A Single Nucleotide Polymorphism in the *MDM2* Gene Disrupts the Oscillation of p53 and MDM2 Levels in Cells

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Abstract

Oscillations of both p53 and MDM2 proteins have been observed in cells after exposure to stress. A mathematical model describing these oscillations predicted that oscillations occur only at selected levels of p53 and MDM2 proteins. This model prediction suggests that oscillations will disappear in cells containing high levels of MDM2 as observed with a single nucleotide polymorphism in the *MDM2* gene (SNP309). The effect of SNP309 upon the p53-MDM2 oscillation was examined in various human cell lines and the oscillations were observed in the cells with at least one wild-type allele for SNP309 (T/T or T/G) but not in cells homozygous for SNP309 (G/G). Furthermore, estrogen preferentially stimulated the transcription of MDM2 from SNP309 G allele and increased the levels of MDM2 protein in estrogen-responsive cells homozygous for SNP309 (G/G). These results suggest the possibility that SNP309 G allele may contribute to gender-specific tumorigenesis through further elevating the MDM2 levels and disrupting the p53-MDM2 oscillation. Furthermore, using the H1299-HW24 cells expressing wild-type p53 under a tetracycline-regulated promoter, the p53-MDM2 oscillation was observed only when p53 levels were in a specific range, and DNA damage was found to be necessary for triggering the p53-MDM2 oscillation. This study shows that higher levels of MDM2 in cells homozygous for SNP309 (G/G) do not permit coordinated p53-MDM2 oscillation after stress, which might contribute to decreased efficiency of the p53 pathway and correlates with a clinical phenotype (i.e., the development of cancers at earlier age of onset in female). [Cancer Res 2007;67(6):2757–65]

Introduction

The tumor-suppressor p53 is known as the "guardian of the genome" because of its pivotal role in coordinating cellular response to genotoxic stressors and in maintaining genomic stability (1, 2). p53 activation by various stresses, such as DNA damage, hypoxia, aberrant oncogene activation, and cellular ribonucleotide depletion, initiates a transcriptional program, leading to apoptosis, cell cycle arrest, or senescence (2–5). The p53 pathway has been shown to be crucial for the tumor prevention. The *p53* gene is mutated in over 50% of all human tumors (6).

As a transcription factor, the p53 protein is kept at low concentrations in cells under nonstressed conditions until activated by stress, which results in transcriptional regulation of many target genes, including the *MDM2* gene. The p53 protein binds to the P2 promoter of *MDM2* gene and up-regulates MDM2 transcription. On the other hand, MDM2 inhibits p53 by regulating its location, stability, and transcriptional activity, which forms an important negative feedback loop to tightly regulate p53 function (4, 7, 8). In turn, decreased p53 activity results in decreased MDM2 levels. This p53-MDM2 feedback loop gives rise to oscillations, which have been observed in various cellular systems in response to stresses, including ionizing irradiation (IR) and UV (9–11). It has been shown that the p53-MDM2 oscillation occurs only when DNA damage signal reaches a threshold level (12). In MCF7 cells, no oscillation was observed at 0.3 Gy of IR, but was observed at a relatively higher dose of IR (5 Gy). The kinetics of p53 induction, the period of oscillation, and the relative induction fold of p53 over basal levels vary widely among different experimental systems. Although analysis of cellular populations reveal dampened oscillations of p53 and MDM2 after IR-induced DNA damage, the oscillations of p53 and MDM2 in single cells exhibits a "digital" response with discrete pulses of p53 and MDM2 (12, 13). The variables regulating these oscillations are not well understood, and it is unclear how prestress, endogenous MDM2 levels affect the p53-MDM2 oscillation.

We recently proposed a mathematical model that explained the digital oscillatory p53-MDM2 activity elicited after IR at the single-cell level. This model (14) replicated the observed experimental results for the p53-MDM2 oscillations at the single-cell level and at the population level. This model also predicted that proper levels of both p53 and MDM2 protein levels were needed to produce oscillations after DNA damage. Under the circumstances of too high or too low levels of p53 and MDM2 proteins, the oscillations would be compromised or would disappear (14).

A recent report identified a single nucleotide polymorphism in the MDM2 promoter (SNP309), a T-to-G change at nucleotide 309 in the first intron of MDM2. As a result of this SNP, there is increased binding affinity of the transcriptional activator, Sp1, resulting in elevated MDM2 expression and attenuated efficiency of the p53 pathway (15). This finding raises a possibility that high levels of MDM2 protein in SNP309 homozygous (G/G) cells may disrupt the regulation of the p53 protein and the observed oscillation. Moreover, SNP309 is located in a region of MDM2 promoter regulated by estrogen signaling (16–21). Because SNP309 increases the binding affinity for Sp1, a coactivator of multiple hormone receptors, it could potentially affect the hormone-dependent regulation of MDM2 transcription and result in further elevation of the MDM2 protein levels (22–24). In humans, SNP309 (G/G) is associated with an early age onset of, and increased risk for, tumorigenesis (15, 25–30). SNP309 (G/G) seems to have a more

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significant effect on accelerating tumor formation in a gender-specific (females) and hormonal-dependent manner (25, 29, 31). Therefore, it is of interest to study whether estrogen and SNP309 affect the regulation of MDM2 levels, which, in turn, may affect the oscillations of p53 and MDM2.

To test the model predictions and further understand the effect of MDM2 SNP309 on the function of the p53 pathway, the kinetics of production of p53 and MDM2 proteins were analyzed after stress in various human cell lines with different SNP309 genotypes (T/T, T/G, G/G). Coordinated oscillations of p53 and MDM2 proteins were observed in cells wild-type for SNP309 (T/T) but not observed in cells homozygous for SNP309 (G/G) after IR. We also checked the effect of SNP309 upon the regulation of MDM2 by estrogen. Estrogen preferentially stimulated the transcription of MDM2 from the SNP309 G allele and increased MDM2 protein levels in estrogen-responsive cell lines in a genotype-specific fashion. Regulation of basal p53 levels by tetracycline in H1299-HW24 cells showed an intermediate range of p53 levels for which the oscillations were observed. Increasing p53 levels in the absence of IR did not generate oscillation, indicating that DNA damage is an important trigger of the p53-MDM2 oscillation. These results suggest that disruption of the tight feedback loop of p53-MDM2 may be an important reason to dampen the efficiency of the p53 pathway and increase susceptibility to cancer in individuals carrying SNP309 (G/G) in the *MDM2* gene.

Materials and Methods

Cells, cell culture, and treatments. Human lung epithelial H460, and human breast cancer epithelial MCF7, T47D, and ZR75.1 cell lines were purchased from American Type Culture Collection (Manassas, VA). Human melanoma A875 cell line and human lymphoma Manca cell line were kindly provided by Drs. Donna George (Department of Genetics, University of Pennsylvania, Philadelphia, PA) and Andrew Koff (Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, NY), respectively. Human lung H1299-HW24 cell line that express wild-type p53 was established as previously described (32). p53 is expressed with low concentrations of tetracycline and is maximally expressed in the absence of tetracycline. H460 and A875 cells were maintained at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), MCF7 cells, T47D, ZR75.1, and Manca cells were maintained at 37°C in RPMI 1640 supplemented with 10% FBS. H1299-HW24 cells were maintained at 37°C in DMEM supplemented with 10% FBS, 250 μg/mL G418, and 1 μg/mL tetracycline. For estrogen treatment, cells were cultured in phenol red–free medium supplemented with 10% charcoal-stripped FBS for 3 days before being treated with various concentrations of 17-β-estradiol (E2; Sigma Chemical Co., St. Louis, MO) for 12 h. Doses of 5 to 50 Gy were used for cell irradiation (CIS BioInternational, Cedex, France; IBL 437C ^{137}Cs-irradiation source; dose rate, 5 Gy/min).

Protein analysis with Western blot. Radioimmunoprecipitation assay buffer was used to prepare total cell extracts, and 30 μg of total protein were separated on a 4% to 20% Tris-glycine gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane. MDM2 monoclonal antibody SMP14, p53 polyclonal antibody FL393, EFP polyclonal antibody H300, and β-actin antibody A5441 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Measurement of endogenous total p53 levels. H1299-HW24 cells, cultured with different concentrations of tetracycline, were collected, counted, and lysed in cell extraction buffer for ELISA (Biosource, Camarillo, CA). The p53 protein levels were measured using human p53 ELISA kit (Biosource) according to the manufacturer's instruction.

Quantitative real-time PCR. Total RNA was prepared from cells using an RNeasy kit (Qiagen, Valencia, CA) and was treated with the DNase I to remove residual genomic DNA. The cDNA was prepared with random primers using Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was done in triplicate with Taqman PCR Mix (Applied Biosystems) for 15 min at 95°C for initial denaturation, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s in the 7000 ABI sequence Detection System. Assay-on-demand for human MDM2 (Hs00242813_m1), human p21 (Hs00355782_m1), and human actin (Hs99999903_m1) were purchased from Applied Biosystems. Expression of MDM2 and p21 was normalized to the housekeeping gene *actin*.

Allele-specific chromatin immunoprecipitation assay. The allele-specific chromatin immunoprecipitation (ChIP) assay (33) was used to detect the transcription of MDM2 from each allele in MCF7, a SNP309 heterozygous cell line. MCF7 cells treated with or without estrogen for 12 h were cross-linked using 1% formaldehyde. Cells were washed, lysed with SDS lysis buffer, and sonicated. Antibodies against phosphorylated RNA polymerase II (Ser^5, MMS-134R clone H14; Ser^3, MMS-129R clone H5; Covance, Philadelphia, PA) were used to immunoprecipitate protein-DNA complexes. After extensive washing, protein-DNA complexes were eluted and cross-links were reversed at 65°C for 4 h. The protein was digested by proteinase K and DNA was purified by phenol-chloroform extraction and precipitated with ethanol. DNA was resuspended in TE buffer and ready for SNaP shot method. For SNaP shot, a section of DNA surrounding SNP309 was amplified by PCR using amplification primers (forward: 5′-CGCGG-GAGTTCAGGGTAAAGGTCA-3′, reverse: 5′-GCCCAATCCCGCCCAGACT-AC-3′). After PCR, residual primers and unincorporated deoxynucleotide triphosphates were removed using Antarctic phosphatase and *Exo*I. Purified PCR products were analyzed using a primer extension method. The extension primer was designed to anneal to the amplified template DNA immediately adjacent to the SNP309 site (5′-TTTTTTTTTTGGGCTGCGGG-GCCGCT-3′). Subsequent extension with DNA polymerase added a single fluorescent dideoxynucleotide triphosphate (ddNTP) complementary to the nucleotide at the polymorphic site. The extended primers, labeled with different fluorescent dyes, were run on an ABI 3730 capillary electrophoresis instrument and analyzed with Gene Mapper 3.0 software (Applied Biosystems). Relative transcription from each allele was calculated by normalizing peak area of each allele from ChIP to that from input.

Results

No p53-MDM2 oscillation in cells homozygous for SNP309 (G/G). Previously, a mathematical model was proposed to explain the coordinated p53-MDM2 oscillation in response to DNA damage caused by IR, and this model was able to recapitulate the p53-MDM2 oscillation observed in individual cells as well as cell populations (14). This model suggested that only when both p53 and MDM2 levels were within an optimal range or ratio the p53-MDM2 oscillation could occur. An increase of 2-fold or more in the basal or nonstressed levels of MDM2 protein would result in no oscillation of p53 and MDM2. Likewise, if the basal levels of p53 protein were too low (0.5-fold less than the level set in the model) or too high (6-fold higher than the level set in the model), the model predicted no detectable oscillation. Figure 1 shows the model predictions of the kinetic changes for p53 and MDM2 protein levels after IR at different p53 basal levels.

A recent report showed that SNP309 (G/G) in MDM2 enhanced transcription and protein levels of MDM2 (15). This raised a possibility that DNA damage–induced oscillations would not occur in cells homozygous for SNP309 (G/G) with higher levels of MDM2. Therefore, the effect of the high levels of MDM2 protein in cell lines homozygous for SNP309 upon the DNA damage-induced p53-MDM2 oscillation was determined. Several cell lines were selected based on their genotypes for SNP309 as described in a previous report (15). A875 and Manca are homozygous for SNP309 (G/G, high levels of MDM2); MCF7 is heterozygous for SNP309 (G/T, intermediate levels of MDM2); and H460 is T/T for SNP309 (low levels of MDM2). All these cell lines express wild-type p53 protein. The basal levels of p53 and MDM2 proteins were shown in Fig. 2*A*.

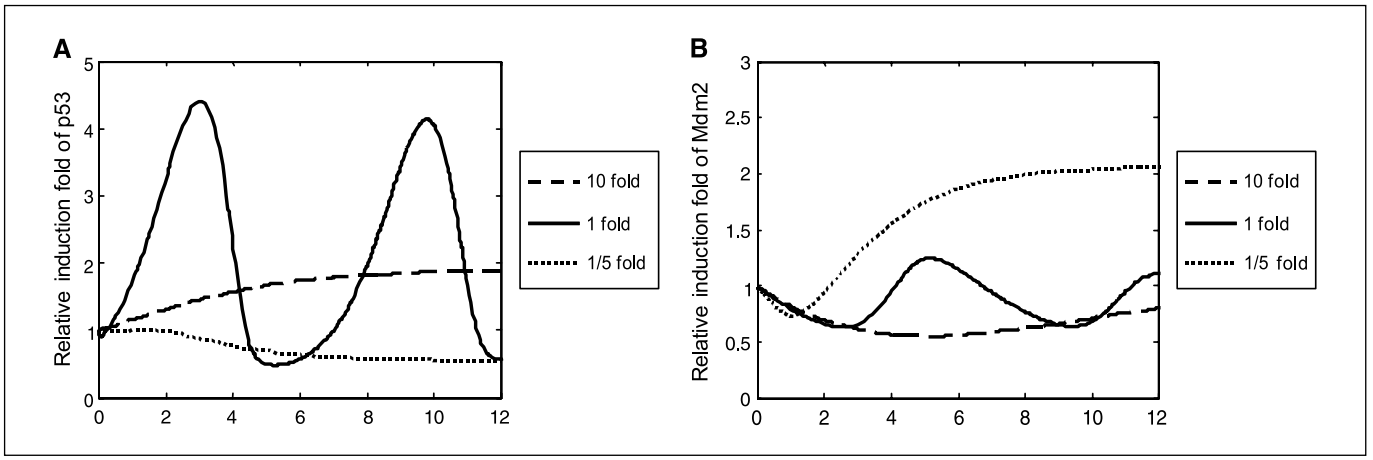
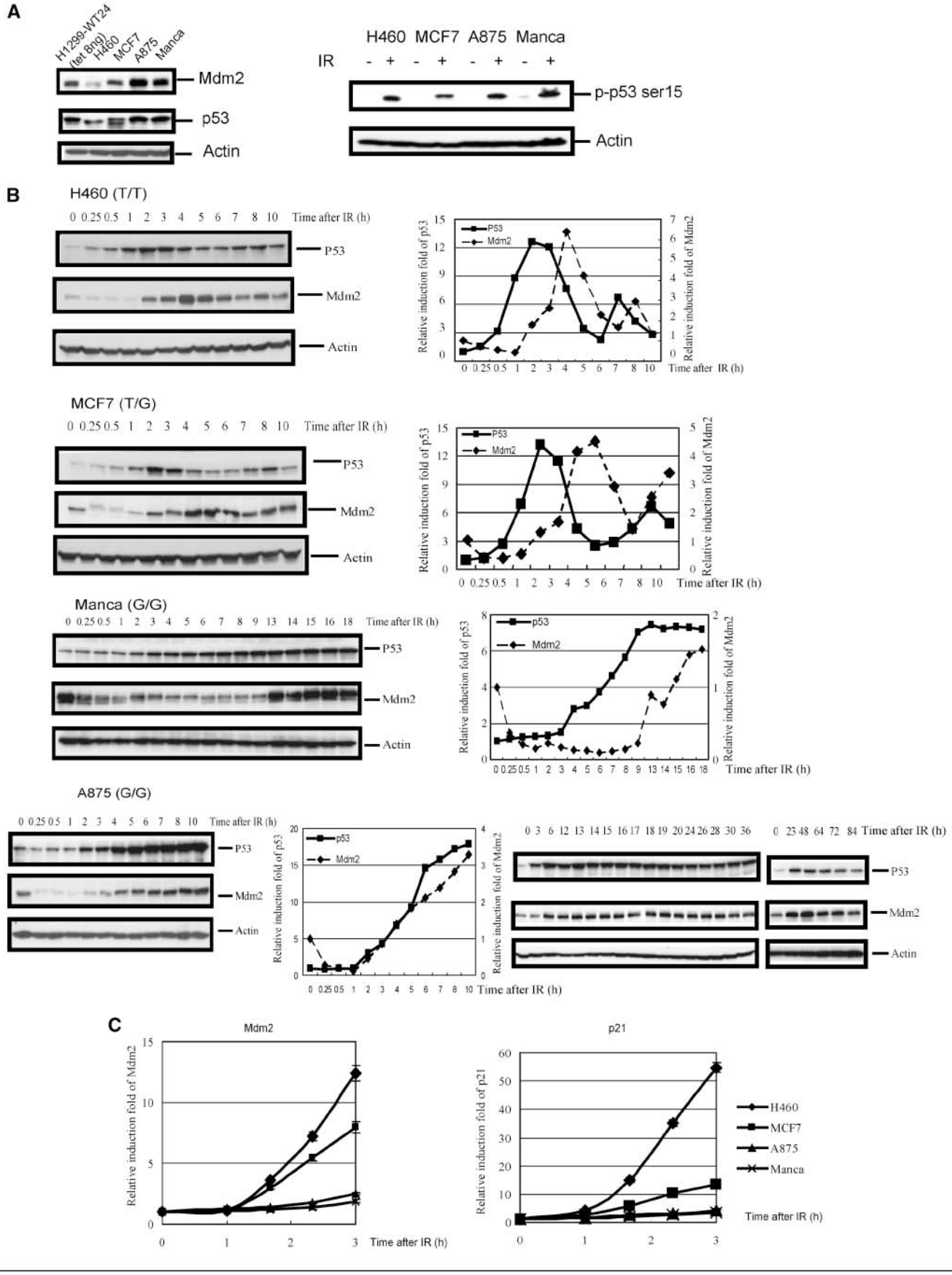


Figure 1. The kinetic changes of p53 and MDM2 after IR at different p53 basal levels predicated by the mathematical model. When basal p53 transcription rate is at its nominal level, both p53 (*A*) and MDM2 (*B*) oscillate after IR (*solid lines*). When p53 transcription rate is at the lower (2-fold down from nominal, *dotted lines*) or higher (10-fold up from nominal, *dashed lines*) levels, oscillation is disabled, and both p53 (*A*) and MDM2 (*B*) tend to stay at the steady-state values different from their basal levels after IR.

Oscillations were checked after exposure to 25 Gy IR, during which cell viability was >80% (10 h for H460 and MCF7, 18 h for Manca, and 84 h for A875 cells). Kinetic changes of p53 and MDM2 proteins were determined in cells wild-type or homozygous for SNP309. Although the levels of the p53 phosphorylation at Ser^{15} were similar among all these cell lines at 1 h after IR (Fig. 2*A*), the kinetic changes of p53 and MDM2 were cell line specific based on their genotypes. As shown in Fig. 2*B*, H460 cells, wild-type (T/T) for SNP309, showed coordinated oscillations, with a very similar pattern as previously reported (12, 13). The p53 protein accumulated rapidly and reached the first peak at ~2 h and the second peak at ~7 h after IR. MDM2 levels first decreased immediately after IR, as previously reported (34), but then increased starting 2 h after IR. There was a 2-h delay in peak accumulation relative to that of p53. Kinetic changes of p53 and MDM2 proteins after IR in cells heterozygous (T/G) for SNP309 (MCF7) showed a similar pattern of oscillation as shown in Fig. 2*B*. However, in cells homozygous (G/G) for SNP309 (Manca and A875), the p53-MDM2 oscillation was not observed within the same time frame (Fig. 2*B*). The levels of p53 showed delayed and slow increase with persistent elevation from 9 h after IR. MDM2 levels decreased for a longer period of time and slowly increased at a much later time after IR. To test if extended oscillation still existed in these cells but with a much broader width of each pulse, kinetic changes of p53 and MDM2 proteins in A875 cells were measured up to 84 h after IR. As shown in Fig. 2*B*, the accumulation of p53 and MDM2 was persistent and the oscillation was not observed within this long time frame. High levels of p53 protein remained 4 days after IR with maximal accumulation observed by 1 day after IR and then gradually declined. The levels of MDM2 reached maximal accumulation by 2 days after IR and then returned to basal levels. It has been reported that a stress signal below a certain threshold would not generate the p53-MDM2 oscillation. To exclude the possibility that cells homozygous for SNP309 (G/G) require high threshold of stress signals to generate the oscillation, the kinetic changes of p53 and MDM2 proteins were also measured in the same cell lines using a range of radiation doses (5–50 Gy). Although oscillations were observed in cell lines wild-type or heterozygous for SNP309 (H460 and MCF7), no oscillation was observed in cell lines homozygous for SNP309 after treatment with different doses of IR (data not shown). These results showed that cells homozygous for SNP309 (G/G) with the high levels of MDM2, p53, and MDM2 accumulation was observed after DNA damage, but coordinated oscillations of p53 and MDM2 were abolished.

The significantly slower rate of p53 activation and MDM2 induction, two important variables regulating the oscillation, in cell lines homozygous for SNP309. Coordinated oscillations of p53 and MDM2 require induction of MDM2 by p53 and, in turn, the negative regulation of p53 by MDM2. To generate the oscillation, variables that govern the p53-MDM2 interaction, such as degradation of p53, delay of MDM2 induction, and levels of p53 and MDM2 proteins, must reside in an optimal window of times and concentrations. It has been reported that the p53 response to DNA damage was attenuated in cells homozygous for SNP309 (G/G), including the compromised transcription induction of p53 target genes after stress (15). As shown in Fig. 2*C*, the induction of *p21*, a well-known p53 target gene, was much slower in the cell lines homozygous (G/G) for SNP309 than in the cell lines wild-type (T/T) or heterozygous (T/G) for SNP309. It is possible that the rate of p53 activation and MDM2 induction after DNA damage are too slow in cells homozygous (G/G) for SNP309, and the change of these two important variables may disturb the oscillation. As shown in Fig. 2*B*, the accumulation of p53 after DNA damage was much slower in cells homozygous (G/G) for SNP309 compared with the cells wild-type (T/T) or heterozygous (T/G) for SNP309. The induction of p53 in both cell lines homozygous for SNP309 (A875 and Manca) was not obvious until 3 h after DNA damage, whereas p53 was clearly induced within 1 h in the cell line wild-type for SNP309 (H460). The rate of p53 induction in MCF7 (heterozygous for SNP309) was intermediate. The induction of MDM2 transcription by IR in these cell lines was determined using real-time PCR. As shown in Fig. 2*C*, the cells wild-type (T/T) for SNP309 showed the fastest induction of MDM2 transcription with >10-fold induction at 3 h after IR. Cell lines homozygous (G/G) for SNP309 showed the slowest induction with ~2-fold induction at 3 h after IR. The induction of MDM2 transcription in the cell line heterozygous (T/G) for SNP309 was found to be intermediate with ~8-fold induction at 3 h after IR. Consistent with these data, the kinetic changes of MDM2 protein parallel the pattern observed for induction of MDM2 transcription. Figure 2*B* showed that the


A
H1299-WT24 (tet 8ng)
H460
MCF7
A875
Manca
Mdm2
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H460 MCF7 A875 Manca
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B
H460 (T/T)
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P53
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Actin
Relative induction fold of p53
Relative induction fold of Mdm2
Time after IR (h)
MCF7 (T/G)
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P53
Mdm2
Actin
Relative induction fold of p53
Relative induction fold of Mdm2
Time after IR (h)
Manca (G/G)
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Actin
Relative induction fold of p53
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Time after IR (h)
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Relative induction fold of p53
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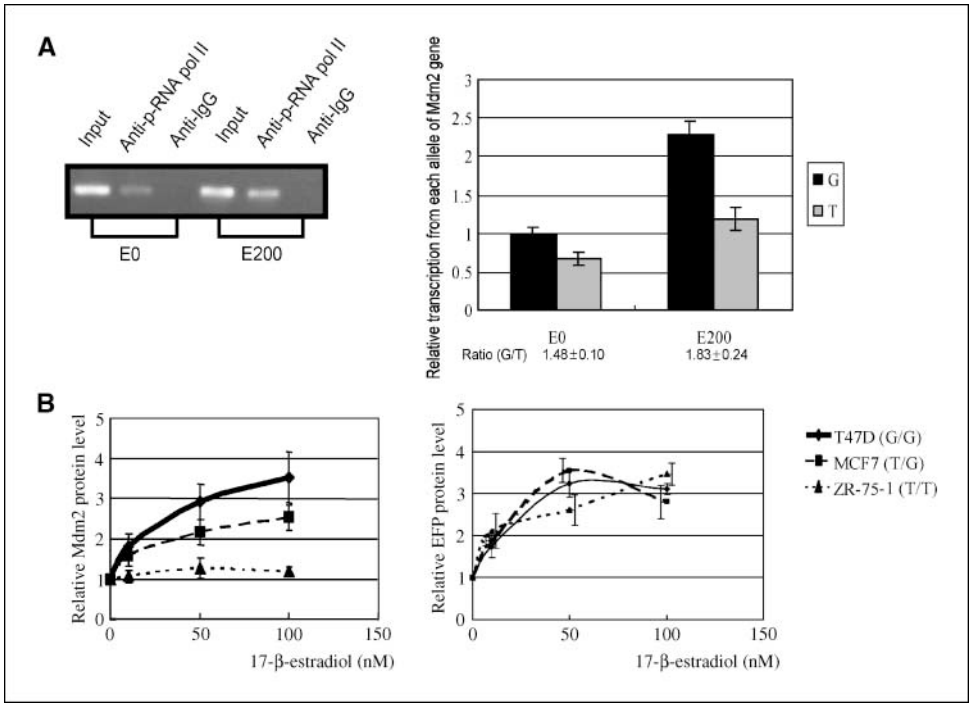

Figure 3. Estrogen preferentially increases MDM2 levels from SNP309 G allele. *A,* allele-specific quantification of RNA polymerase loading on the MDM2 promoter region was done in MCF7 cells with and without estrogen treatment. *Left,* MCF7 cells were cultured with (*lanes 1–3*) or without (*lanes 4–6*) estrogen for 12 h followed by ChIP assay with antibody against phosphorylated RNA polymerase II (*lanes 2* and *4*) or antibody against IgG (*lanes 3* and *5*). *Right,* the transcription of each allele was determined by SNaP shot using the immunoprecipitated DNA fragment from ChIP assay as template. An extension primer annealed to the DNA template immediately adjacent to the SNP309 site was used to add a single fluorescent ddNTP complementary to the nucleotide at the SNP site. The extension products were run on an ABI capillary electrophoresis instrument and relative transcription from each allele was calculated by normalizing peak area of G and T alleles from ChIP to that from input. *Columns,* average of at least three independent experiments; *bars,* SD. *B,* three ER^+ breast cancer cell lines with different genotypes for SNP309 were treated with various concentrations of E2 for 12 h. The MDM2 (*left*) and the EFP (*right*) protein levels were measured by Western blot and normalized to the levels of β-actin. *Points,* average of three independent experiments; *bars,* SD.

induction of the MDM2 protein was much slower in both cell lines homozygous (G/G) for SNP309 compared with H460 (T/T). The transcription of the MDM2 was also monitored at the very early time points after IR (15 min to 1 h), and no decrease of MDM2 transcription after IR was observed (data no shown). This suggested that the immediate decrease of the MDM2 protein levels after DNA damage was due to increased degradation of the MDM2 protein instead of decreased transcription of MDM2 (34). These data together showed the clear difference in the rate of p53 activation and MDM2 induction between cells homozygous (G/G) and wild-type (T/T) for SNP309. The rate of p53 activation and MDM2 induction were significantly slower in cells homozygous (G/G) for SNP309, and appeared to fall out of the proper range in which our model predicted oscillation could occur.

Estrogen preferentially increased MDM2 levels in SNP309 homozygous cells. Several reports have shown that estrogen receptor–positive (ER^+) breast tumors and breast cancer cell lines have higher MDM2 mRNA and protein levels (16–20). It has been reported that a region of P2 promoter is involved in regulation of MDM2 expression by estrogen (21). Interestingly, SNP309 is located only 10 bp away from the potential ER-responsive region of the promoter. Because SNP309 increases the binding affinity toward Sp1, a coactivator of hormone receptor, it could potentially affect the transcriptional regulation of MDM2 by estrogen signaling. Two approaches were used to test this possibility. One approach used is allele-specific quantification of RNA polymerase loading because the amount of phosphorylated RNA polymerase II associated with the promoter region is related to the transcriptional activity of the corresponding genes (33, 35, 36). MCF7, an ER^+ breast cancer cell line heterozygous (T/G) at SNP309, was used to compare the transcription of MDM2 from each allele after estrogen treatment. Cells were treated with estrogen; the DNA and protein were cross-linked; and then an antibody against phosphorylated RNA polymerase II was used to immunoprecipitate the cross-linked transcription complex. The DNA fragment containing SNP309 polymorphic site was amplified by PCR. As shown in Fig. 3*A*,

Figure 2. The kinetic changes of the levels of p53 and MDM2 proteins and RNA after IR in cells with different genotypes at SNP309. *A,* endogenous levels of p53 and MDM2 protein in various cell lines were measured by Western blot (*left*). These cell lines include H460 (wild-type for SNP309, T/T), MCF7 (heterozygous for SNP309, T/G), A875 and Manca (homozygous for SNP309, G/G), and H1299-WT24 cultured with 8 ng/mL tetracycline. p53 Ser^{15} phosphorylation 1 h after IR was measured in H460, MCF7, A875, and Manca (*right*). *B,* H460, MCF7, Manca, and A875 cells were irradiated with 25 Gy of IR and harvested at the indicated time points after irradiation. The p53 and MDM2 levels were checked by Western blot and normalized to the levels of β-actin, and the induction fold of p53 and MDM2 after IR was calculated for each cell line at each time point. *C,* the levels of MDM2 (*left*) and p21 (*right*) transcription at different time points after IR in cells wild-type for SNP309 (T/T, H460), heterozygous for SNP309 (T/G, MCF7), and homozygous for SNP309 (G/G, A875, and Manca) were checked by quantitative real-time PCR. All values were normalized to the levels of actin, and the averages of three independent experiments are represented.

estrogen treatment increased the amount of immunoprecipitated DNA fragment containing SNP309 polymorphic site, indicating the increased transcription of MDM2. The transcription levels of each allele were then determined by SNaP shot using the amplified DNA fragment as template. As shown in Fig. 3*A*, in cells cultured in estrogen-depleted medium, the relative transcription levels from SNP309 G allele were higher than that from the wild-type allele. The ratio of SNP allele/wild-type allele (G/T) was 1.48 $\pm$ 0.1, demonstrating that the SNP309 G allele was preferentially transcribed. This is consistent with our previous finding that SNP309 G allele increases the binding affinity of Sp1, a conclusion drawn by comparing cells with different SNP309 genotypes (15). The relative transcription levels from SNP G allele was further preferentially increased by estrogen treatment, and G/T ratio was significantly increased to 1.83 $\pm$ 0.24 ($P < 0.005$), suggesting that estrogen could preferentially increase transcription from SNP G allele. We confirmed these results with a second approach using three ERα^+ breast cancer cell lines with different genotypes on SNP309: G/G for T47D, G/T for MCF7, and T/T for ZR75.1. The effect of estrogen on the MDM2 protein levels was examined by Western blot analysis. As shown in Fig. 3*B*, estrogen treatment preferentially increased MDM2 levels in cells homozygous for SNP309 (T47D), whereas there was only a marginal increase of MDM2 levels in cells wild-type for SNP309 (ZR75.1) and an intermediate increase in cells heterozygous for SNP309 (MCF7). By contrast, the induction of *EFP*, an estrogen-responsive gene (37), by estrogen was similar in these three cell lines (Fig. 3*B*). These results suggested that the difference in the regulation of MDM2 levels by estrogen was influenced by SNP309 status and Sp1 binding located nearby and MDM2 levels were preferentially increased by estrogen in ER$^+$ SNP309 (G/G) homozygous cells. This may contribute to accelerated tumorigenesis in humans carrying SNP309 (G/G) in a gender-specific and estrogen-dependent manner (15, 31).

The p53-MDM2 oscillation occurred only when endogenous MDM2 and p53 levels were in the proper range. To further test how different levels of endogenous MDM2 and p53 proteins affect p53-MDM2 oscillation, H1299-HW24, the H1299 cell line expressing wild-type p53 under a tetracycline-regulated promoter, was used (32). Upon withdrawal of tetracycline, p53 protein was induced, reaching maximal levels 24 h after tetracycline withdrawal. Although low concentrations of tetracycline allowed for partial expression of p53 and partial induction of p53 target genes, including MDM2, complete withdrawal of tetracycline induced higher expression of p53 and MDM2 (Fig. 4*A*). Different concentrations of tetracycline (20, 8, and 0 ng/mL) were chosen to allow p53 expression at different levels in these cells for the following experiments. p53 protein levels were measured by ELISA assay. As shown in Fig. 4*B*, the levels of p53 were 0.57×10^5 molecules per cell at 20 ng/mL tetracycline, increased by $\sim$3-fold (1.9×10^5 molecules per cell) at 8 ng/mL tetracycline, and reached the highest expression (5.9×10^5 molecules per cell) at 0 ng/mL tetracycline. For MDM2, the protein levels were $\sim$2.5-fold higher at 8 ng/mL tetracycline, and over 8-fold higher in the absence of tetracycline, compared with that at 20 ng/mL tetracycline. Cells with different p53 expression levels by culturing at different concentrations of tetracycline were exposed to IR, and the levels of p53 and MDM2 proteins were then determined at different time points after IR. As shown in Fig. 4*C*, the levels of p53 increased in all irradiated cells but had the fastest induction in the cells cultured with 8 ng/mL tetracycline. MDM2 protein decreased immediately after IR and then gradually came back in all cells cultured with different concentrations of tetracycline. However, the oscillation of p53 (Fig. 4*C*) and MDM2 (Fig. 4*D*) was only observed in the cells cultured with 8 ng/mL tetracycline, but not in the cells cultured with 0 or 20 ng/mL tetracycline. These data suggested that radiation-induced p53-MDM2 oscillation can only occur when the endogenous p53 and MDM2 protein levels were within the certain (intermediate) range, which was predicted by our model (14). Basal p53 and MDM2 levels in cells cultured with 8 ng/mL tetracycline were relatively close to that in H460 and MCF7 cells, two cell lines that showed radiation-induced p53-MDM2 oscillations (Fig. 2*A*). Moreover, observed kinetic changes of p53 and MDM2 protein levels after IR were very similar with those predicted by the model shown in Fig. 1. Together, these results suggested that variables accounted for in the model adequately describe the oscillation observed under these experimental conditions: Too high or too low levels of p53 or MDM2 failed to generate the oscillation, whereas intermediate levels could generate the oscillation.

DNA damage is required to generate the p53-MDM2 oscillation. Thus far, the p53-MDM2 oscillation was only observed in cells after DNA damage (12). Upon DNA damage, p53 was activated and thus MDM2 was induced, which, in turn, enhanced the degradation of p53. To test whether the p53-MDM2 oscillation can occur without DNA damage, the H1299-HW24 cells were used. The cells were first cultured with 8 ng/mL tetracycline for 24 h to induce p53 and MDM2 to the levels at which the p53-MDM2 oscillation occurred after IR. Tetracycline was then completely removed from the culture medium to further induce p53 and then MDM2, and the kinetic changes of p53 and MDM2 proteins were examined. As shown in Fig. 5, after complete tetracycline withdrawal, the levels of p53 and MDM2 were increased with MDM2 kinetics showing a 1-h delay. However, the increase of MDM2 did not result in reciprocal decrease of p53 levels and, thus, no p53-MDM2 oscillation was observed. Increasing p53 levels by tetracycline withdrawal only instead of IR-induced DNA damage does not generate the p53-MDM2 oscillation. These results suggested that DNA damage was an important factor required for generating the p53-MDM2 oscillation. This could be due to the requirement for a protein modification of p53 and/or MDM2, which results from an ataxia telangiectasia mutated or checkpoint kinase 2 activation after DNA damage. Clearly, additional events are required in a cell to initiate the oscillations of p53 and MDM2 proteins. This behavior was also suggested by our model in ref. 38, in which we showed that a minimum amount of phosphorylated p53 is necessary to trigger oscillations.

Discussion

Intracellular activity of p53 is regulated through a feedback loop involving its transcriptional target, MDM2. Upon stress, the phosphorylation on p53 and rapid degradation of MDM2 result in the activation of p53. p53 transcriptionally activates MDM2, which then reduces p53 levels at later times (8, 34). This p53-MDM2 oscillation has been observed in response to various stress signals, including IR and UV (9–11). Although analysis of cell populations showed that p53 and MDM2 undergo dampened oscillation after DNA damage, analysis of single cell showed that the average number of oscillation increased with the amount of DNA damage but the average height and duration of each oscillation were roughly fixed (13). These results suggest that p53

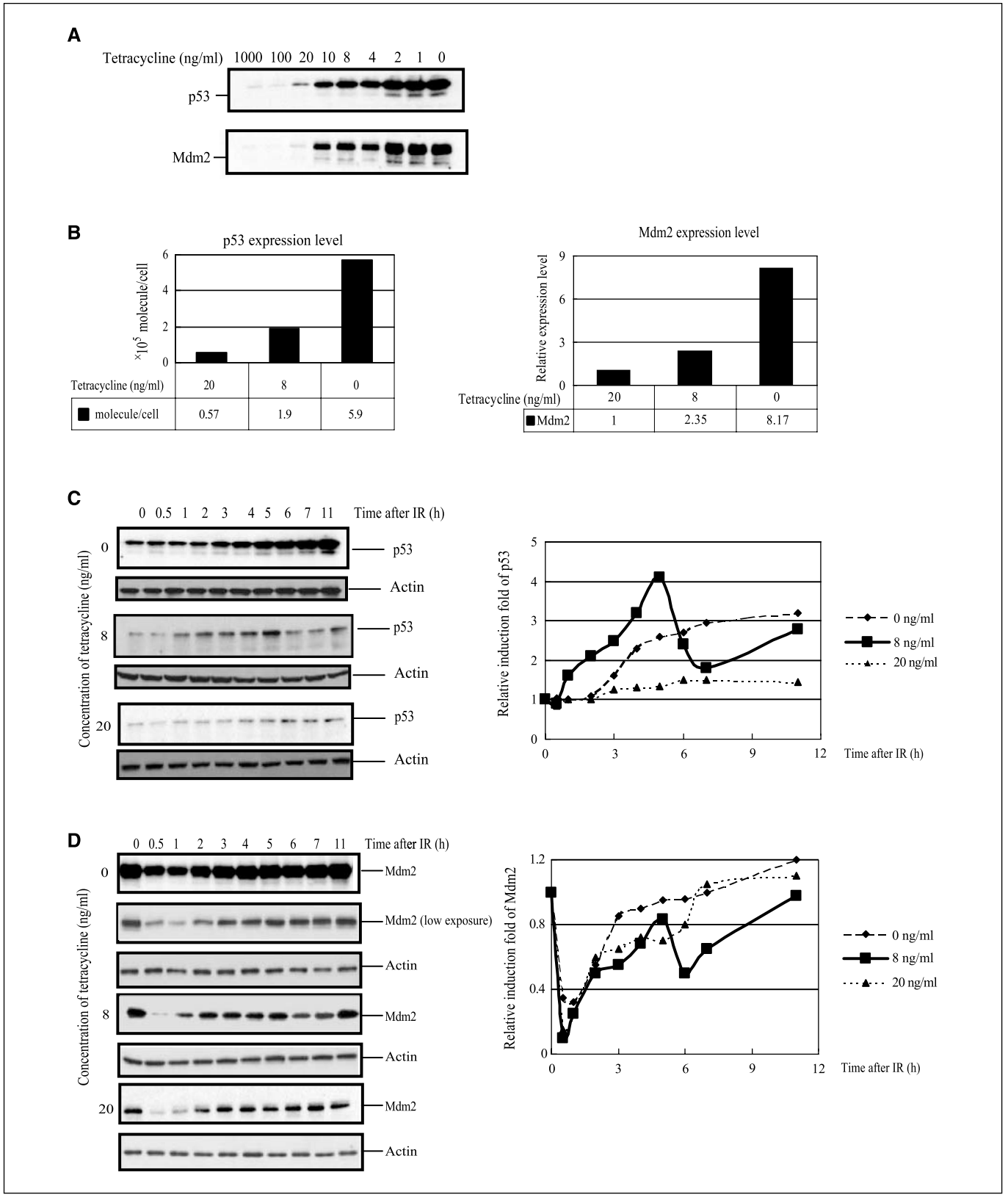

Figure 4. The proper range of endogenous p53 protein levels is required for the p53-MDM2 oscillation. *A,* H1299-HW24 cells were cultured in the presence of various concentrations of tetracycline for 24 h, and the protein levels of p53 and MDM2 were checked by Western blot. *B,* three different concentrations of tetracycline (20, 8, and 0 ng/mL) were chosen for further experiments. H1299-HW24 cells were cultured in the presence of 20, 8, and 0 ng/mL of tetracycline for 24 h; the levels of p53 were determined by p53 ELISA kit; and the relative expression of MDM2 was measured by Western blot (*B*). Cells cultured with these three different concentrations of tetracycline were then irradiated with 25 Gy of IR and harvested at the indicated time points after irradiation. The p53 (*C*) and MDM2 (*D*) levels were measured by Western blot and normalized to the levels of β-actin, and the induction fold of p53 and MDM2 proteins after IR was calculated at each time point.

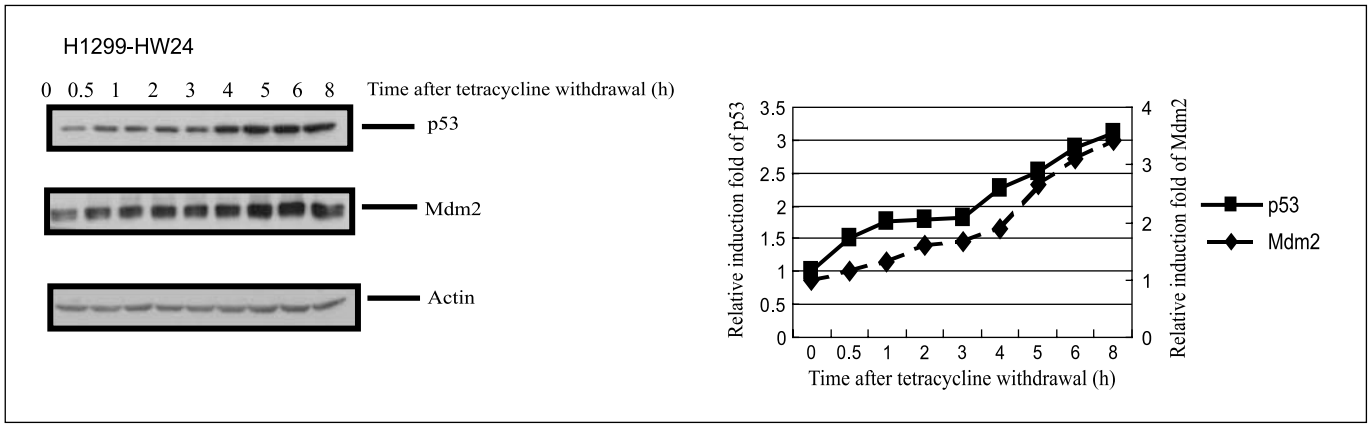

Figure 5. DNA damage is required for the p53 and MDM2 oscillation. H1299-HW24 cells were cultured in the presence of 8 ng/mL tetracycline for 24 h; tetracycline was then completely removed from the medium and cells were collected at the indicated time points after tetracycline withdrawal. The p53 and MDM2 levels were checked by Western blot and normalized to the levels of β-actin. The induction fold of p53 and MDM2 after tetracycline withdrawal was calculated at each time point.

and MDM2 are produced in cells in a pulsatile (digital) manner over time rather than continuously (analogue) after stress. This digital behavior of the p53-MDM2 oscillation can program p53 activity and rapidly terminate the p53 response once the stress signal has been effectively dealt with. This reduces the risk of having too much p53, leading to inappropriate death. On the other hand, if the extent of DNA damage is excessive, increased number of p53 peaks may trigger an irreversible response resulting in apoptosis.

Although the presence of the p53-MDM2 oscillation is widely recognized, the biological functions of the oscillation and the rules that govern its outcome remain unclear. We have designed a mathematical model that reproduced the experimentally observed oscillations (14, 38). Based on this model, we predicted that a selected range of endogenous p53 and MDM2 levels is required to generate the oscillations. To test this prediction, in this report, the effect of endogenous or prestress MDM2 levels on this oscillation was studied by using cell lines with SNP309 in the MDM2 gene. SNP309 is located in the first intron of the *MDM2* gene with a T-to-G change. The G allele creates a better binding site for Sp1 and leads to higher expression of the MDM2 transcript (on average 2- to 4-fold) and MDM2 protein levels (on average 2- to 4-fold) in cell lines homozygous (G/G) for SNP309 (15). Cell lines with the SNP309 (G/G) genotype have weaker p53 responses upon DNA damage, including low apoptotic responses and poor cell cycle arrest. After DNA damage, p53 showed a slow induction with the extended and slow accumulation in cells homozygous for SNP309 (G/G). There was an exaggerated delay in increase in MDM2 levels in these cells. After reaching their highest levels, p53 and MDM2 sustained rather than exhibiting pulsatile change over time. Therefore, no oscillation was observed. These results suggest that the functions of the p53 pathway may be impaired in cells for which there is no p53-MDM2 oscillation, even when p53 is activated and its target genes, including MDM2, can be induced. We further compared the rate of p53 activation and induction of MDM2, two important variables regulating the p53-MDM2 oscillation in cells with or without SNP309. The rate of p53 activation was much slower, and the induction fold was much less in cells homozygous for SNP309 (G/G), which may cause a disruption of the coordinated oscillation. After a stress signal such as IR is applied, the high levels of MDM2 protein in SNP309 (G/G) cells declined, but still stayed at a much higher level than that observed in wild-type (T/T) cells. Thus, the fold induction of MDM2 by p53 was much lower in SNP309 (G/G) cells than in wild-type (T/T) cells and this could be important (the ratio of basal to induced levels) for the oscillation.

Many reports have shown that MDM2 levels can be regulated by estrogen signaling (20, 21). In breast tumors positive for the ER, MDM2 levels are higher than that in ER-negative tumors (16, 19). Because SNP309 is located in a promoter region regulated by estrogen signaling, and SNP309 increases the binding affinity of Sp1, a coactivator of this hormone receptor, this SNP might further affect the regulation of MDM2 by estrogen. The results presented in this study showed that estrogen preferentially stimulated transcription of MDM2 from the SNP309 G allele and preferentially increased MDM2 levels in SNP 309 homozygous cells, leading to further attenuation of p53 pathway. These results are consistent with a report demonstrating that the SNP309 G/G genotype is significantly enriched in female patients diagnosed with breast cancers or lymphomas below the age of menopause (31), and provide a possible molecular mechanism how SNP309 accelerates tumor formation in a gender-specific and estrogen-dependent manner.

By using the H1299-HW24 cell line expressing wild-type p53 under a tetracycline-regulated promoter, we manipulated the expression of p53 and MDM2 to different levels. This permitted further testing of predictions of our model. The results showed that only when p53 and MDM2 basal levels were in the certain range could stress-induced oscillations be observed. Too high or too low levels of p53 or MDM2 failed to generate the oscillations, whereas only the intermediate levels generated the oscillations. These results are consistent with the model prediction and are also consistent with our finding that the p53-MDM2 oscillation did not occur after stress in SNP309 cells with heighten basal MDM2 levels. An alternative explanation, not necessarily at odds with the tenets of the mathematical model, is that in this system (which uses cells containing p53 expression plasmids), the endogenous MDM2 protein might not be able to keep up with the highly induced p53 levels, resulting in the loss of the oscillation at the high p53 levels. However, this argument fails to explain why oscillations were not observed when p53 levels were low and MDM2 could keep up with the p53 induction. We favor the explanation provided by the analysis of the p53-MDM2 feedback loop as a dynamic system in

ref. 38, in which we showed that the oscillations at intermediate values of p53 result from the loss of the stability that characterizes this dynamic system at high and low values of p53.

Using the same cell line, we further tested if the accumulation of p53 protein by tetracycline withdrawal in the absence of radiation-induced DNA damage could generate oscillations. We found that oscillations did not occur under this condition. This result is consistent with a previous report that oscillations occurred only when DNA damage reached a certain threshold (not observed at 0.3 Gy IR, but observed at 5 Gy IR), demonstrating that the oscillation depended on DNA damage signals (12). DNA damage may trigger the p53-MDM2 oscillation through damage detection or repair pathways that are separate from the p53 pathway. This could, in turn, be mediated by posttranslational modifications of p53 and MDM2. It is known that modifications of p53 and MDM2 proteins are stress specific. By examining the kinetic changes of p53 and MDM2 induced by various stresses, we can further understand the variables regulating the p53-MDM2 oscillation.

In summary, this study shows that higher basal levels of MDM2 associated with SNP309 (G/G) or an altered p53/MDM2 ratio does not support coordinated p53-MDM2 oscillations after stress. Although altered p53/MDM2 ratio in cells does not prevent p53 activation and p53-dependent transcription, it reduces or eliminates the oscillation of these proteins, decreases levels of p53-mediated transcription, and reduces the level of apoptosis. These observations correlate with a clinical phenotype; that is, the development of cancers at earlier age of onset in populations carrying SNP309.

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