CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Mouse Double Minute 2 Actively Suppresses p53 Activity in Oocytes during Mouse Folliculogenesis

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The p53 signaling network is indispensable in cellular stress responses and tumor suppression. Negative regulations of p53 by mouse double minute 2 (MDM2) and its homolog MDM4 are an integrated component of the network and have been implicated in regulating the stress responses and the maintenance of normal development and homeostasis of multiple somatic cell lineages. However, the regulatory role of MDM2 on p53 and stress responses in female germ cells remains undetermined. Here, we used the Cre-loxP system to delete *Mdm2* in oocytes at different stages of folliculogenesis in mice. *Mdm2* deletion resulted in a clear p53 nuclear accumulation in the oocytes and impeded fertilities with early follicular loss in mice, resembling human premature ovarian failure phenotypes. These phenotypes were fully rescued by concurrent deletion of *p53* in mice. In addition, Nutlin-3, a small molecule compound that inhibited the binding of MDM2 to p53, also promoted p53-dependent oocyte death. Although cancer therapeutic agents 5-fluorouracil and doxorubicin could not induce a robust p53 activation in the wild-type oocytes, they induced p53 nuclear accumulation in the *Mdm2* and *Mdm4* double heterozygous oocytes. These results demonstrated a critical prosurvival role for MDM2 in the oocytes. Moreover, they suggested a more tightened and rigorous regulatory mode for the MDM2/MDM4-p53 network in female germ cells under stress situations. (*Am J Pathol* 2017, 187: 339–351; <http://dx.doi.org/10.1016/j.ajpath.2016.09.023>)

Folliculogenesis is a tightly regulated and continuous process, and it can be easily disrupted by inner or outside influences, which may lead to ovarian dysfunction, even infertility. Premature ovarian failure (POF), which affects approximately 1% of women,¹ is defined as the occurrence of amenorrhea due to cessation of ovarian function in women younger than 40 years. Because many environmental factors have been linked to POF,² understanding the mechanisms of stress responses in female germ cells, especially in the meiotically arrested oocytes in the primordial follicles, may help to improve our knowledge of this disease and to develop new strategies for better intervention.

The protein p53 is known for its roles as a tumor suppressor and a master regulator of cellular stress responses. It plays a crucial role in maintaining genomic stability and in suppressing tumor development in various organs.³ In somatic cells, p53 is maintained at low levels and is almost

undetectable mainly through mouse double minute 2 (MDM2)-mediated ubiquitination and subsequent degradation.^{4,5} MDM4, a homolog of MDM2, plays a nonoverlapping and synergistic role with MDM2 in inhibiting p53.⁶ In response to various stresses, including DNA damage, ribosome stress, hypoxia, and oncogenic activation, the associations between p53 and MDM2/MDM4 become disrupted, leading to p53 stabilization and transcriptional activation,⁷ which resulted in cell cycle arrest, DNA repair, senescence, and apoptosis through expression of targeted genes such as *p21*, *Puma*, *Noxa*, and *Bax*.^{8–11}

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In females, p53 was found to regulate maternal reproduction by influencing blastocyst implantation through its target, leukemia inhibitory factor.^{12,13} However, how p53 regulates stress responses and female fertility in the germ cells is not completely understood. The ancient function of the p53 family might be the regulation of germ cell apoptosis. Studies in *Caenorhabditis elegans* indicated that p53 homolog CEP-1, which was most closely related to mammalian p53 family member p63, regulates germ cell apoptosis in response to DNA damage and meiotic recombination failure.^{14,15} In mice, p63 also appears to be required for apoptosis in oocytes of primordial follicles induced by irradiation or chemotherapeutic agents, such as cisplatin.^{16–19} Another study proposed that cisplatin was able to induce apoptosis of oocytes in the arrested primordial follicles by triggering a signaling network involving all three p53 family members (p53, p63, and p73), which was controlled by p63.²⁰ More recently, it was shown that both the meiotically programmed and exogenously induced DNA damage elicit a checkpoint requiring CHK2-mediated activation of both p63 and p53, with the deletion of *P63* alone insufficient for the longer term survival of the oocytes and rescue of infertility.²¹ Thus, these studies pointed to a combinatorial role of p63 and p53 in regulating apoptosis of the oocytes, quite distinct from the dominant role of p53 in somatic cells. The regulation of p53 functionality in the oocytes thus asks for further investigation.

In the present study, given the prominent role of MDM2 in regulating p53 activity in somatic cells, we aimed to examine the function of MDM2 in mouse oocytes at different stages of folliculogenesis and its impact on p53 signaling. We generated mutant mice with *Mdm2*-specific deletion in oocyte at different stages of folliculogenesis. These mice exhibited impaired fertility and early follicular loss phenotypes that were completely dependent on p53. We also found that MDM2 inhibitor Nutlin-3 was able to induce p53-dependent oocyte death. In addition, cancer therapeutic drugs 5-fluorouracil (5-FU) and doxorubicin were only able to activate p53 in oocytes in the presence of a combined reduction of gene dosages of *Mdm2* and *Mdm4*. These results suggested the possibility of a more tightened control of the MDM2-p53 network in oocytes during folliculogenesis.

Materials and Methods

Mice and Animal Care

C57BL/6J-*Gdf9-Cre* and C57BL/6J-*Zp3-Cre*^{22,23} mice obtained from the Nanjing University-Nanjing Biomedical Institute (Nanjing, China) were crossed to *Mdm2*^{FM}, *Mdm4*^{+/-} mice,^{24,25} and *p53*^{Flox} (*p53*^F) mice.²⁶ All of the *Mdm2*^{FM}, *Mdm4*^{+/-} and *p53*^{Flox} mice were backcrossed to C57BL/6 background for five generations. Mice were bred and maintained under specific pathogen-free conditions, and

experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the animal facility of Model Animal Research Center of Nanjing University, China.

Fertility Studies of Female Mice

To evaluate reproductive performance of the mice, six of each knockout and control group females were individually caged and bred to wild-type (WT) C57BL/6J males of known fertility at 5 or 6 weeks of age. The numbers of litters and pups produced were recorded over a 6-month period.

Serum Hormone Analysis

To evaluate serum hormone levels, mice were anesthetized by tribromoethanol, and blood was collected from the retro-orbital of the mice. Blood of adult female *Gdf9-Cre*; *Mdm2*^{FM/FM} and *Zp3-Cre*; *Mdm2*^{FM/FM} mice at 2 to 4 months of age were collected randomly due to the lack of regular estrus cycles; blood of littermate control female mice were collected at the proestrus stage based on vaginal smears.²⁷ Serum was prepared using blood collection tubes and stored at -20°C until assayed. Follicle-stimulating hormone (FSH) levels were assayed by the FSH Enzyme-Linked Immunosorbent Assay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols.

Oocyte Collection

To collect intact oocytes, oocytes from postnatal day (P) 7 to P14 mice were isolated by mechanical disruption of ovaries and follicles, followed by incubation in M2 medium (Sigma-Aldrich, St. Louis, MO) with trypsin (1 mg/mL; Sangon Biotech, Shanghai, China), collagenase (1 mg/mL; Sigma-Aldrich), hyaluronidase (1 mg/mL; Sigma-Aldrich), and DNaseI (10 µg/mL; Sigma-Aldrich) at 37°C for at least 10 minutes. Full-grown, germinal-vesicle-intact stage oocytes were collected by puncturing ovarian follicles from 3-week-old C57BL/6J female mice with a 30-gauge needle in M2 medium. The surrounding granulosa cells were separated from the oocytes by pipetting. For each sample, at least 20 oocytes were collected for immunofluorescent staining or placed in 1 mL of TRIzol (Invitrogen, Carlsbad, CA), snap-frozen, and stored at -20°C for RNA analysis.

Gonadotropin-Induced Superovulation

For superovulation experiments, four to five 3-week-old females (with the weight of 12 to 14 g) were injected intraperitoneally with 5 IU of pregnant mare serum gonadotropin (Ningbo Sansheng Pharmaceutical Co., Ltd., Ningbo, China) and 46 hours later with 5 IU human chorionic gonadotropin (Ningbo Sansheng Pharmaceutical Co., Ltd.). Cumulus-oocyte complexes were isolated from the ampulla 16 hours after human chorionic

gonadotropin injection and collected in M2 medium containing 1 mg/mL hyaluronidase (Sigma-Aldrich) to dissociate cumulus cells. The number of retrieved oocytes per female was recorded.

RNA Extraction and qPCR Analysis

RNA was isolated from the oocytes with TRIzol. For the RNA precipitation step, 20 µg of glycogen (Invitrogen) was added into 0.5 mL isopropyl alcohol and incubated at -20°C overnight. RNA was reverse transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. The paired primers were as follows: *Gapdh*, 5'-TCCAC-CACCCTGTTGCTGTA-3' and 5'-TGAACGGGAAGCT-CACTGG-3'; *Puma*, 5'-GCACCTAGTTGGGCTCCATT-CTG-3' and 5'-GTACGAGCG-GCGGAGACAAG-3'; *p21*, 5'-AGGCAGCGTATATCAGGAG-3' and 5'-CCTGACA-GATTTCTATCACTCCA-3'; *Mdm2*-exon5, 5'-ACGAGA-AGCAGCAGCACATT-3' and 5'-ACTCGGGACTCCAA-ACACAT-3'; *Bax*, 5'-AGACAG-GGGCCTTTTGTCTAC-3' and 5'-AATTCGCCGGAGACACTCG-3'; *Noxa*, 5'-TC-GCAAAGAGCAGGATGAG-3' and 5'-CACTTTGTCT-CCAATCCTCCG-3' (forward and reverse, respectively). The real-time quantitative PCRs (qPCRs) were performed with SYBR Green reagents (Invitrogen) on an ABI Prism Step-One Plus instrument (Applied Biosystems, Foster City, CA). *Gapdh* was used as endogenous control. The relative amount of target gene expression in knockout mice was calculated as fold change relative to littermate controls, after normalized to *Gapdh*.

Quantification of Ovarian Follicles and Histologic Analysis

Quantification of ovarian follicles was performed as previously described.²⁸ Briefly, ovaries were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. To count the numbers of follicles, paraffin-embedded ovaries were serially sectioned at 6-µm thickness and stained with hematoxylin and eosin for morphologic observation. Every fifth section was then analyzed by light microscopy for the presence of primordial, primary, secondary, and antral follicles. Only those follicles in which the nucleus of the oocyte was clearly visible were scored. For those values obtained by evaluating every fifth section, the cumulative follicle counts were multiplied by a factor of 5 to account for the fact that four-fifths of the ovary was not analyzed. Three to five ovaries were analyzed per genotype and per age.

Immunofluorescence and Immunohistochemical Analyses

Oocytes for immunofluorescence staining of p53 were collected and fixed in 2% paraformaldehyde for 30 minutes at 4°C . Then they were transferred to membrane

permeabilization solution [0.3% bovine serum albumin, 0.1% Triton X-100 in phosphate-buffered saline (PBS)] for 10 minutes. After permeabilization, oocytes were washed three times in $1\times$ PBS with 0.1% Tween-20, 5 minutes each. Then the oocytes were placed in blocking solution (0.3% bovine serum albumin, 0.1% Tween-20, PBS) for 2.5 hours. Oocytes were incubated with primary antibodies against p53 (dilution 1:500; CM5; Novocastra, Newcastle, United Kingdom) overnight at 4°C . Then oocytes were washed and followed by secondary antibodies conjugated with cyanine 3/fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hours at room temperature; omission of the primary antibody served as control in which case no signal was observed. The DNA was stained with DAPI (1 mg/mL; D6584; Sangon Biotech). The oocytes were then washed and mounted. For immunofluorescent analysis of p53 with frozen sections, mouse ovaries were washed with cold PBS and fixed in 4% paraformaldehyde for 1 hour at 4°C . The samples were washed three times in $1\times$ PBS, 10 minutes each at 4°C . Then ovaries were washed in 30% sucrose ($1\times$ PBS) at 4°C until they sunk to the bottom of the vial. The processed ovaries were then embedded in OCT and sectioned (10 µm in thickness). For immunofluorescent staining, frozen sections were incubated at 4°C overnight with antibodies for p53 (dilution 1:500; CM5; Novocastra). Fluorescence was evaluated using a Leica (Wetzlar, Germany) TCS SP2 confocal laser scanning microscope or Olympus (Tokyo, Japan) FV 1000 confocal microscope.

For immunofluorescence/immunohistochemical analysis on paraffin sections, antigen retrieval was processed by incubating the slides in 0.01 mol/L sodium citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0, 0.05% Tween 20) for 25 minutes in a steamer. Primary antibodies were as follows: anti-p53 (dilution 1:500; CM5; Novocastra), anti-cleaved caspase-3 (dilution 1:200; Asp 175; Cell Signaling Technology, Danvers, MA), anti-DEAD (Asp-Glu-Ala-Asp) box polypeptide 4/MVH (dilution 1:200; ab13840; Abcam, Cambridge, MA), anti-proliferating cell nuclear antigen (PCNA) (dilution 1:200; PC10; Cell Signaling Technology).

Western Blot Analysis

Mouse ovaries were lysed in RIPA buffer supplemented with protease and phosphatase inhibitor cocktail tablets (Roche, Basel, Switzerland). The membranes were incubated with anti-PCNA (dilution 1:1000; PC10; Cell Signaling Technology), and appropriate secondary antibody (dilution 1:3000; Sigma-Aldrich) sequentially.

In Vitro Ovarian Culture

Ovaries were removed from P5 female mice and placed on a piece of Millicell-CM membrane inserts (0.4 µm; Millipore, Bedford, MA) covered with only a thin film of culture medium. The medium for organ culture was Dulbecco's

modified Eagle's medium/F12 (HyClone, Logan, UT) supplemented with 1 mg/mL bovine serum albumin (Sigma-Aldrich), 1 mg/mL Albumax (Gibco BRL, Grand Island, NY), 50 µg/mL ascorbic acid (Sigma-Aldrich), 5 U/mL penicillin/5 µg/mL streptomycin (HyClone), and 27.5 mg/mL transferrin (Sigma-Aldrich). The organ cultures were maintained at 37°C and 5% CO₂ in modular incubation chambers (Thermo Scientific, Rockford, IL). For those cultures lasting >2 days, the medium was removed and fresh medium was added every 2 days. Three to five ovaries per treatment were cultured for each experiment. Experiments were repeated 3 times.

Statistical Analysis

Data were expressed as means ± SEM. For comparisons between two groups, statistical significance was determined using unpaired two-tailed *t*-test. *P* < 0.05 was considered statistically significant, and *P* < 0.01 and *P* < 0.001 were statistically highly significant.

Results

Deletion of *Mdm2* in Oocytes of the Primordial Follicles Results in Infertility in Female Mice

To determine the function of MDM2 in oocyte survival and growth, we crossed *Mdm2*^{FM/FM} mice, in which the exon 5 and 6 of the *Mdm2* gene were flanked by loxP sites,²⁴ with *Gdf9-Cre* transgenic mice. Expression of *Gdf9-Cre* is first detected in the oocytes of primordial follicles at P3.²² Oocytes from *Gdf9-Cre; Mdm2*^{FM/FM} females displayed >80% decrease in *Mdm2* mRNA levels compared with *Mdm2*^{FM/FM} controls at P7 (Supplemental Figure S1A). To investigate whether the deletion of *Mdm2* in oocytes influenced the reproductive performance of the mice, females were bred to WT males beginning at 5 weeks of age and continuously for 6 months. There were a steady average number of pups per litter (Figure 1A) and increased cumulative number of offspring produced by the *Mdm2*^{FM/FM} females (Figure 1B). In contrast, only two of six *Gdf9-Cre; Mdm2*^{FM/FM} females were able to give birth to a small number of offspring in the first month of mating, with all of the six mice producing no offspring thereafter (Figure 1, A and B). Consistently, we also found drastically reduced ovarian size (Figure 1C) and weight (Figure 1D) in *Gdf9-Cre; Mdm2*^{FM/FM} females at the age of 6 weeks, when the female mice normally reach their sexual maturity. Ovaries of *Mdm2*^{FM/FM} mice contained both growing follicles and numerous primordial follicles that would preserve their fertility for the rest of the reproductive life. In comparison, there was a complete lack of follicular structures in the ovaries of *Gdf9-Cre; Mdm2*^{FM/FM} mice with only corpus luteum remaining (Figure 1C). Moreover, elevated level of FSH, which was used for diagnosis of POF in humans,²⁹ was found in sera of 2- to 3-month-old

Gdf9-Cre; Mdm2^{FM/FM} mice (Figure 1E). Collectively, these results showed that deletion of *Mdm2* in oocytes of primordial follicles led to a phenotype resembling human POF in mice.

Rapid Depletion of Primordial Follicles in *Gdf9-Cre; Mdm2*^{FM/FM} Mice

The follicular resource available in the ovary is a direct determinant of female reproductive life span.^{30,31} To investigate how the absence of *Mdm2* in oocytes gradually led to infertility, ovarian structures of *Gdf9-Cre; Mdm2*^{FM/FM} and *Mdm2*^{FM/FM} females at P5, P8, and P25 were examined. Ovaries of both *Gdf9-Cre; Mdm2*^{FM/FM} and *Mdm2*^{FM/FM} females at P5 contained similar numbers of follicles, including mostly primordial follicles with small oocytes surrounded by flattened pregranulosa cells and a few activated follicles with enlarged oocytes surrounded by one or more layers of cubic granulosa cells (Figure 2, A and B). However, at P8, *Gdf9-Cre; Mdm2*^{FM/FM} ovaries contained few primordial follicles and a significantly reduced number of activated follicles, including primary and secondary follicles (Figure 2, C and D). At P25, the *Gdf9-Cre; Mdm2*^{FM/FM} ovaries appeared much smaller with the markedly reduced numbers of follicles. Healthy-looking primordial follicles and primary and secondary follicles in *Gdf9-Cre; Mdm2*^{FM/FM} ovaries were scarce, corresponding to 1.9%, 0.4%, 2.2% of those in *Mdm2*^{FM/FM} ovaries, respectively (Figure 2, E and F). Consistently, the weight of *Gdf9-Cre; Mdm2*^{FM/FM} ovaries was significantly reduced compared with the controls at both P25 and 3 months of the age (Figure 2G).

To study the rapid depletion of primordial follicles, immunohistochemical analysis of cleaved caspase-3 was performed to evaluate apoptosis. Apoptosis, particularly in oocytes of primordial follicles, was significantly elevated in ovaries of *Gdf9-Cre; Mdm2*^{FM/FM} mice (Figure 2, H and I).

Deletion of *Mdm2* in Oocytes of the Primary Follicles Leads to Follicular Loss and Decreased Fertility in Mice

The rapid depletion of primordial follicles in *Gdf9-Cre; Mdm2*^{FM/FM} ovaries makes it difficult to address the function of MDM2 in oocytes at other stages of folliculogenesis. To circumvent this problem, we generated mice with specific *Mdm2* deletion in oocytes of growing follicles using *Zp3-Cre* allele. The *Zp3-Cre* activity, which is first detected in P5 mouse ovaries, is restricted in oocytes of primary and further developed follicles.³² qPCR analysis showed that oocytes from *Zp3-Cre; Mdm2*^{FM/FM} females displayed a 48% and 89% decrease in *Mdm2* mRNA levels at P7 and P12, respectively (Supplemental Figure S1B).

Fertility testing was performed on *Zp3-Cre; Mdm2*^{FM/FM} and *Mdm2*^{FM/FM} control female mice. Compared with control females, *Zp3-Cre; Mdm2*^{FM/FM} females gave birth

Figure 1 *Mdm2* deletion mediated by *Gdf9-Cre* resulted in infertility in female mice. **A** and **B**: Six *Gdf9-Cre; Mdm2^{FM/FM}* and *Mdm2^{FM/FM}* female mice were individually caged and bred to WT C57BL/6J males of known fertility over a 6-month period. Average number of pups per litter from the mating in each month (**A**) and the cumulative number of pups over a 6-month period (**B**) are shown. **C**: H&E-stained ovary sections from 6-week-old *Gdf9-Cre; Mdm2^{FM/FM}* and *Mdm2^{FM/FM}* mice. **Inset**: images of ovaries by a light microscope. **D**: Average weight of ovaries obtained from 6-week-old *Gdf9-Cre; Mdm2^{FM/FM}* and *Mdm2^{FM/FM}* mice. **E**: FSH levels in sera of young adult (2- to 3-month-old) *Gdf9-Cre; Mdm2^{FM/FM}* and *Mdm2^{FM/FM}* mice. Data are expressed as means ± SEM. *n* = 6 (**A**, **B**, and **D**); *n* = 9 (**E**). ****P* < 0.001. Scale bar = 200 μm (**C**). FSH, follicle-stimulating hormone; H&E, hematoxylin and eosin; WT, wild-type.

to a significantly reduced number of pups per litter and total number of pups in the 6-month period of breeding (Figure 3, A and B). *Zp3-Cre; Mdm2^{FM/FM}* females also showed a significant reduction in ovarian weight compared with control mice at both P25 and 3 months of age (Figure 3C), although the phenotype was less severe than the *Gdf9-Cre; Mdm2^{FM/FM}* mice (Figure 2G). Follicular development of *Zp3-Cre; Mdm2^{FM/FM}* and control mice was further examined. The number of primordial follicles was slightly higher in *Zp3-Cre; Mdm2^{FM/FM}* mouse ovaries at P8, P25, and 3 months of age, although the difference was not statistically significant (Figure 3, D–I). At P8, the numbers of primary and secondary follicles were only slightly decreased (Figure 3, D and E). At P25, except for primordial follicles, the numbers of other follicles were all decreased in *Zp3-Cre; Mdm2^{FM/FM}* mice, with the primary follicles showing a milder reduction than the secondary follicles, corresponding to 41% and 4.7% of those in control ovaries, respectively (Figure 3, F and G). In addition, *Zp3-Cre; Mdm2^{FM/FM}* ovaries contained many degenerating oocytes and atretic follicles (Figure 3F). At 3 months of age, plenty of primary follicles were still found in the outer region of the medulla of the *Zp3-Cre; Mdm2^{FM/FM}* ovary, yet only few secondary and antral follicles remained (Figure 3, H and I). Collectively, these results suggested the

possibility that either the progression of the primary follicles to the secondary follicles was inhibited in the *Zp3-Cre; Mdm2^{FM/FM}* mice or that the oocytes of primary follicles were less sensitive to *Mdm2* deletion than those of the secondary follicles. In either case, the follicular development and survival beyond the stage of primary follicles were severely affected in *Zp3-Cre; Mdm2^{FM/FM}* mice. Consistent with their ovarian dysfunction, *Zp3-Cre; Mdm2^{FM/FM}* mice also displayed a significant increase in the serum FSH levels (Figure 3J).

It is well known that the status of oocyte closely influences the growth and survival of their surrounding granulosa cells.^{33,34} There was reduced proliferation of granulosa cells in the growing follicles of *Zp3-Cre; Mdm2^{FM/FM}* ovaries, as shown by immunohistochemical staining for PCNA in the ovarian sections (Supplemental Figure S2A). Western blot analysis also confirmed a dramatic decrease of PCNA in the *Zp3-Cre; Mdm2^{FM/FM}* ovaries (Supplemental Figure S2B). Meanwhile, apoptosis of granulosa cells, which was closely associated with follicular atresia,³⁵ was also found increased in the P25 *Zp3-Cre; Mdm2^{FM/FM}* ovaries (Supplemental Figure S2C). These results collectively suggested a suppression of follicular growth and an increase in follicular atresia in the *Zp3-Cre; Mdm2^{FM/FM}* mice.

Figure 2 Deletion of *Mdm2* in oocytes led to a rapid depletion of primordial follicles in the *Gdf9-Cre; Mdm2^{FM/FM}* mice. **A, C, and E:** Morphologic analysis of ovaries from *Gdf9-Cre; Mdm2^{FM/FM}* and *Mdm2^{FM/FM}* littermates. Ovaries from P5 (**A**), P8 (**C**), and P25 (**E**) *Gdf9-Cre; Mdm2^{FM/FM}* and *Mdm2^{FM/FM}* mice were embedded in paraffin, and serial sections of 6- μ m thickness were prepared and stained with H&E. Follicles were clearly identified by immunofluorescence staining for oocyte marker DDX4 (**right panel**, red). **Arrows in E** depict primordial follicles. **B, D, and F:** The numbers of follicles per ovary in P5 (**B**), P8 (**D**), and P25 (**F**) *Gdf9-Cre; Mdm2^{FM/FM}* and *Mdm2^{FM/FM}* mice were quantified as described in [Materials and Methods](#). **G:** Average weight of ovaries obtained from P25 and 3-month-old *Gdf9-Cre; Mdm2^{FM/FM}* and *Mdm2^{FM/FM}* mice. **H:** Representative micrographs of immunohistochemical staining of cleaved caspase-3 from ovaries of P5 *Gdf9-Cre; Mdm2^{FM/FM}* and *Mdm2^{FM/FM}* mice. **Arrows in H** depict apoptotic oocytes. **I:** Quantification of cleaved caspase-3–positive oocytes. Data are expressed as means $\pm$ SEM. $n = 3$ (**B, D, and I**); $n = 4$ (**F**); $n = 6$ (**G**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 100 μ m (**A, C, and E**); 20 μ m (**H**). DDX4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; H&E, hematoxylin and eosin; P, postnatal day.

p53 Activation Is Responsible for the Follicular Loss and Impeded Fertility Caused by the Deletion of *Mdm2* in Mice

MDM2 is the main negative regulator of p53 in somatic cells. To investigate whether loss of *Mdm2* resulted in

p53 activation in female germ cells, immunofluorescence analyses were performed with both the ovarian sections ([Figure 4A](#)) and isolated oocytes ([Figure 4, C and E](#)). Although p53 expression was undetectable in the oocytes of the control group, *Mdm2* loss resulted in significant p53 nuclear accumulation in oocytes from primordial and

Figure 3 Deletion of *Mdm2* in oocytes of the primary follicles led to follicular loss and decreased fertility in the *Zp3-Cre; Mdm2^{FM/ FM}* mice. **A** and **B**: *Zp3-Cre; Mdm2^{FM/ FM}* and *Mdm2^{FM/ FM}* female mice were individually caged and bred to WT C57BL/6J males of known fertility over a 6-month period. Average number of pups per litter from the matings in each month (**A**) and the cumulative number of pups over a 6-month period (**B**) are shown. **C**: Average weight of ovaries obtained from P25 and 3-month-old *Zp3-Cre; Mdm2^{FM/ FM}* and *Mdm2^{FM/ FM}* mice. **D**, **F**, and **H**: Morphologic analysis of ovaries from *Zp3-Cre; Mdm2^{FM/ FM}* and *Mdm2^{FM/ FM}* mice at P8 (**D**), P25 (**F**), and 3 months of age (**H**). Follicles are clearly identified by immunofluorescent staining for oocyte marker DDX4 (right panel, red). Arrows in **F** depict oocytes undergoing apoptosis or degeneration. Arrows in **H** depict primary follicles. **E**, **G**, and **I**: Quantification of follicle numbers in ovaries of P8 (**E**), P25 (**G**), and 3-month-old (**I**) *Zp3-Cre; Mdm2^{FM/ FM}* and *Mdm2^{FM/ FM}* mice as described in *Materials and Methods*. **J**: FSH levels in sera of 3- to 4-month-old *Zp3-Cre; Mdm2^{FM/ FM}* and *Mdm2^{FM/ FM}* mice. Data are expressed as means $\pm$ SEM. $n = 6$ unless specified otherwise. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bar = 200 μ m. FSH, follicle-stimulating hormone; P, postnatal day; WT, wild-type.

growing follicles of the *Gdf9-Cre; Mdm2^{FM/ FM}* mice (Figure 4, A–D). An increase in the nuclear intensity of p53 was also found in most oocytes isolated from the *Zp3-Cre; Mdm2^{FM/ FM}* mice (Figure 4, E and F).

Consistent with the p53 activation, qPCR analysis showed increased expression of proapoptotic genes *Puma*, *Noxa*, *Bax*, and the cell cycle inhibitory gene *p21*, all of which are p53 transcriptional target genes, in ovaries of both

Gdf9-Cre; Mdm2^{FM/ FM} and *Zp3-Cre; Mdm2^{FM/ FM}* mice (Figure 4, G and H).

To explore whether p53 activation in oocytes is responsible for the ovarian phenotypes displayed in the *Gdf9-Cre; Mdm2^{FM/ FM}* and *Zp3-Cre; Mdm2^{FM/ FM}* mice, we generated oocyte-specific *Mdm2* and *p53* double knockout mice. Fertility testing showed that the drastically reduced fertility caused by the *Mdm2* deletion was completely rescued in

Figure 4 Up-regulated p53 activity in oocytes of *Gdf9-Cre; Mdm2^{F/M}* and *Zp3-Cre; Mdm2^{F/M}* female mice. **A:** Representative images of p53 immunofluorescence (green) in frozen sections of ovaries from P7 *Gdf9-Cre; Mdm2^{F/M}* and *Mdm2^{F/M}* mice. **B:** Quantification of p53-positive-stained oocytes in the ovarian sections. **C:** Representative images of p53 immunofluorescence (green) in oocytes isolated from P7 *Gdf9-Cre; Mdm2^{F/M}* and *Mdm2^{F/M}* mice. **D:** Quantification of p53-positive-stained oocytes. **E:** Representative images of p53 immunofluorescence (green) in isolated oocytes from P12 *Zp3-Cre; Mdm2^{F/M}* and *Mdm2^{F/M}* mice. **F:** Quantification of p53-positive-stained oocytes. **G:** qPCR analysis of mRNA expression in oocytes isolated from P7 *Gdf9-Cre; Mdm2^{F/M}* and *Mdm2^{F/M}* mice. Approximately 60 oocytes were collected in each group. **H:** qPCR analysis of mRNA expression in oocytes isolated from P12 *Zp3-Cre; Mdm2^{F/M}* and *Mdm2^{F/M}* mice. Approximately 40 oocytes were collected in each group. Data are expressed as means $\pm$ SEM. $n = 3$. ** $P < 0.01$, *** $P < 0.001$. Scale bars: 25 μ m (**A**); 10 μ m (**C** and **E**). P, postnatal day; qPCR, real-time quantitative PCR.

both *Gdf9-Cre; Mdm2^{FM/FM}; p53^{F/F}* and *Zp3-Cre; Mdm2^{FM/FM}; p53^{F/F}* mice (Supplemental Figure S3, A and B). There were no differences in either ovarian size or structure of these two groups of mice compared with the *Mdm2^{FM/FM}; p53^{F/F}* control group at both P25 (Supplemental Figure S3, C and D) and even 8 months of age (Supplemental Figure S3, E and F), indicating normal follicular development in these mice. p53 was undetectable in the *Gdf9-Cre; Mdm2^{FM/FM}; p53^{F/F}* and *Zp3-Cre; Mdm2^{FM/FM}; p53^{F/F}* oocytes, further validating the specificity of p53 nuclear staining found in the *Gdf9-Cre; Mdm2^{FM/FM}* and *Zp3-Cre; Mdm2^{FM/FM}* oocytes (Supplemental Figure S3G). Meanwhile, expression levels of *Puma*, *Noxa*, *Bax*, and *p21* in the *Gdf9-Cre; Mdm2^{FM/FM}; p53^{F/F}* and *Zp3-Cre; Mdm2^{FM/FM}; p53^{F/F}* oocytes were also comparable with those in control littermates (Supplemental Figure S3, H and I). Collectively, these results demonstrated that the defects in the follicular development and survival observed in the *Gdf9-Cre; Mdm2^{FM/FM}* and *Zp3-Cre; Mdm2^{FM/FM}* mice were completely dependent on p53, indicating that regulation of p53 by MDM2 is indispensable during mouse folliculogenesis.

p53-Dependent Oocyte Death Induced by Nutlin-3

Nutlin-3 is a small molecule chemical compound that binds tightly and selectively to the p53-interaction domain of MDM2.³⁶ Treatment with Nutlin-3 has been shown to restore p53 activation for subsequent induction of apoptosis, senescence, or reversible cell cycle arrest in various model systems.^{37,38} However, the potential side effect of Nutlin-3 on female reproduction is still unclear. To determine whether Nutlin-3 could induce oocyte death similarly as genetic deletion of *Mdm2*, WT mouse ovaries at P5 were cultured with various doses of Nutlin-3 (2 to 10 $\mu\text{mol/L}$) for 4 days. With 2 and 5 $\mu\text{mol/L}$ of Nutlin-3 treatment, most of the primordial follicles were intact, and a few unhealthy follicles could be distinguished by pyknosis of granulosa cells and intense eosinophilic staining of oocytes (Figure 5A). Treatment with 10 $\mu\text{mol/L}$ of Nutlin-3, however, eliminated most of the follicles with only rare healthy oocytes remaining (Figure 5A). Forty-eight hours after treatment with 10 $\mu\text{mol/L}$ Nutlin-3, there was a significant increase in cleaved caspase-3—positive oocytes (Figure 5B). p53 was also found to be accumulated in the nucleus of the oocyte in the Nutlin-3—treated ovaries (Figure 5C).

To validate the function of p53 in oocyte in Nutlin-3—induced follicular loss, ovaries with oocyte-specific deletion of *p53* were also cultured in the presence of 10 $\mu\text{mol/L}$ Nutlin-3. Four days after the treatment, the prominent loss of normal primordial and primary follicles was effectively suppressed by the *p53* deletion (Figure 5, D and E). Although only 6.8% of primordial follicles remained after 4 days of 10 $\mu\text{mol/L}$ Nutlin-3 treatment in the control group, approximately 67% of primordial follicles with oocyte-specific *p53* deletion survived. Nutlin-3 treatment

also eliminated 93% of primary follicles in the control mouse ovaries. In contrast, the number of primary follicles was not significantly different in the untreated and treated *Gdf9-Cre; p53^{F/F}* ovaries (Figure 5E). These results suggested that drugs that directly disrupted the p53-MDM2 interaction were able to activate p53 in the oocytes and to induce follicular loss in mice.

5-FU and Doxorubicin Are Able to Activate p53 in *Mdm2* and *Mdm4* Double Heterozygous Oocytes

Many chemotherapeutic drugs, such as 5-FU and doxorubicin, have the ability to attenuate the MDM2-p53 interaction to activate p53 in somatic cells through various signaling mechanisms. To investigate whether these drugs could activate p53 in oocytes in the primordial and growing follicles, WT and control mice at P10 were treated with 5-FU and doxorubicin. Twenty-four hours after a single i.p. injection of either 5-FU (15 mg/kg) or doxorubicin (20 mg/kg), there was a significant increase in p53-positive immunostaining in the granulosa cells of the follicles (Figure 6, A and B). However, no p53-positive staining was detected in the oocytes of these mice (Figure 6, A and B), suggesting that these drugs were insufficient in mounting a robust p53 response in the short term.

Previous studies reported that MDM2 and MDM4 play synergistic roles in p53 inhibition.^{39,40} To investigate whether p53 could be activated by chemotherapeutic drugs in the presence of reduced gene dosages of *Mdm2* and *Mdm4*, mice double heterozygous for *Mdm2* and *Mdm4* in oocytes (*Gdf9-Cre; Mdm2^{FM/+}; Mdm4^{+/-}*, referred as *Gdf9-Cre; MM*) were derived by crossing *Gdf9-Cre* mice with the *Mdm2^{FM/FM}; Mdm4^{+/-}* mice and analyzed for p53 activation and sensitivity to drug treatment. Both the p53 immunostaining (Supplemental Figure S4A) and expression of p53 target genes (Supplemental Figure S4B) in the oocytes were not altered in the *Gdf9-Cre; MM* mice without the drug treatments. In addition, the *Gdf9-Cre; MM* mice displayed normal superovulation rates at 3 weeks of age (Supplemental Figure S4C). Analysis on ovarian structure also showed normal follicular development in these mice (Supplemental Figure S4D). However, 24 hours after a single i.p. injection of either 5-FU or doxorubicin, there was increased p53 accumulation in both granulosa cells and nucleus of oocytes in the P10 *Gdf9-Cre; MM* mice but not in oocytes of the control littermates, including *Gdf9-Cre; Mdm2^{FM/+}; Mdm2^{FM/+}; Mdm4^{+/-}*, and *Mdm2^{FM/+}* mice (Figure 6, A and B). Moreover, accelerated oocyte death or degeneration was found in the 5-FU—treated *Gdf9-Cre; MM* mice, whereas most of the oocytes remained healthy in the control mice with the same treatment (Figure 6C). Cleaved caspase-3 staining also confirmed that oocytes from the small follicles of *Gdf9-Cre; MM* mice were prone to apoptosis by the 5-FU treatment (Supplemental Figure S5). These results suggested that a combined and dose-dependent inhibition of p53 by MDM2 and MDM4 may

Figure 5 Nutlin-3 induced oocyte death required p53. **A:** H&E-stained sections of P5 WT ovaries cultured for 4 days with either DMSO or Nutlin-3 (2 $\mu\text{mol/L}$, 5 $\mu\text{mol/L}$, and 10 $\mu\text{mol/L}$, respectively). **B:** Immunofluorescent staining of cleaved caspase-3 (red) in paraffin sections of P5 WT ovaries cultured for 48 hours with either DMSO or Nutlin-3 (10 $\mu\text{mol/L}$). **C:** Representative images of p53 immunofluorescence (green) in frozen sections of P5 WT ovaries cultured for 48 hours with either DMSO or Nutlin-3 (10 $\mu\text{mol/L}$). **D:** H&E-stained sections of ovaries (**left and middle panels**) from P5 $p53^{F/F}$ and $Gdf9\text{-Cre}; p53^{F/F}$ mice cultured for 4 days with either DMSO or Nutlin-3 (10 $\mu\text{mol/L}$). Follicles are clearly identified by immunofluorescent staining for oocyte marker DDX4 (**right panel**, red). **E:** The average number of primordial and primary follicles per section is quantified in $Gdf9\text{-Cre}; p53^{F/F}$ and $p53^{F/F}$ ovaries cultured with DMSO or Nutlin-3 (10 $\mu\text{mol/L}$). Data are expressed as means $\pm$ SEM. $n = 4$ $Gdf9\text{-Cre}; p53^{F/F}$ ovaries (**E**); $n = 4$ $p53^{F/F}$ ovaries (**E**). $**P < 0.01$, $***P < 0.001$. Scale bars: 100 μm (**A** and **D**); 25 μm (**B** and **C**). DDX4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; DMSO, dimethyl sulfoxide; H&E, hematoxylin and eosin; P, postnatal day; WT, wild-type.

determine the sensitivity of oocytes to 5-FU and doxorubicin treatment.

Discussion

Control of p53 activity by MDM2 and MDM4 in somatic cells is essential for embryogenesis and organogenesis to

progress normally.^{41,42} Importantly, various stress stimuli activate p53 by relieving the negative regulations imposed by these factors. Thus, the negative regulations of p53 are a critical determinant for the execution of p53-mediated cellular responses. The differential role of p53 on DNA damage in mouse oocytes promotes the investigation of the control of the MDM2-p53 network in female germ cells. In this study, we demonstrated that the complete disruption of

Figure 6 Treatments of 5-FU and doxorubicin induced p53 activation in oocytes of *Gdf9-Cre; MM* mice. **A** and **B**: Immunofluorescent staining of p53 (green) in ovarian frozen sections of P10 *Gdf9-Cre; MM* mice and the control littermates 24 hours after a single intraperitoneal injection of 5-FU (**A**) or doxorubicin (**B**). **Arrows** depict positive stained nuclei. **C**: H&E-stained sections of ovaries from P12 *Gdf9-Cre; MM* mice and the control littermates 72 hours after a single intraperitoneal injection of 5-FU. **Arrows** depict intact oocytes in the **upper panel** and apoptotic oocytes in the **lower panel**. Scale bars: 50 μ m (**A** and **B**); 100 μ m (**C**). 5-FU, fluorouracil; H&E, hematoxylin and eosin; P, postnatal day.

the MDM2-p53 network in mouse oocytes resulted in impeded fertility and POF phenotypes. Thus, tight regulation of p53 by MDM2 is absolutely required for oocytes survival and folliculogenesis. Further study also suggested a dose-dependent regulatory role of MDM2 and MDM4 in inhibiting p53 activation in oocytes in the presence of chemotherapeutic drugs 5-FU and doxorubicin.

Oocyte-specific deletion of *Mdm2* resulted in a rapid loss of primordial follicles in the *Gdf9-Cre; Mdm2^{FM/FM}* females. Because the number of primordial follicles remaining in the ovary represents the ovarian reserve that is the direct determinant of the reproductive life span,⁴³ the early and rapid depletion of the primordial follicles due to oocyte death led to the infertility and POF phenotypes. In the subsequent study, *Mdm2* was deleted in the growing follicles in the *Zp3-Cre; Mdm2^{FM/FM}* mice. Although primordial follicles remained intact, there was a drastic loss of secondary and more mature follicles in these mice, leading to progressive reproductive failure and eventually the POF phenotypes. The follicular loss in this context may be due to oocyte degeneration and defective developmental progression of the follicles, eventually leading to follicular atresia. Transition from primary to secondary follicles is driven by local intraovarian autocrine and/or paracrine factors such as growth factors, cytokines, and steroids that are produced by individual component of the follicles, the oocyte, granulosa cells, and theca cells.³³ The

increased expression of growth inhibitory molecules due to *Mdm2* deletion may initiate the deterioration of the oocytes and may further disrupt the communications between the oocyte and granulosa cells, resulting in the loss of follicles, especially the secondary follicles. Therefore, the *Mdm2* deletion at different stages of folliculogenesis resulted in mouse phenotypes resembling human POF phenotypes with either early depletion of ovarian reserve or defects in follicular growth and maturation, respectively.

Increased nuclear accumulation of the p53 protein and the activation of p53 downstream target genes in the *Mdm2*-deleted oocytes were consistent with the role of MDM2 as a main negative regulator controlling p53 stability and activity. Importantly, concurrent deletions of *Mdm2* and *p53* rescued the reproductive defects in the *Gdf9-Cre; Mdm2^{FM/FM}* and *Zp3-Cre; Mdm2^{FM/FM}* mice, further demonstrating the conserved nature of the p53-MDM2 network in both somatic and germ cells. Therefore, MDM2 is actively suppressing p53 activity in the mouse oocyte for the survival and development of follicles. These results also suggested that MDM2 may not be regulating p63 and p73 as some other studies in somatic cells have suggested.^{44–47} Although both p63 and p73 or their orthologues have been implicated in monitoring germlines in various species, the regulation of p53 by MDM2 seemed to be highly specific and conserved in both

somatic cells and germ cells.⁴⁸ Nutlin-3 might represent an alternative to the current cytotoxic chemotherapy in particular for pediatric tumors and hematologic malignancies and is currently in a phase 1 clinical trial for the treatment of retinoblastoma.⁴⁹ Our findings here suggested that measures might need to be taken to preserve the fertility of the female patients when necessary with Nutlin-3 treatment.

A recent advance indicated a combinatorial role of both p63 and p53 in the response to DNA damage in the oocyte of primordial follicles because only the *p63/p53* double-deficient oocytes survived in the long term after the irradiation treatment.²¹ Although oocytes with *p53* deletion succumbed to rapid apoptosis, oocytes with *p63* deletion gradually died by 7 days,²¹ suggesting the possibility of a relatively slow dynamics of p53 activation in oocytes on stresses. Indeed, in sharp contrast to the granulosa cells, p53 is hardly detectable in oocytes even in the presence of chemotherapeutic drugs 5-FU and doxorubicin. However, both the treatment with Nutlin-3, which directly antagonizes p53-MDM2 binding, and genetic deletion of *Mdm2* in oocytes resulted in acute p53 activation and robust oocyte death, especially in primordial follicles, representing the full potential of p53 activation in oocytes. Moreover, 5-FU and doxorubicin treatment was able to promote significant p53 nuclear accumulation only in the oocytes of *Gdf9-Cre; MM* mice. *Mdm2* and *Mdm4* haploinsufficiency is known to lead to increased p53 activity in somatic cells.^{39,40} These results strongly suggested that the negative regulation of p53 is a key determinant of p53 activity and might be more strengthened in the oocytes versus somatic cells. Because 5-FU and doxorubicin could induce DNA damage as well as ribosome stress, both conditions of which are known to attenuate p53-MDM2 interactions in somatic cells^{50,51} via various signaling mechanisms, it is also possible that the stress-induced signaling pathways that led to the release of negative regulations on p53 might be less efficient in the oocytes. Future studies are required to address the exact mechanisms of such regulations in oocytes. Nonetheless, this study pointed to the conserved negative regulatory mechanism of p53 not only operative for the maintenance of the follicular reserve and their development but also linked to the degree of stress responses as dictated by p53 in oocytes. In addition, because p63 expression is restricted in the oocytes of small follicles, this study also revealed the effects of p53 activation in the deterioration and atresia of growing and large follicles. Given the obvious p53 activation in the granulosa cells of the large follicles in the presence of chemotherapeutic drugs, a combined effect of p53 in both oocytes and somatic cells may strongly influence the maturation of follicles and may lead to infertility under such circumstances.

Collectively, although p63 and p53 share the responsibility of monitoring germline in the presence of stresses, this study provided evidence that p53 alone is able to induce oocyte apoptosis, which is tightly and actively

suppressed by its inhibitors. The tightened control of p53 activity in germ cells in the presence of stresses may explain its predominant role in regulating stress responses of somatic cells to reduce the risk of malignant transformation.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.09.023>.

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