2F1, when complexed with Rb during the G1 phase of the cell cycle, can mediate the transcriptional repression of S phase genes (28-31). E2F1 thus provides both positive and negative control of S phase entry.

Since MDM2 binds E2F1 and stimulates its activity in S-phase, we asked whether this interaction was responsible for the cell cycle defects induced by MDM2 overexpression in mammary epithelial cells. To test *in vivo* the hypothesis that the BLG*mdm2* phenotype was due to increased E2F1-mediated transactivation of S phase genes, we generated mice carrying the BLG*mdm2* transgene in an *E2F1* null background. Histological analysis and examination of S phase demonstrated that E2F1 is not required for the development of the BLG*mdm2* phenotype. In addition, we generated bitransgenic mice by crossing mice that overexpress a BLG*E2F1* transgene in mammary epithelial cells which causes hyperplasia and increased apoptosis, with BLG*mdm2* mice. The mammary glands in mice overexpressing both the *E2F1* and *mdm2* transgenes showed a combination of the individual phenotypes histologically, and did not demonstrate a significant change in the amount of inappropriate DNA synthesis. The above experiments define an alternative pathway for MDM2 function *in vivo*, in addition to known interactions with p53 and E2F1.

Results

Increased Cyclin A, but Not D or E, Expression in BLG*mdm2* Mammary Tissue.

The phenotype of the BLG*mdm2* transgenic mouse was initially identified as a lack of cellular proliferation in the mammary gland during pregnancy and lactation (9). Additionally, a large percentage of the cells in the gland had enlarged, often multiple, nuclei, and were polyploid. This phenotype was most apparent at mid-lactation, a time when the wild-type gland has already completed proliferation and differentiation, and is at full capacity for milk production. In contrast, the BLG*mdm2* transgenic gland produced little milk, and the epithelial cells also displayed signs of continued DNA synthesis, as determined by BrdU incorporation (Fig. 2). To elucidate the molecular nature of the transgenic phenotype, we asked whether there were any alterations in the production of various cell cycle-regulated proteins at different stages of mammary gland development. Because cyclin expression is tightly regulated in various phases of the cell cycle, we focused on cyclin D1 (G1), cyclin E (G1/S), cyclin A1 (S/G2), and cyclin B1 (G2/M). To that end, we performed immunohistochemistry on wild type and BLG*mdm2* transgenic mammary glands at mid-pregnancy and mid-lactation, using antibodies raised against the various cyclins.

Two G1 cyclins tested, cyclin D1 and cyclin E, showed similar production patterns in both wild type and transgenic mammary glands. Specifically at day 14 of pregnancy, when growth factors are stimulating cyclin D1 production (15), mammary epithelial cells from both wild type and transgenic mice were positive for cyclin D1 (Fig. 2). This expression decreased somewhat in animals of either genotype by day 10 of lactation, when the cells are no longer receiving proliferation signals from the extracellular

matrix (Fig. 2). Cyclin E, a G1/S phase cyclin, was produced in most cells of the mammary gland of either genotype at day 14 of gestation (data not shown), or at day 10 of lactation (Fig. 2).

Immunohistochemistry performed with an antibody to the S/G2-specific cyclin A revealed an interesting difference between the wild-type and the BLG*mdm2* transgenic mammary gland. Cyclin A was detectable in both tissues during pregnancy, but during lactation only the BLG*mdm2* transgenic gland continued to be positive for cyclin A (Fig. 2).

We also tested for cyclin B production using two different cyclin B antibodies, but were unable to detect a noticeable level of expression (data not shown). The lack of cyclin B expression in the cells of the BLG*mdm2* mammary gland suggests that the cells do not receive signals for M phase.

BLG*mdm2* and BLG*mdm2/E2F1-/-* Mammary Glands Are Histologically Similar. Mice carrying the BLG*mdm2* transgene demonstrate a p53-independent function for MDM2. Specifically, in the presence or absence of p53, the mammary epithelial cells of BLG*mdm2* transgenic mice are hypoproliferative and become large, multinucleate, and polyploid. Because E2F1 is bound and activated by MDM2 in tissue culture (11), we examined whether E2F1 contributed to the transgenic phenotype (see Fig.1 for model). BLG*mdm2* mice were crossed with mice heterozygous for a null allele of *E2F1* (25). BLG*mdm2/E2F1*+/- F1 progeny were then backcrossed to *E2F1*+/- mice. The resulting BLG*mdm2/E2F1-/-* females were mated and sacrificed at day 10 of lactation. The mammary glands of these mice were then compared with the mammary glands of wild-

type, BLG*mdm2*, and *E2F1-/-* mice to determine whether there were histological differences, changes in cyclin gene expression, and BrdU incorporation which shows the extent of DNA synthesis.

The general hypoplasia and enlarged or multiple nuclei present in BLG*mdm2* transgenic mice were also present in BLG*mdm2* transgenic *E2F1* null mice but not in wild type or *E2F1* null mice (Fig. 3). Additionally, the extent of DNA synthesis was roughly equivalent in tissues carrying the BLG*mdm2* transgene regardless of the *E2F1* genotype (Fig. 3, Table I). In contrast, the wild-type and *E2F1-/-* cells of the mammary glands, which are histologically similar, showed virtually no BrdU incorporation (Fig. 3, Table I). Investigation into changes in expression of cyclin genes yielded no differences between genotypes, with the exception of cyclin A. As in the BLG*mdm2* tissue, cyclin A was produced in some, but not all, of the BLG*mdm2/E2F1-/-* cells, whereas there was no appreciable cyclin A production in the wild-type and *E2F1-/-* cells (Fig. 3). Surprisingly, cyclin E levels were not decreased in the absence of *E2F1*, as might be expected of an *E2F1* transcriptional target (Fig. 3) (17). These data demonstrated that there was no significant difference between BLG*mdm2* transgenic glands in the presence or absence of *E2F1*.

Overexpression of E2F1 Does Not Alter the Effects of MDM2

Overproduction. To extend the above observations, we further tested the hypothesis of E2F1 involvement in the BLG*mdm2* phenotype. If the ability of E2F1 to promote S phase is increased in the presence of MDM2, then simultaneous over production of E2F1 and MDM2 should exacerbate the BLG*mdm2* phenotype. To test this possibility, we utilized

mice that overexpress an *E2F1* transgene in the mammary gland (BLG*E2F1*) and display a hyperproliferation of cells and increased apoptosis (32).

To create mice that overproduce both E2F1 and MDM2, we crossed mice carrying the BLG*E2F1* transgene with BLG*mdm2* transgenic mice. Females carrying one allele of each transgene were sacrificed 10 days after giving birth, and the mammary glands in these bitransgenic mice were compared with those from mice carrying either of the two single transgenes. Histological examination of H&E-stained sections indicated that the bitransgenic mammary tissue had a unique mixture of the two single transgenic phenotypes (Fig. 4). Both small, hyperproliferative cells, indicative of E2F1 overproduction, and large, multinucleate cells, indicative of MDM2 overproduction, were apparent. Additionally, inappropriate DNA synthesis as measured by BrdU incorporation is present in bitransgenic mammary tissue at similar levels as either single transgenic gland (Fig. 4, Table I). No changes in the levels of cyclins A or E were observed in the bitransgenic compared to the single transgenic glands (Fig. 4), although increased cyclin E might be expected in the glands of mice carrying the BLG*E2F1* transgene. These data thus indicate that increased levels of E2F1 did not alter the phenotype of BLG*mdm2* transgenic glands, and further support the finding that E2F1 function is not required for the cell cycle defects in BLG*mdm2* transgenic mammary glands.

Discussion

Transgenic mice overproducing MDM2 exhibit a disruption of normal cellular proliferation in the mammary gland, in a p53-independent manner (9). This phenotype is marked by a decrease in mammary epithelial cell number, and the cells tend to contain enlarged or multiple nuclei that undergo multiple rounds of inappropriate DNA synthesis and can become polyploid. A molecular explanation for this phenotype is difficult to formulate, as very little is known about MDM2 function outside of its ability to inhibit p53 function. The first step in further characterization of the BLG*mdm2* phenotype was to identify the defective cell cycle stage by investigating cyclin gene expression, which pinpointed S phase as a likely candidate.

Analysis of cyclin gene expression in the mammary epithelial cells of wild type mice in comparison to the BLG*mdm2* transgenic mice revealed significant differences only in cyclin A levels. Cyclin A was produced at high levels in many cells of the transgenic mammary gland, but not the wild-type gland. These data reinforce the notion that DNA synthesis is aberrant in the MDM2-overexpressing cells and indicate that the cells are either stranded in S phase, perhaps because they are unable to recognize an exit signal, or are hypersensitive to signals which cause them to enter S phase inappropriately. Interestingly, a transgenic mouse overexpressing cyclin A in the mammary gland has been generated using the same β-lactoglobulin promoter used in this study (33). The overexpression of cyclin A caused nuclear abnormalities such as multinucleation and karyomegaly, and an increased number of apoptotic cells as compared to normal mammary epithelial cells. The absence of apoptosis in the mammary epithelial cells of the

BLG*mdm2* transgenic mice described here, even in the presence of high levels of cyclin A, may be due to the ability of MDM2 to inhibit p53-dependent apoptosis (34).

Rather surprisingly, cyclin E was present in virtually every epithelial cell of either wild-type or transgenic glands, possibly because the cells are arrested (or trying to arrest, in the case of the BLG*mdm2* transgenic gland) at the G1 phase of the cell cycle, or because cyclin E levels do not noticeably oscillate in this tissue.

Several proteins besides p53 have been identified that interact with MDM2. MDM2 binds $p19^{ARF}$, one of the proteins encoded by the *INK4a* locus (35-37). Since this interaction causes the degradation of MDM2, it is unlikely to be responsible for the phenotype of the BLG*mdm2* mice, which is due to the overproduction of MDM2. MDM2 has more recently been shown to bind Numb, a protein that participates in cell fate specification (38), but the functional significance of this interaction is not clear. The interaction of MDM2 with two other proteins, Rb and E2F1, could, however, result in the BLG*mdm2* phenotype. MDM2 can bind Rb and ultimately decrease its ability to arrest cells in G1 in tissue culture (39). Although it would be of interest to test genetically the relevance of this interaction in generating the BLG*mdm2* phenotype, the *Rb-/-* mouse dies during embryogenesis (40, 41), and there are no available mice overproducing Rb in the appropriate tissue. MDM2 can also bind E2F1/DP1 and increase its ability to activate transcription of S-phase genes in tissue culture cells (11). We therefore tested the possibility of an MDM2 interaction with E2F1 as the likely factor in creating the BLG*mdm2* phenotype. By crossing BLG*mdm2* mice with mice either lacking or overexpressing *E2F1*, we were able to determine that there was no requirement for E2F1 in the BLG*mdm2* phenotype.

MDM2 could regulate E2F1 function by a variety of mechanisms (see Fig. 1 for model). For example, it could lift the Rb/E2F1-mediated transcriptional repression of S-phase genes; it could encourage dissociation of Rb from E2F1; or it could act directly with E2F1 to activate S-phase genes. The ultimate result of all of these activities would be the promotion of S phase, one of the key findings in the mammary gland of mice overproducing MDM2. Therefore, we considered it quite possible that MDM2 was acting on E2F1 to cause inappropriate DNA synthesis. However, no changes were identified in BLG*mdm2* mice in the presence or absence of *E2F1* (see Fig. 3), indicating that the promiscuous activation of endogenous E2F1 by MDM2 was not a critical factor in the development of the BLG*mdm2* phenotype. Due to the multiplicity of E2F transcription factors, it is possible that endogenous E2F1 is not an essential regulator of the cell cycle in the epithelial cells of the mammary gland, but that a different family member performs such a function.

Because E2F1 appears to have dual functions and too little or too much E2F1 can lead to cell cycle defects, we also tested whether overproduction of E2F1 in conjunction with MDM2 could exacerbate the phenotype of BLGmdm2 transgenic mice. We tested this possibility *in vivo* by generating mice that overproduce both MDM2 and E2F1. Again, we did not see an increase in the severity of the BLG*mdm2* phenotype, nor did we see any major differences in the BLG*E2F1* phenotype (see Fig. 4). Instead, we saw a unique mixture of the two phenotypes. This observation is interesting because it demonstrates that neither protein is more dominant than the other. If the two were in the same pathway, then one would expect to see a preponderance of one or the other phenotype. These data, in combination with the lack of alterations of the BLG*mdm2*

phenotype in either the absence or overexpression of *E2F1* described in this report, leads to the conclusion that these two cell cycle regulators act independently of each other in this tissue.

While we have focused our discussion on MDM2-interacting proteins, the possibility also exists that it is the ability of MDM2 to function as a transcription factor that results in the BLG*mdm2* phenotype. MDM2 has many characteristics of transcription factors, including an acidic region, a nuclear localization signal, and a RING finger (42) and it can activate transcription when fused to the Lex A DNA binding domain (43). Thus, the overexpression of *mdm2* in the mouse mammary gland may result in the alteration of gene expression.

MDM2 therefore appears to be acting through a novel mechanism, when overproduced in the mammary gland of mice during pregnancy and lactation, to induce the phenotype of hypoproliferation, unregulated DNA synthesis, and polyploidy. Our findings effectively rule out interactions with both p53 and E2F1 as causes. What remains to be defined is the pathway by which MDM2 disrupts the normal proliferation and development of the mammary gland. Experiments addressing potential MDM2 transcriptional regulation or identifying novel MDM2-interacting proteins would possibly clarify this currently unanswered question.

Materials and Methods

Mouse Breeding and Genotyping. BLG*mdm2* transgenic mice (line TG3640) (9) were maintained as hemizygotes by crossing to C57Bl/6J wild type mice. 129/Sv-C57BL/6 mice heterozygous for a mutant E2F1 allele (*E2F1+/-*) were obtained from L. Yamasaki (25) and crossed with mice carrying the BLG*mdm2* transgene. BLG*mdm2/E2F1+/-* progeny were backcrossed with *E2F+/-* mice to generate offspring that were BLG*mdm2/E2F1-/-*. BLG*E2F1* transgenic mice (line TG3604) were crossed with BLG*mdm2* mice (line TG3640) to create mice that carried both transgenes in a hemizygous state.

The presence of the BLG*mdm2* transgene was determined by a dominant coat color marker that had been coinjected with the transgene and by PCR with transgene-specific primers BLG and MDM2 as described previously (9). *E2F1* heterozygous and homozygous mutant animals were identified by PCR as described previously (25), with the following exceptions. PCR was performed for both wild-type and mutant alleles together using 16 pmol each of L26 and L28 primers and 32 pmol of L31 primer per reaction. The PCR reactions (25 μl) were amplified using AmpliTaq (Perkin-Elmer) for 1 cycle (94°C 5 min), 35 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 1 min) and 1 cycle (72°C for 7 min). BLG*E2F1* transgenic mice were determined by PCR as described (32).

Nulliparous females of the appropriate genotypes were mated and sacrificed at day 14 or 18 of gestation or at day 10 of lactation. If necessary, pups born to BLG*mdm2* females were removed to a foster mother and replaced with slightly older pups to allow for continued nursing. Most animals were given an intraperitoneal injection of 100 μg of

BrdU in PBS/g body weight approximately 2 hours before sacrifice. The first abdominal (#4) mammary glands on both sides of the animal were dissected for analysis.

Immunohistochemistry. Mammary gland tissues were fixed in 0.4% paraformaldehyde in PBS at 4°C overnight, washed twice in PBS, and dehydrated through a graded series of ethanols, from 70% to 100%, according to standard procedures. The tissue was then incubated in xylene for 30 min. before it was embedded in paraffin. Sections were cut at 7-μm and placed on lysine-coated slides. After rehydration, the slides were incubated in 0.01 M citrate buffer pH 7.0 in a steamer for 25 min. for antigen retrieval and then soaked in 0.3% hydrogen peroxide in methanol for 15 min. After rinsing in PBS, the slides were blocked with serum from the Vectastain kit (Vector Labs, Burlingame, CA) and then incubated with the appropriate antibody for 1 hour at 37°C in a humidified chamber. MDM2 (9312) and cyclin E (1014) antibodies were rabbit polyclonal antisera raised by our laboratory and used at a 1:250 dilution. Cyclin A (C-19; polyclonal; 1:250) and cyclin D1 (72-13G; monoclonal; 1:100) were obtained from Santa Cruz Biotechnology. All immunohistochemistry was performed with the Elite Vectastain Kit for mouse or rabbit (Vector Labs) according to the manufacturer's instructions. Staining was detected with the substrate DAB (Vector Labs). All immunostained slides were counterstained with Nuclear Fast Red (Vector Labs) before dehydrating and mounting with Permount. BrdU immunostaining was performed using the BrdU Staining kit (Zymed, San Francisco, CA) according to the manufacturer's instructions.

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FIGURE LEGENDS

Fig. 1. Model for MDM2 involvement in the Rb/E2F1 pathway. MDM2 might prevent E2F1 function during G1-phase transcriptional repression when associated with Rb, or influence the Rb/E2F1 interaction itself, or promote the ability of free E2F1 to activate S-phase gene transcription.

Fig. 2. Analysis of cell cycle gene expression in wild type or BLG*mdm2* transgenic mammary glands. Wild-type and BLG*mdm2* mammary glands at day 10 of lactation were stained with H&E and photographed at 200X magnification. Immunohistochemical analysis was performed on wild-type or BLG*mdm2* mammary glands to measure the levels of BrdU and cyclins D, E, and A. All immunohistochemical analysis was performed on mammary glands taken at day 10 of lactation, with the addition of samples at day 14 of gestation for cyclin D and cyclin A. Microscopy for all immunohistochemistry was performed at 400X magnification.

Fig. 3. Histological comparison between BLG*mdm2* and BLG*mdm2/E2F1-/-* mammary glands. Mammary glands were taken at day 10 of lactation from mice with the following genotypes: wild-type, BLG*mdm2*, *E2F1-/-*, and BLG*mdm2/E2F1-/-*. The glands were then fixed, embedded, and sectioned. Immunohistochemical analysis for BrdU, cyclin E, and cyclin A was performed on sections of glands from mice of each genotype and photographed at 400X magnification.

Fig. 4. Histological comparison between BLG*E2F1* and BLG*mdm2*/BLG*E2F1* mammary glands. Mammary glands taken from mice transgenic for either BLG*E2F1* or BLG*mdm2*/BLG*E2F1* at day 10 of lactation were fixed, embedded, and sectioned. H&E staining as well as immunohistochemical analysis for BrdU, cyclin E, and cyclin A were performed. H&E sections were photographed at 200X magnification, and all sections for immunohistochemical analysis were photographed at 400X magnification.

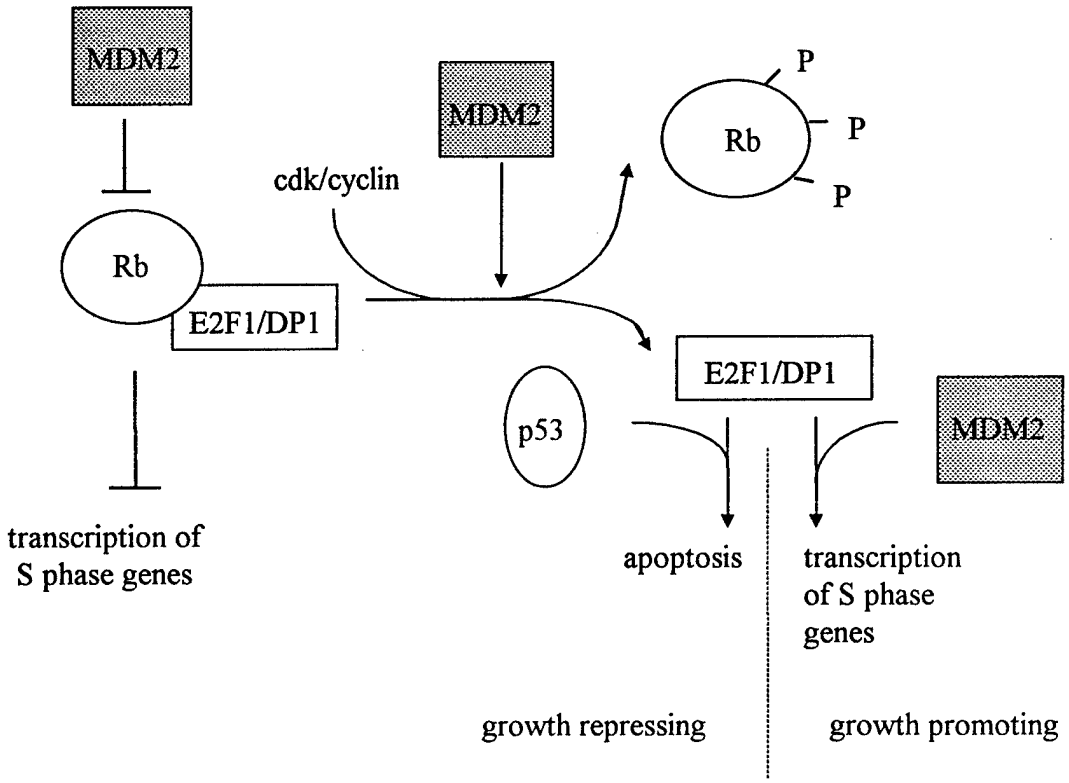
Table 1. Quantitation of BrdU incorporation[a]

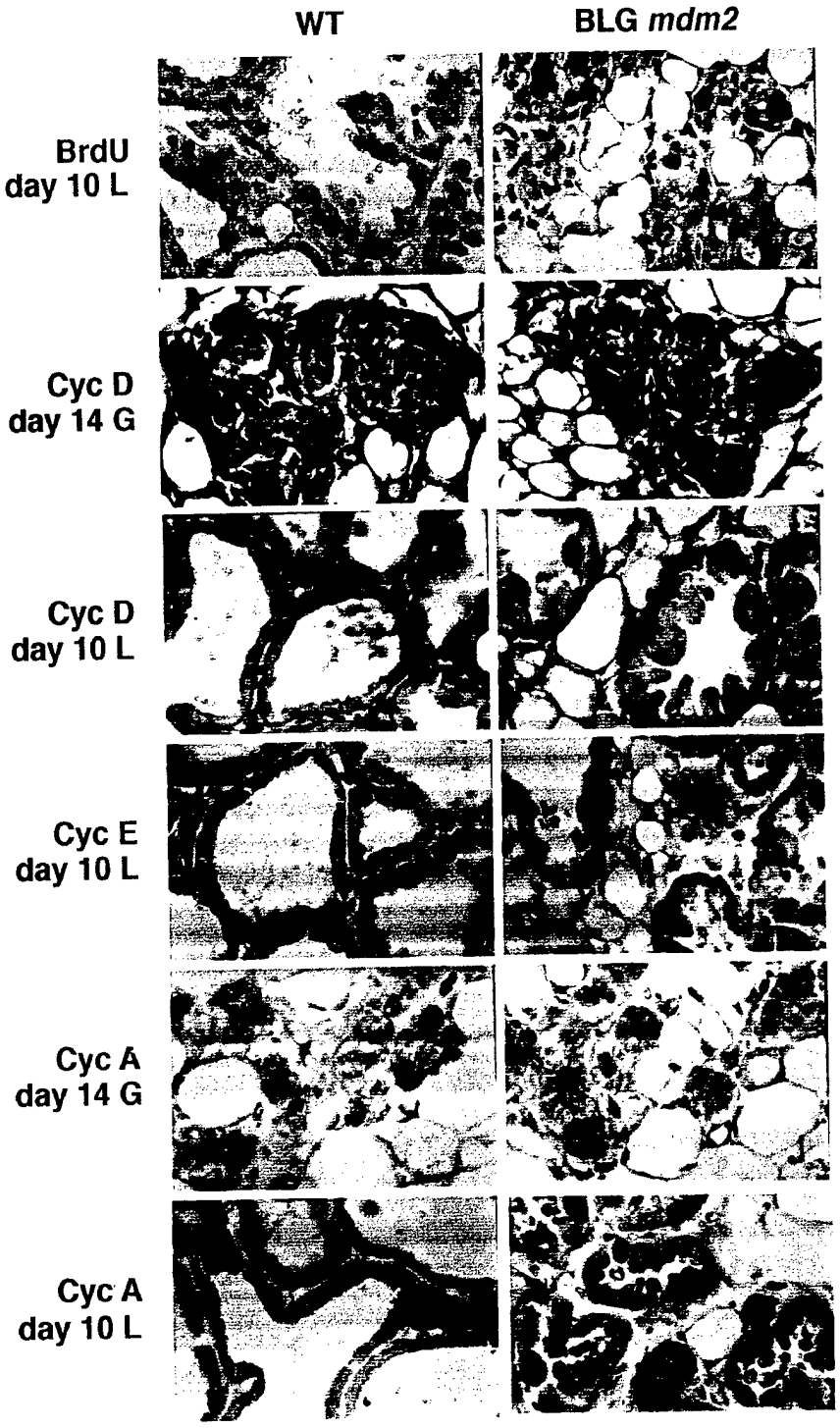
Genotype	No. of BrdU-positive cells/total[b]	% BrdU-positive cells
Wild-type	3/734	0.4
BLG*mdm2*	62/677	9.2
E2F1-/-	1/304	0.3
BLG*mdm2*/*E2F1-/-*	25/239	10.4
BLG*E2F1*	43/563	7.6
BLG*mdm2*/BLG*E2F1*	37/516	7.2

[a]Sections of mammary glands taken at day 10 of lactation from mice with the indicated genotypes were stained for BrdU incorporation.

[b]Cells from 2 to 5 fields for each genotype were counted and the number of BrdU-positive cells was divided by the total number of cells to determine the percentage of BrdU-positive cells.

FIGURE 1. Model for MDM2 and E2F1 interaction *in vivo*




WT
BLG mdm2
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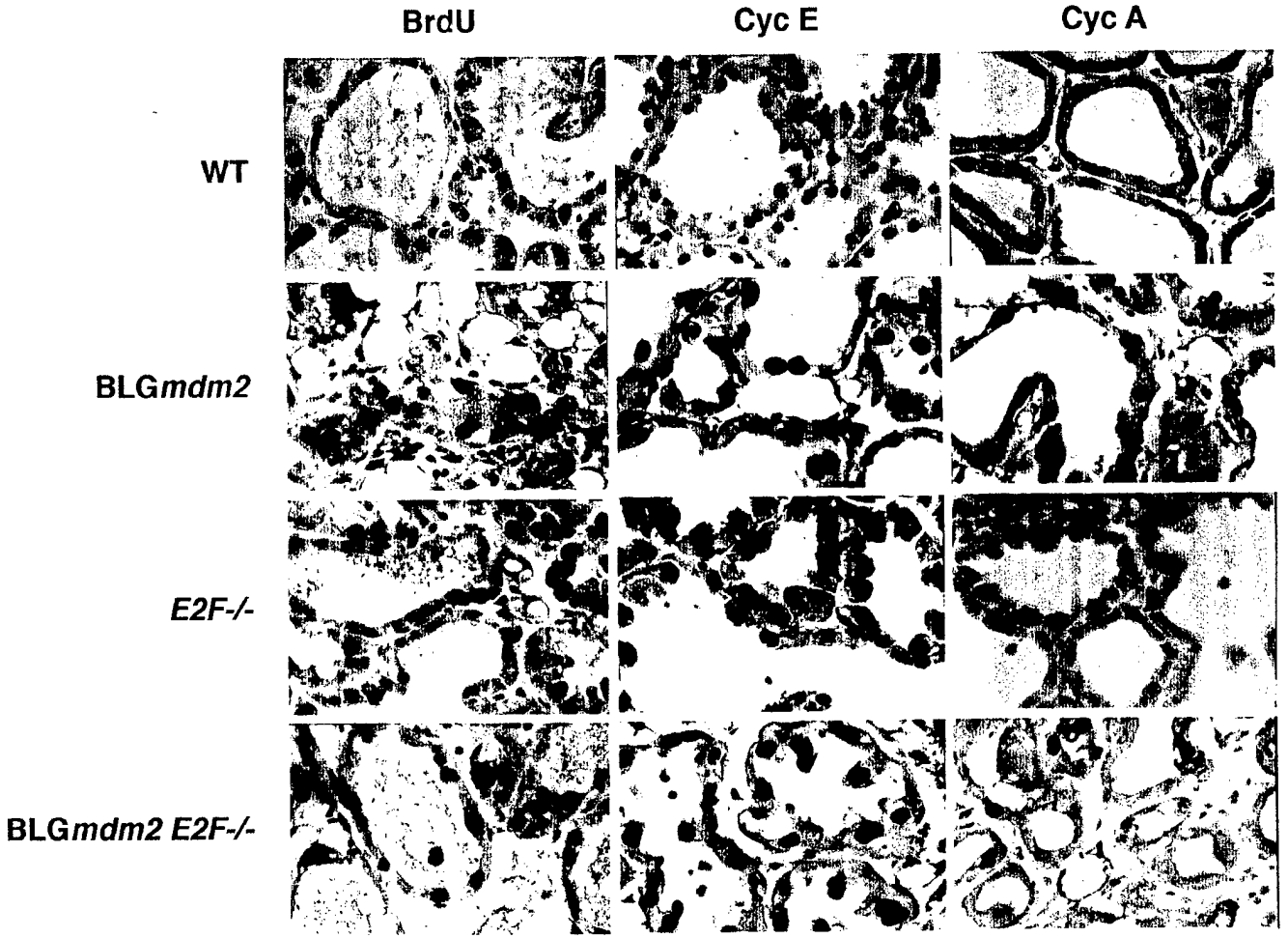
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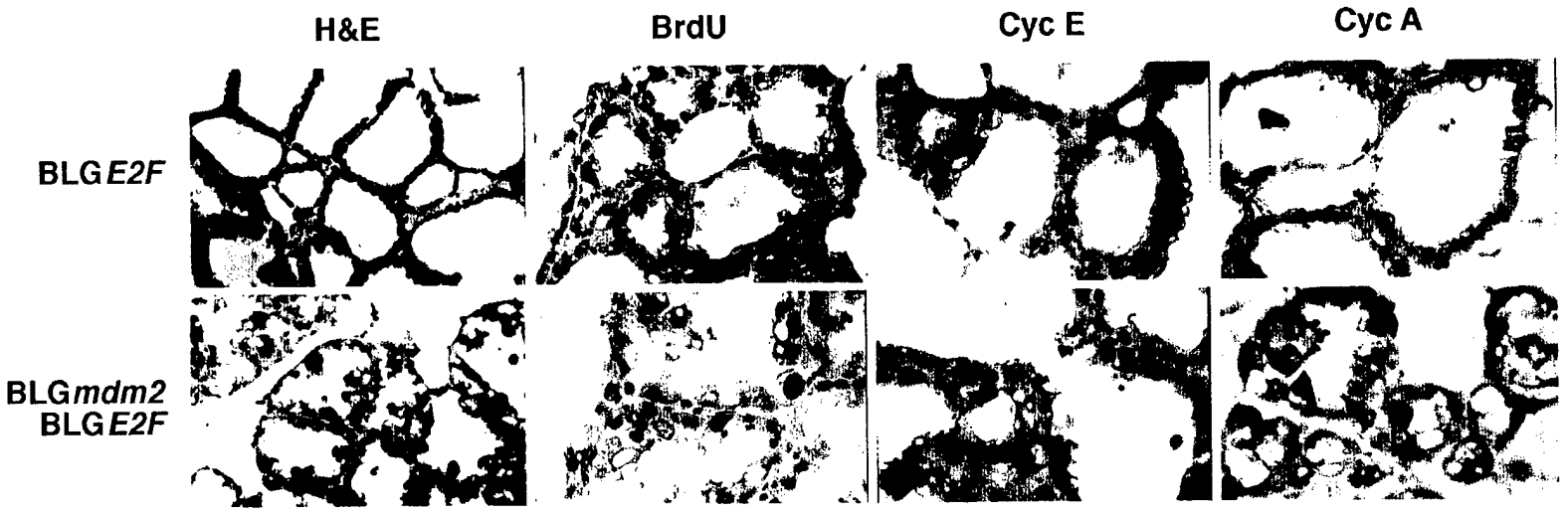
H&E
BrdU
Cyc E
Cyc A
BLG*E2F*
BLG*mdm2*
BLG*E2F*