Delayed and stage specific phosphorylation of H2AX during preimplantation development of γ -irradiated mouse embryos

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Abstract

Within minutes of the induction of DNA double-strand breaks in somatic cells, histone H2AX becomes phosphorylated in the serine 139 residue at the damage site. The phosphorylated H2AX, designated as γ -H2AX, is visible as nuclear foci in the irradiated cells which are thought to serve as a platform for the assembly of proteins involved in checkpoint response and DNA repair. It is known that early stage mammalian embryos are highly sensitive to radiation but the mechanism of radiosensitivity is not well understood. Thus, we investigated the damage response of the preimplantation stage development by analyzing focus formation of γ -H2AX in mouse embryos γ -irradiated *in utero*. Our analysis revealed that although H2AX is present in early preimplantation embryos, its phosphorylation after 3 Gy γ -irradiation is hindered up to the two cell stage of development. When left *in utero* for another 24–64 h, however, these irradiated embryos showed delayed phosphorylation of H2AX. In contrast, phosphorylation of H2AX was readily induced by radiation in post-compaction stage embryos. It is possible that phosphorylation of H2AX is inefficient in early stage embryos. It is also possible that the phosphorylated H2AX exists in the dispersed chromatin structure of early stage embryonic pronuclei, so that it cannot readily be detected by conventional immunostaining method. In either case, this phenomenon is likely to correlate with the lack of cell cycle arrest, apoptosis and high radiosensitivity of these developmental stages.

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Introduction

Higher eukaryotic cells activate a series of damage response, when challenged with DNA damage (Zhou & Elledge 2000). This damage response is evolved to assure the genomic integrity and the survival of the cells, and failure of any of the damage response pathways leads to mutation and cancer (Bartek & Lukas 2001). Preimplantation stage mouse embryos are known to be highly sensitive to radiation (Russel & Sailors 1963, Goldstein *et al.* 1975, Matsuda *et al.* 1989). This high radiosensitivity is likely due to the peculiar damage response of the early metazoan embryogenesis in general. For example, they undergo rapid DNA replication and cell division until the midblastula transition stage, without activating cell cycle checkpoint responses even in the presence of DNA damage and under-replicated DNA (Epel 2003). In fact, aphidicolin treatment of *Xenopus* and *Drosophila* embryos decreases their DNA content, but they nevertheless progress to the midblastula transition

stage (Clute & Masui 1997, Raff & Glover 1988). We also have demonstrated that mouse zygotes fertilized with irradiated sperm do not show G1/S and G2/M checkpoint responses (Shimura *et al.* 2002b). It may even be envisaged that the early stage embryos are devoid of some DNA repair function so that DNA damage can be amplified through subsequent cell cycling which eventually result in apoptotic cell death; the most effective way to eliminate potentially deleterious cells. Indeed, mouse embryos fertilized with irradiated sperm were shown to progress successfully to the implantation stage and eliminated by apoptosis thereafter, leaving only healthy fetus (Shimura *et al.* 2002b).

Damage response is composed of three components; damage sensors, signal transducers, and effectors to transmit signals from DNA damage to the machineries executing any of the three functions of damage response, cell cycle arrest, apoptosis and DNA repair networks (Zhou & Elledge 2000). ATM and ATR kinases are responsible for early steps of damage sensing and signal

transduction. The former is pivotal in damage response to ionizing radiations and activated by the conformational changes of the chromatin brought about by DNA damage (Kastan & Bartek 2004). ATM kinase phosphorylates a variety of target proteins including histone H2AX.

H2AX is one of the H2A variants with a conserved Ser-Gln-Glu motif at the carboxyl-terminal of the protein, and the serine 139 of this motif is rapidly phosphorylated by ATM within minutes after irradiation (Rogakou *et al.* 1998, Redon *et al.* 2002, Sedelnikova *et al.* 2003). The phosphorylated form of H2AX is designated as γ -H2AX, and forms foci at the site of DNA damage which then serve as a platform to recruit various repair and cell cycle checkpoint proteins (Fernandez-Capetillo *et al.* 2004). The γ -H2AX focus formation is used as a marker of DNA double strand breaks in irradiated cells, and they are visible even at break and exchange points of metaphase chromosomes (Rogakou *et al.* 1998, Rothkamm *et al.* 2003, Forand *et al.* 2004). γ -H2AX is most likely to play important roles in DNA repair and chromatin remodeling during DNA damage response. It has been suggested that γ -H2AX promotes recombination and conformational changes of chromatin (Fernandez-Capetillo *et al.* 2003, Reina-San-Martin *et al.* 2003); hence chromatin reorganization by γ -H2AX could prevent the premature separation of broken ends, a function that would safeguard against harmful chromosome rearrangements.

Although numerous reports have been made on the kinetics of H2AX phosphorylation in irradiated tissue culture cells, no such study was made in early embryonic cells after irradiation. To gain an insight into high radiosensitivity of early stage embryonic development, we examined mouse preimplantation embryos for the formation of γ -H2AX foci after exposure to γ -rays. The results demonstrate that 3 Gy irradiation of one-cell stage and two-cell stage embryos poorly induced phosphorylation of H2AX. Irradiated embryos showed delayed phosphorylation of H2AX when they progressed to the morula stage. In contrast, γ -H2AX foci were immediately induced after irradiation of post-compaction stage embryos. These results demonstrate that one of the ATM pathways operates in a stage specific manner during preimplantation stage development of mouse embryos.

Materials and Methods

Animals

The animal care and handling were done according to the Kyoto University Guidelines for animal experimentation. Eight- to ten-week-old ICR mice were used in this study and they were maintained under the controlled conditions of temperature (23 ± 2 °C), and light (12 h light: 12 darkness cycle) with standard diet and water *ad libitum*. ICR mice were used since the

female of this strain had large numbers of ovulation of around 20 without hormonal stimulation.

In vivo fertilization, irradiation, embryo recovery and assessment

Female mice were mated with male mice on the day of estrous. Mating was allowed for 1 h and the day of successful mating as assessed by vaginal plugs was designated as day 0. Pregnant mice were irradiated at various times after mating with ^{60}Co γ -rays at a dose rate of 1.12 Gy/min (Gammacell 40, MDS Nordion, Kanata, Ontario, Canada) to expose embryos of particular preimplantation stages. Irradiation was done to the abdominal portion by covering other part of the body by lead plates. Animals were killed humanely at specified intervals after the exposure. Females of the ICR strain yielded a large number of fertilized embryos (approximately 14) and they were collected from the oviduct until day 2.5 and later from the uterine horn by gentle flushing with PBS. Embryos were examined for their morphology, cell number, compaction and blastocyst formation.

Immunofluorescence analyses

Embryos were washed with PBS and the zona pellucida was removed by digestion with actinase E (0.5%) for 5 min. The embryos were fixed with 4% paraformaldehyde in PBS for 30 min and then permeabilized for 30 min at room temperature with PBS containing 0.1% (vol/vol) Triton X-100 and 3 mg/ml bovine serum albumin. They were then stained with anti-phospho-Histone H2AX antibody (1:100 dilution, Upstate Biotechnology, Upstate, NY, USA), anti-Histone H2AX antibody (1:100 dilution) or antiphospho-ATM antibody (1:1000 dilution, Rockland, Gilbertsville, PA, USA) for 1 h at 37 °C followed by treatment with second antibody conjugated with fluorescein isothiocyanate (FITC) (antirabbit IgG, 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or Cy-3 (anti-mouse IgG, 1:200 dilution, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). The immunostained embryos were washed with PBS containing 0.1% triton X, counterstained with propidium iodide or 4', 6-diamidino-2-phenylindole (DAPI), and observed under fluorescent microscope. In the present study, we examined at least 50 embryos from more than five pregnant female mice per data point and the experiments were repeated to confirm the reproducibility of the results.

TUNEL Assay

Embryos were washed with PBS and placed onto a cover slip precoated with 0.1% poly L-lysine. Embryos were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. TUNEL assay was performed using

commercial kit (Takara Biomedicals, Kusatsu, Japan). Briefly, embryos were permeabilized for 10 min, washed three times with PBS and incubated at 37 °C for 1 h, with the reaction buffer containing dUTP-FITC and terminal deoxynucleotidyl transferase. Embryos were then washed three times in PBS, stained with DAPI and observed under fluorescent microscope for TUNEL positive cells.

λ -phosphatase treatment

Embryos recovered from the irradiated mice were washed and zona pellucida was removed as described above. After fixation, the embryos were washed with λ -phosphatase buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM Na₂ EDTA, 5 mM dithiothreitol, 0.01% Brij 35 and washed again with λ -phosphatase buffer supplemented with 2 mM MnCl₂. The irradiated embryos were treated with 100 units of λ -phosphatase (New England Biolabs, Beverly, MA, USA) for 30 min at 37 °C. Control embryos were treated with buffer supplemented with MnCl₂ but without λ -phosphatase. The embryos were washed with PBS containing BSA, and stained with γ -H2AX antibody as described above.

Statistical analysis:

Statistical significance was assessed using one-way analysis of variance and $P < 0.05$ was considered significant.

Results

Developmental outcome of embryonic irradiation

As shown below, we observed a strong developmental stage dependence of H2AX phosphorylation in response to irradiation. Since radiation can alter embryonic development which would indirectly modify the radiation effect itself, we first examined the developmental outcome of 3 Gy irradiation before analyzing H2AX phosphorylation. Embryos exposed to 3 Gy γ -rays *in utero* at 8 h after fertilization were recovered 64 h later (72 h after fertilization) and then examined for the total cell number. When not exposed, these embryos had on average 39 blastomeres at 72 h after fertilization (Table 1). One-cell stage embryos were most sensitive to 3 Gy γ -rays, and the cell number of the embryos at 72 h after fertilization was around 24. Later stage embryos were less sensitive to radiation for the reduction of the cell number. It is interesting to note that these irradiated embryos with the reduced cell numbers nevertheless implanted successfully and the numbers of placenta were unchanged (Adiga, unpublished observation). In addition, *in vitro* proliferation of inner cell mass of blastocysts on MEF feeder layer was suppressed

Table 1 Total cell number in mouse embryos irradiated at different developmental stages.

Stage of irradiation (3 Gy)	N	Total cell number ($\pm$ S.E.M.) at 72 h
Control	66	39.4 $\pm$ 2.6
One cell stage (8 h post fertilization)	52	24.3 $\pm$ 1.7*
Two cell stage (24 h post fertilization)	64	32.7 $\pm$ 1.8†
Six–eight cell stage (48 h post fertilization)	58	28.7 $\pm$ 2.2†
Blastocyst (72 h post fertilization)	50	39.1 $\pm$ 2.6

* $P < 0.001$, † $P < 0.01$ (one-way ANOVA).

for embryos with less than 20 blastomeres on day 3.5 (Adiga, unpublished observation).

Lack of H2AX phosphorylation in irradiated one-cell stage mouse embryos

One-cell stage embryos were irradiated *in utero* 8 h after fertilization with 3 Gy of γ -ray. They were recovered 30 min later and examined for the formation of γ -H2AX foci. No fluorescence signal was found in either of unirradiated and 3 Gy irradiated zygotes when stained with the anti- γ -H2AX antibody (Fig. 1A). At the same time, we noted that the second polar body of the 3 Gy irradiated embryos was brightly stained for γ -H2AX. These suggest that the H2AX protein is present in one-cell stage embryo. Thus, either phosphorylation of H2AX is hindered in the pronuclei of irradiated zygotes or γ -H2AX was by some reason escaped detection by conventional immunostaining.

In one-cell stage embryos, the polar body DNA is highly condensed, whereas the chromatin in zygotic pronuclei is loosely organized. Hence, we examined whether or not phosphorylation of H2AX is dependent on chromatin configuration of the embryos. One-cell stage embryos were recovered at 8 h after fertilization and cultured *in vitro* for 16 h in the presence of 0.04% colcemid and the metaphase arrested embryos were immunostained for γ -H2AX. Interestingly, γ -H2AX staining was observed in metaphase chromosomes regardless of 3 Gy irradiation (Fig. 1B). Immunostaining with anti-H2AX antibody also confirmed the presence of H2AX in unirradiated one-cell stage embryos (Fig. 1C).

ATM kinase phosphorylates H2AX and therefore, poor staining for γ -H2AX might be due to the absence of the ATM kinase activity in one-cell stage mouse embryos. However, the ATM kinase activity is present in the irradiated zygotes, since we detected a faint but definite nuclear staining for the phosphorylated form of ATM in the pronuclei of 3 Gy irradiated one-cell stage embryos (compare the nuclear staining of 0 and 3 Gy samples of Fig. 1D). Since the ATM kinase activity was present, we then investigated the level of the damage required for the phosphorylation of γ -H2AX. One-cell stage embryos were irradiated with 5, 10 and 15 Gy, and immunostained

Figure 1 Immunostaining of *in vivo* irradiated one-cell stage mouse embryos. (A) One-cell stage embryos were irradiated *in utero* at 8 h after fertilization and examined 30 min later for γ -H2AX. The pictures are those for the control and 3 Gy irradiated embryos. Note the strong staining of polar body in irradiated embryos. (B) Colcemid arrested metaphase of one-cell stage embryos were examined. Mitotic chromosomes were strongly stained for γ -H2AX regardless of 3 Gy irradiation to the embryos. (C) Presence of H2AX protein in one-cell stage embryos was confirmed by immunostaining with antiH2AX antibody. (D) One-cell stage embryos were examined for phosphorylation of ATM. The 3 Gy irradiated embryos were stained positive for phosphorylated ATM in pronuclei and polar bodies. (E) Staining for γ -H2AX was observed in 15 Gy irradiated one-cell stage embryos (upper panels). Note the homogeneous staining of the pronuclei which was completely abolished by treatment with λ -phosphatase (lower panels).

30 min thereafter. The doses of 5 and 10 Gy were ineffective in inducing readily detectable H2AX phosphorylation in the nuclei. However, nuclear staining for γ -H2AX was clearly visible when the γ -ray dose was 15 Gy (Fig. 1E). The staining of pronuclei was homogeneous and differed from that of the foci like pattern observed in tissue culture cells (Rogakou *et al.* 1999). This staining was clearly detecting phosphorylated form of H2AX and not the cross-reacting proteins since the λ -phosphatase treatment of the 15 Gy irradiated embryos completely abolished the nuclear staining. This observation indicates that a high level of DNA damage did lead to phosphorylation of H2AX even in the zygotic stage embryos.

H2AX phosphorylation in later stage embryos

Since earlier studies have shown that γ -H2AX focus formation is a common feature of irradiated somatic cells, we speculated that embryonic cells after certain stages of development would become sensitive to radiation induction of γ -H2AX focus formation. Thus, we examined mouse embryos of 8 h (one-cell stage), 24 h (two-cell stage), 48 h (six–eight cell stage) and 72 h (early blastocyst) for radiation induction of γ -H2AX focus formation. One-cell stage embryos were devoid of γ -H2AX foci when examined 30 min after irradiation, as

shown in Fig. 1A. However, when these irradiated embryos were left *in utero* for another 64 h (72 h after fertilization), a small fraction of blastomeres in the embryos became positive for γ -H2AX foci (Fig. 2A and B). The time course analysis for the appearance of γ -H2AX foci demonstrated that the irradiated one-cell stage embryos exhibited γ -H2AX positive blastomeres at 72 h after fertilization, but the fraction of the positive blastomeres were rather small (13.3%) (Fig. 3A). Similarly, two-cell stage embryos exposed to 3 Gy and examined 30 min later were devoid of γ -H2AX foci. A large fraction of blastomeres of those two-cell stage irradiated embryos became positive for γ -H2AX when left further for 24 h *in utero* reached six–eight cell stage (24 h after fertilization) (Fig. 2C and D). The time course analysis demonstrated that the number of γ -H2AX foci positive nuclei increased to 79% at 48 h after fertilization, declined thereafter to 0.4% at the end of 72 h (Fig. 3B). In contrast to one-cell stage and two-cell stage embryos, a rapid phosphorylation of H2AX was observed in six–eight-cell stage embryos (48 h post fertilization) when they were exposed to 3 Gy γ -rays (Fig. 3C). The γ -H2AX foci were visible in most of the blastomeres within 30 min (approximately 85% positive nuclei/embryo); the foci persisted for at least another 12 h and disappeared completely 24 h post irradiation at 72 h after fertilization (Fig. 3C).

The early blastocyst stage embryos of 72 h post fertilization behaved similarly to the six–eight-cell

Figure 2 Delayed phosphorylation of H2AX in γ -irradiated one-cell stage and two-cell stage embryos. (A) One-cell stage embryos irradiated with 3 Gy *in utero* 8 h after fertilization and examined for γ -H2AX after 30 min. (B) One-cell stage irradiated embryos were kept *in utero* for another 64 h and then examined for γ -H2AX. (C) Two-cell stage embryos irradiated with 3 Gy *in utero* 24 h after fertilization and examined for γ -H2AX after 30 min. (D) Two-cell stage irradiated embryos were further kept *in utero* for another 24 h and examined.

stage embryos in that γ -H2AX foci appeared within 30 min in almost all of the blastomeres. The staining increased further to 100% of the blastomeres and began fading at 6 h (Fig. 3D). We could not follow the embryos further due to onset of embryonic implantation.

Apoptotic response of embryos irradiated at the zygotic stage

We studied apoptosis of embryos directly irradiated at the zygotic stage. The 8 h zygotes were irradiated with 3 Gy of γ -rays *in utero*, and embryos were recovered from the mother at 24, 48 and 72 h after fertilization. The embryos were then analyzed for the TUNEL positive cells. As is clear from Fig. 4, the cell numbers of the 8 h irradiated embryos were suppressed when compared with the corresponding unirradiated embryos and they did not reach the compaction stage even at 72 h after fertilization (Fig. 4, the 3rd panel from the left). These embryos were TUNEL negative when they were examined at 24, 48 and 72 h after fertilization. In contrast, the embryos irradiated at 48 h after fertilization nevertheless reached the compaction stage and were positive for TUNEL (Fig. 4, the 4th panel from the left). The TUNEL positive cells were particularly frequent in the inner cell mass of the 72 h embryos irradiated at 48 h. These results indicate that radiation induction of apoptosis occurs only in the cells of the inner cell mass after they reach the blastocyst stage.

Discussion

Lack of phosphorylation in one-cell stage embryos

The present study demonstrates the peculiarity of damage response in mouse preimplantation stage embryos. In particular, the pattern of H2AX phosphorylation undergoes dynamic changes during preimplantation stage development. Although one-cell stage embryos carried H2AX protein, its phosphorylation by 3 Gy irradiation seemed to escape detection with the conventional immunofluorescence analysis in zygotic pronuclei and in nuclei of two-cell stage embryos. These irradiated embryos when kept *in utero* for another 64 h became γ -H2AX focus positive. Similarly, two-cell stage embryos were again devoid of γ -H2AX foci but they became positive when left another 24 h *in utero*. In contrast, γ -H2AX foci were readily induced in the post morula stage embryos and the rapid phosphorylation and the focus like staining pattern of the protein is similar to those observed in various somatic cells. In addition to the changes in the H2AX phosphorylation, we have also noted that apoptosis does not take place in the preimplantation stage embryos but was induced readily in the cells of inner cell mass after the embryos reach the blastocyst stage.

H2AX is known to be phosphorylated by ATM kinase (Rogakou *et al.* 1998, Kobayashi 2004), and the lack of phosphorylation of H2AX could be due to the inefficiency of the ATM kinase activity. However, this is

Figure 3 Time course of γ -H2AX positive cells in irradiated embryo. The time course was analyzed for embryos irradiated at the one cell stage (A), two cell stage (B), six–eight cell stage (C) and the early blastocyst stage (D). Arrow indicates the time of irradiation during preimplantation development. Open circles: unirradiated control; open squares: 3 Gy irradiated embryos. Approximately one hundred embryos were used for each data point.

unlikely since phosphorylated form of ATM was detected in 3 Gy irradiated one-cell stage embryos. In addition, polar body of irradiated one-cell stage embryos was highly positive for radiation induction of γ -H2AX. We also observed that a high dose such as 15 Gy induced phosphorylation of H2AX in zygotic pronuclei, although the pattern of the staining in these embryos was somewhat different from that of the later stage embryos. Furthermore, metaphase chromosomes of one-cell stage embryos were stained for γ -H2AX even without irradiation. Such staining for γ -H2AX of metaphase chromosomes without external DNA damage was reported to occur in meiotic cells as well as in HeLa cells (Fernandez-Capetillo *et al.* 2004, Forand *et al.* 2004, Turner *et al.* 2004, Ichijima *et al.* 2005), and this damage independent phosphorylation required ATM activity (Ichijima *et al.* 2005). Taken together, these results indicate that ATM kinase is present in one-cell and two-cell stage embryos.

There are three possible explanations for the apparent lack of γ -H2AX foci. The early embryos have a large amount of cytoplasmic protein and minute quantity of γ -H2AX might not be detected by the conventional immunological staining. However, γ -H2AX is stained

Figure 4 Apoptosis in the *in vivo* irradiated one-cell stage mouse embryos. Embryos were 3 Gy irradiated *in utero* at 8 h after fertilization and recovered at 24, 48 and 72 h. Embryos were also irradiated at 48 h after fertilization and recovered at 72 h. The TUNEL assay was performed after fixation. The upper panels are those stained with DAPI and the lower panels for TUNEL staining. Note that only TUNEL positive cells were found in embryos irradiated at 48 h after fertilization and recovered at 72 h.

efficiently in metaphase chromosomes in one-cell stage embryos and this tends to argue that our method is good enough to stain the embryos. Therefore, the lack of staining has to be explained either by weak phosphorylation of the protein by ATM or by difficulty in detecting the phosphorylated protein as foci.

Possible biological significance:

Extensive chromatin remodeling occurs in the zygote during the first cell cycle after fertilization and this appears to be the determining factor for the normal onset of gene expression (Renard 1998). This remodeling is accompanied by alterations in DNA methylation, histone modification and recruitment of various chromatin proteins into the pronuclei. The processes of histone modification and methylation affect male and female pronuclei differently (Adenot *et al.* 1997, Mayer *et al.* 2000). It is also known that DNA damage in the paternal genome by an anticancer drug interferes with chromatin modification in the subsequent preimplantation stage embryos (Barton *et al.* 2005). This modified state of chromatin is likely to be the reason for the lack of γ -H2AX foci.

Pronuclei of early embryos are far larger than those in somatic cells and therefore chromatin is much loose conformation. On one hand, this may result in poor ATM mediated phosphorylation of H2AX and this could be the reason why there was no foci like, staining of γ -H2AX. On the other hand, phosphorylation of H2AX may take place normally, but the resulting γ -H2AX is dispersed too loosely to be detected as foci.

Phosphorylation of H2AX is one of the earliest steps of damage response and γ -H2AX is a platform for many damage response proteins/complexes to assemble. These proteins/complexes are characterized by the motifs of BRCT and FHA which are shared by MDC1, 53BP, BRCA1 and MRN (Mre11-Rad50-Nbs1; van den

Bosch *et al.* 2003, Kobayashi 2004). These proteins/complexes are involved in cell cycle checkpoint and DNA repair. Therefore, the poor phosphorylation of H2AX and/or γ -H2AX focus formation is likely to result in defective damage response, leading to improper cell cycle arrest and DNA repair. In fact, there exists some clue to suggest involvement of H2AX phosphorylation in cell cycle checkpoint. B lymphocytes from H2AX^{-/-} mice were found not to exhibit G2/M arrest after 1 Gy irradiation although this insensitivity to G2/M checkpoint was not observed for doses higher than 2 Gy (supplemental Fig. 4 of reference Celeste *et al.* 2002). In consistent with this observation, we reported that one-cell stage mouse embryos fertilized with X-irradiated sperm are devoid of G1 and G2 arrest (Shimura *et al.* 2002a, 2002b). The lack of cell cycle checkpoint in one-cell stage embryos is compensated by a unique p53 dependent S phase checkpoint (Shimura *et al.* 2002b, Toyoshima *et al.* 2005).

The preimplantation stage mouse development has long been known to lack G1 phase (Mukherjee 1976). The lack of cell cycle phases and damage response has well been documented in other organisms. For example, in *Drosophila* and zebrafish, inhibition of embryonic DNA synthesis by aphidicolin has little effect on nuclear division and cytokinesis until the mid blastula transition at the tenth cleavage division (Raff and Glover 1988, Ikegami *et al.* 1997). In addition, cleavage stage human embryos may lack checkpoint function which contribute to extremely high incidence of post-zygotic chromosome abnormalities (Handyside & Delhanty 1997).

Delayed and stage specific phosphorylation of H2AX and its possible implication

Rapid focus formation of γ -H2AX in post-morula stage embryos was observed when irradiated at 6–8 cell, morula and blastocyst stages while irradiation of one-cell stage and two-cell stage embryos resulted in delayed γ -H2AX focus formation at 72 and 48 h postfertilization, respectively. Thus, embryos have to progress to certain developmental stages such as morula and blastula stages where a full set of factors are ready to execute a variety of damage responses. The dynamic patterns of stage-specific gene activity fall into two major phases, one up to the oocyte-to-embryo transition and one after cellular differentiation (Hamatani *et al.* 2004, Wang *et al.* 2004). In fact, early embryonic development of *Xenopus* is punctuated by so called midblastula transition in which when a burst of transcription and prolonged cell cycle time take place (Newport & Kirschner 1982). Similar midblastula transition exists in mice and a surge of transcription was reported to occur at the morula stage (Jeong *et al.* 2005). It is interesting to note that the morula is the stage where mouse embryos start to show cleavage delay when fertilized by irradiated sperm (Adiga, unpublished observation).

Indeed, as mentioned above, embryonic cells before the morula stage was devoid of G1 phase of cell cycle (Mukherjee 1976). Thus, the efficient phosphorylation and/or focus formation of H2AX coincides with this midblastula transition of mouse development.

Altogether, preimplantation stage development can be categorized into two: the pre-morula stage without proper damage response and the post-morula stage with full set of damage response including cycle checkpoint. DNA damage in the first period is not repaired properly and one exception in this stage is the repair system of error free homologous recombination between sister chromatids which occurs in a p53-dependent fashion (Toyoshima, unpublished observation). Any damage which cannot be repaired by the error free system is likely to be further amplified by rapid replication without accompanying damage response. The second stage of preimplantation stage has a full set of damage response which includes apoptotic response as observed in the present study. The blastomeres carrying amplified damage during the first stage of the development are efficiently eliminated by apoptosis especially when they reach to the blastocyst stage. We have observed apoptosis of cells in the inner cell mass of embryos fertilized by irradiated sperm. Thus, the poor phosphorylation of H2AX in one-cell stage and two-cell stage embryos may serve as a mechanism to eliminate deleterious cells from subsequent fetal development and thus avoid malformation (Norimura *et al.* 1996).

Conclusions

In summary, H2AX does exist in the preimplantation stage mouse embryos and the formation and disappearance of the nuclear γ -H2AX foci after irradiation is embryonic stage related. In addition, the embryos do not undergo apoptosis unless they undergo first differentiation at compaction. These suggest that DNA damage response is significantly suppressed during early embryogenesis.

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