Analysis of p53 Dependent Damage Response in Sperm-irradiated Mouse Embryos

Megumi TOYOSHIMA*

p53 dependent S-phase checkpoint/Mouse embryo/Pre-implantation/p53/p21/Apoptosis.

Ionizing radiation activates a series of DNA damage response, cell cycle checkpoints to arrest cells at G1/S, S and G2/M, DNA repair, and apoptosis. The DNA damage response is thought to be the major determinant of cellular radiosensitivity and thought to operate in all higher eukaryotic cells. However, the radiosensitivity is known to differ considerably during ontogeny of mammals and early embryos of mouse for example are much more sensitive to radiation than adults. We have focused on the radiation-induced damage response during pre-implantation stage of mouse embryo. Our study demonstrates a hierarchy of damage responses to assure the genomic integrity in early embryonic development. In the sperm-irradiated zygotes, p53 dependent S-phase checkpoint functions to suppress erroneous replication of damaged DNA. The transcription-dependent function is not required and the DNA-binging domain of the protein is essential for this p53 dependent S-phase checkpoint. p21 mediated cleavage arrest comes next during early embryogenesis to prevent delayed chromosome damage at morula/blastocyst stages. Apoptosis operates even later only in the cells of ICM at the blastocyst stage to eliminate deleterious cells. Thus, early development of sperm-irradiated embryos is protected at least by three mechanisms regulated by p53 and by p21.

INTRODUCTION

Ionizing radiation induces DNA double-strand breaks (DSB) and inflicts a variety of DNA damage responses which include induction of cell cycle checkpoint and apoptosis.1,2) p53 tumor suppressor gene plays a crucial role in the damage responses of mammalian somatic cells.3) Damage signals are transduced by ATM and related sensor kinases to a variety of target proteins and p53 is one such protein which then executes the various functions of checkpoint and apoptosis.

In contrast to later ontogenic stages, embryonic development in mouse is quite sensitive to radiation4,5) It has been reported that p53 was activated upon irradiation of mouse embryos at the E6.5 stage and that the high level of p53 was correlated to radiation induction of apoptosis without cell cycle arrest of cells in the fetal stage mouse development, and p53-deficient (p53KO) mice are extremely susceptible to radiation-induced teratogenesis.6) Thus, p53-dependent apoptosis was proven to be the major mechanism of suppressing radiation-induced teratogenesis. Interestingly, Mdm2-, Mdmx- and Rad51-deficient embryos dies shortly after implantation, but this phenotype was shown to be rescued by p53 deficiency.7–10) These studies demonstrate the importance of p53 function in the post-implantation stages of mouse development.

In much earlier stages of mouse development, cell cycle checkpoints and apoptosis are absent or are compromised even though mouse zygotes possess an extremely high level of p53.11–17) It was reported that mouse embryonic stem (ES) cells are highly sensitive to genotoxic stresses and do not undergo p53-dependent G1/S checkpoint upon DNA damage.18,19) However, the mechanisms of the high radiosensitivity is poorly understood to this day.

One difficulty in studying the damage response in conventional cellular systems is that the effect of DNA damage itself and the effect of cellular responses are hard to distinguish in irradiated cells. In mouse zygote, genomes of the incoming sperm and residing oocyte form two separate pronuclei. DNA synthesis occurs in these two pronuclei, which thereafter merge before the entry to M phase. This gives a unique opportunity to analyze the mechanism of damage responses separated from the effect of DNA damage itself.

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Thus, DNA damage can be delivered through irradiated sperm while the responses can be analyzed in damage-free female pronuclei. Using this system, our previous study suggested clearly that DNA damage induced by sperm irradiation causes genomic instability and somatic reversion for the maternally derived allele in F1 mice. In the present review, unique features of damage response of early stage mouse embryos using this system are described.

**Suppression of DNA synthesis by p53-dependent S-phase checkpoint in sperm-irradiated mouse zygote**

In order to study damage response of 6 Gy sperm-irradiated mouse zygotes, S phase progression was monitored by pulse-labeling with $[^3]H\text{TdR}$ and subsequent grain counting of the samples. Pronuclear DNA synthesis began at 8 h after fertilization for both control and sperm-irradiated zygotes. The rate of DNA synthesis was suppressed throughout S phase in sperm-irradiated zygote, and the degree of suppression was found to be similar between irradiated male and unirradiated female pronuclei. While the uptake of $[^3]H\text{TdR}$ lasted both in control and sperm-irradiated zygotes from 8 to 21 h after fertilization, some of the latter zygotes exhibited a tendency in the delay of S phase by taking up relatively a larger amount of the isotope at later stages of S phase such as at 21 h. These results show that irradiation of sperm did not trigger G1/S checkpoint in mouse zygotes but suppressed DNA synthesis. In addition, this suppression of DNA synthesis was the result of DNA damage response, and not the result of DNA damage itself since unirradiated female pronuclei exhibited a similar level of the suppression. Suppression of DNA synthesis was also observed in zygotes irradiated directly with 3 Gy X-rays.

Involvement of p53 in suppression of DNA synthesis was tested by using p53-deficient zygotes (p53KO zygotes). Fertilization of p53 null oocytes with 6 Gy irradiated p53 null sperm was analyzed for the uptake of $[^3]H\text{TdR}$. DNA synthesis was found not to be suppressed in p53KO zygotes. This was confirmed by the uptake of bromodeoxyuridine (BrdU) and 6 Gy sperm irradiated p53KO zygotes exhibited anti-BrdU staining as strong as for control unirradiated p53KO zygotes. These results demonstrate that there exists a novel p53 S-phase checkpoint in one cell stage mouse embryos.

It is well known that p21 is transactivated by p53 and induces the cell cycle checkpoint in somatic cells. The lack of G1/S checkpoint indicates that p21 may not be functional at the zygotic stage. In addition, p21-deficient zygotes (p21KO zygotes) exhibited the same level of suppression of DNA synthesis in sperm-irradiated mouse zygotes. Therefore, p53 protein itself is likely to be executing the suppression of DNA synthesis in sperm-irradiated mouse zygotes.

In order to explore the direct involvement of p53 protein, GST-p53 fusion protein was prepared from E. coli and microinjected into the cytoplasm of p53KO zygotes. While microinjection of the fusion protein has no effect on DNA synthesis of unirradiated control p53KO zygotes, the same treatment of sperm-irradiated p53KO zygotes suppressed DNA synthesis strongly, thus restoring the p53 dependent S-phase checkpoint. These results of the sperm-irradiated mouse zygotes indicate that the zygotic stage embryos lack G1/S checkpoint, but possess a novel p53-dependent S-phase checkpoint which is not dependent on the transactivation function of p53, but on the function of the protein itself.

**Contribution of ATM pathway but not ATM-mediated phosphorylation of p53**

Phosphorylation of p53 by sensor kinases, such as ATM and ATR, is pivotal to the DNA damage response of the irradiated cells. Therefore, the effect of two kinase inhibitors, caffeine and wortmannin, was tested. Caffeine inhibits both ATM and ATR, while wortmannin inhibits ATM and DNA-PK but not ATR. Incubation of the p53 wild-type zygotes with two inhibitors had no effect on the DNA synthesis. The suppression of DNA synthesis in sperm-irradiated zygotes was abrogated by caffeine and wortmannin, both of which inhibit the activity of ATM.

In order to study the possible role of DNA-PK in the p53-dependent S-phase checkpoint in a more direct way, we have tested zygotes from SCID mice for the effect of sperm irradiation. DNA synthesis was suppressed in sperm-irradiated SCID zygotes to a similar extent as p53 wild-type zygotes, and this implies that DNA-PK is not involved in the p53 dependent S-phase checkpoint in zygotic stage mouse embryos. In addition, our unpublished data demonstrated the activation of ATM both in male and female pronuclei, as detected by anti-phospho-ATM antibody. These imply that ATM is likely to be involved in the p53 dependent S-phase checkpoint in sperm-irradiated zygotes. Phosphorylation by ATM of either p53 or some other target protein(s) might be essential for the suppression.

Phosphorylation plays important roles for the stability and the activity of p53. Human p53 is phosphorylated at serine residues of 15, 20, 46, and 315 after ionizing radiations and UV by a series of kinases including ATM. ATM phosphorylation site mutants of p53 were tested for their ability to suppress DNA synthesis in sperm-irradiated zygotes. Microinjection experiments demonstrated that mutants of these phosphorylation sites were still capable of suppressing DNA synthesis in sperm-irradiated p53KO zygotes. This is contrast to the expectation drawn from the kinase inhibitor experiments, but can be reconciled by postulating the involvement of p53 protein itself in conjunction with yet to be identified target proteins whose phosphorylation by ATM is needed for the suppressive function.

**Transcription-independent suppression of DNA synthesis by p53 in sperm-irradiated mouse zygotes**

Further analyses were made to elucidate the detail of the
p53 function in the suppression of DNA synthesis in sperm-irradiated zygotes.}

In the former, p53 serves as a transcription factor activating a series of genes involved in cell cycle arrest and apoptosis. The postulated target protein could be one of those activated by p53. The p53 mutant L22Q/W23S which completely lacks the transcription function was tested for its ability to suppress DNA synthesis in sperm irradiated zygotes. Microinjection of this protein did not affect DNA synthesis of control p53 wild-type zygotes, control p53KO zygotes, and sperm-irradiated p53 wild-type zygotes. Furthermore, this transactivation mutant protein was capable of suppressing DNA synthesis in sperm-irradiated p53KO zygotes.

Additionally, the suppression of DNA synthesis in sperm-irradiated zygotes was not affected by α-amanitin, which is an inhibitor of RNA polymerase. Thus the transcription-dependent function is not required for suppression of DNA synthesis in sperm-irradiated zygotes.

Involvement of the DNA-binding domain of p53 in the suppression

The interaction of p53 protein with DNA replication machinery might be essential for the suppression of DNA synthesis in sperm-irradiated mouse zygotes. The p53 protein has a regulatory domain which interacts with DNA and other proteins. Two DNA-binding domain mutants were tested. R175H mutant carried arginine to histidine mutation at residue 175, which made the protein incapable of binding to DNA due to its denatured conformation. Another R273H mutant carried arginine to histidine mutation at residue 273, which is known as a hot spot of mutation in tumors, but retains transactivation activity partially. These two mutant proteins abrogated the suppression of DNA synthesis of sperm-irradiated p53 wild-type zygotes in a dominant-negative manner. As was expected, microinjection of these proteins had no effect on DNA synthesis of sperm-irradiated p53KO zygotes. These data suggest that the DNA-binding domain of p53 is essential for the novel S-phase checkpoint in sperm-irradiated mouse zygotes.

p53 is known to accumulate after the treatment of cells with aplanocin and hydroxyurea, which induce DNA replication block. In addition, p53 co-localizes with BLM, Rad51 and 53BP1 at the sites of stalled DNA replication fork. Recent studies have demonstrated that p53 binds to and attenuates the activity of BLM and WRN, and the R273H mutation abrogates these two activities. We speculate that p53 protein is likely to serve as a platform for assembly of a functional protein complex that can regulate DNA synthesis in response to DNA damage.

In somatic cells, the involvement of the ATM pathway in S-phase checkpoint has been well documented. However, the most studies in the past have excluded the involvement of p53 from this important checkpoint. It was reported that p53 was accumulated by the treatment of aplanocin and hydroxyurea, which block DNA replication. Recently, it was reported that p53 was effectively activated and induced a prolonged S-phase arrest. The role of p53 in S-phase checkpoint and the functional difference of p53 between in somatic cells and embryonic cells is yet to be elucidated at present.

Biological function of p53-dependent S-phase checkpoint

In contrast to somatic cells, some of surveillance mechanisms such as cell cycle checkpoint and apoptosis are either lacking or restricted during the pre-implantation stage of embryo. p53-dependent G1/S checkpoint is absent in embryonic stem (ES) cells even though the protein is expressed abundantly. The apoptotic function of p53 is also not expressed during early cleavage of mouse embryo.

In our study, the novel p53-dependent S-phase checkpoint was discovered. To explore this biological function, pre-implantation development of sperm-irradiated embryos was analyzed. p53 wild-type zygotes fertilized with irradiated sperm develop normally to the implantation stage despite the fact that half of them carried a sub-2N amount of DNA. These embryos progressed normally to the eight-cell stage and then implanted successfully, as evidenced by roughly the same number of implantation sites, but the subsequent fetal development was suppressed as evidenced by the number of fetus by sperm irradiation (Table 1). In contrast, sperm-irradiated p53KO zygotes exhibited an abnormal segregation of chromosomes at the first mitosis and those embryos carried numerous micronuclei in the subsequent stages. They failed to undergo further cleavage and degenerated before reaching the eight cell stage. As a consequence, no implantation sites were detected for 6 Gy sperm-irradiated zygotes.

<table>
<thead>
<tr>
<th>Dose to male (Gy)</th>
<th>p53+/+, p21+/+</th>
<th>p53–/–, p21+/+</th>
<th>p53+/+, p21–/–</th>
<th>p53–/–, p21–/–</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of placentas</td>
<td>No. of fetuses</td>
<td>No. of placentas</td>
<td>No. of fetuses</td>
</tr>
<tr>
<td>0</td>
<td>13.8 ± 0.8</td>
<td>13.8 ± 0.7</td>
<td>12.2 ± 1.3</td>
<td>12.0 ± 1.5</td>
</tr>
<tr>
<td>3</td>
<td>13.0 ± 0.8</td>
<td>8.3 ± 1.3</td>
<td>11.0 ± 1.7</td>
<td>6.5 ± 1.5</td>
</tr>
<tr>
<td>6</td>
<td>13.0 ± 2.2</td>
<td>6.2 ± 1.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>11.3 ± 1.3</td>
<td>1.5 ± 0.3</td>
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</table>

irradiated p53KO embryos. Thus, the p53-dependent S phase checkpoint could be a surveillance mechanism that repairs chromosome damage in zygotes.

**Delayed activation of other radioresponses in sperm-irradiated embryos**

The pre-implantation stage of early embryogenesis can be divided into the cleavage stage, morula stage and blastocyst stage. Microarray profiling of pre-implantation stage mouse embryos was conducted which failed to detect p21 until embryos reached the eight cell stage. The pre-implantation development especially, morula and blastocyst stage of control and sperm-irradiated embryo was analyzed.

Sperm-irradiated embryos developed normally for the first 2.5 days, but started to exhibit developmental delay at day 3.5. The average cell number per embryos was less in sperm-irradiated embryos at day 3.5, because of the presence of delayed embryos. The delayed embryos with fewer blastomeres failed to undergo blastocyst formation. Cell cycling study suggested that the delayed sperm-irradiated embryos of day 3.5 were arrested at the G2 phase of the cell cycle. This was confirmed by the presence of G2-M specific phosphorylated histone H3. p21 plays an important role in G2/M arrest and the expression of p21 was examined. When sperm-irradiated embryos were analyzed by real time RT-PCR, the p21 levels in days 1.5 and 2.5 embryos were higher by 1.6- and 1.7-fold over the controls, respectively. However, at day 3.5, sperm-irradiated embryos with delayed morphology exhibited a much higher level of the 17-fold increase. As for the sperm-irradiated embryos with normal morphology, the activation was merely 3.9 fold. These demonstrated that p21 was activated at the blastocyst stage much more readily than the earlier stages.

To confirm the role of p21 in embryogenesis, further analyses were performed in p21-deficient embryos (p21KO embryos). As was expected, sperm-irradiated p21KO embryos progressed normally until day 3.5 and were morphologically indistinguishable from non-irradiated control embryos of the same stage. These results confirm that p21 is indeed responsible for the cleavage delay in a fraction of sperm-irradiated day 3.5 embryos.

p21 can inhibit apoptosis by interacting with proapoptotic molecules. Indeed, wild-type and p21KO embryos revealed no apoptosis before day 2.5. A few apoptotic cells were seen in day 3.5 embryos by 6 Gy sperm irradiation. However, a drastic increase in apoptosis was noted in the sperm-irradiated embryos at day 4.0. In addition, the effect of sperm irradiation in induction of apoptosis was much more strong in day 3.5 p21KO embryos, and even stronger increase of apoptosis was observed at day 4.0 of these embryos. Thus, apoptosis is more prominent in the absence of p21.

**p53- and p21-mediated protection of embryonal and fetal development**

The effect of sperm irradiation on embryonic development was investigated using three genetic backgrounds, the wild-type, p53KO and p21KO, by counting implantation sites (or placentas) as an indicator of successful implantation and fetuses as successful fetal development at day 18 (Table 1). Without sperm irradiation, almost all the implanted embryos developed to live fetus. However, sperm irradiation suppressed the implantation and fetal development in a genotype-dependent fashion. In the wild-type embryos, the implantation was not affected, but the fetal development was suppressed by 6 Gy of sperm irradiation to approximately 50% of the control. In contrast, 6 Gy sperm-irradiated
Early Development of Sperm-Irradiated Embryos is Protected at least by three Mechanisms, p53-Dependent S-phase Checkpoint, p21-Dependent G2/M Checkpoint and p21-Mediated Apoptosis

p53KO embryos failed to reach even the implantation stage. Analyses of the p21KO sperm-irradiated embryos yielded somewhat intermediate results. This implies that early development of sperm-irradiated embryos is protected by a series of mechanisms; the earlier one is regulated by p53 (not via p21) and the other is by p21.

The hierarchy of damage responses during early embryonic development

Finally, our present study demonstrates a hierarchy of damage response operating at different stages of development in order to assure best outcome of successful birth. Sperm-irradiated mouse zygotes were devoid of G1/S arrest, but p53 dependent S-phase checkpoint functions to suppress erroneous replication of damaged DNA. This S-phase checkpoint operates independently of transactivation function of p53, but dependent on the function of the DNA binding domain of the protein. p53KO embryos lacking this S-phase checkpoint degenerate before implantation. p21 mediated G2/M arrest comes next to prevent delayed chromosome damage which starts operating at the morula to blastocyst stage and thereafter. Apoptosis operates even later to eliminate deleterious cells of ICM which forms the future fetus. Thus, early development of sperm-irradiated embryos is protected at least by three mechanisms regulated by p53 and by p21.

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