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RESEARCH ARTICLE

Cellular responses to ionizing radiation change quickly over time during early development in zebrafish

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Abstract

Animal cells constantly receive information about and respond to environmental factors, including ionizing radiation. Although it is crucial for a cell to repair radiation-induced DNA damage to ensure survival, cellular responses to radiation exposure during early embryonic development remain unclear. In this study, we analyzed the effects of ionizing radiation in zebrafish embryos and found that radiation-induced γH2AX foci formation and cell cycle arrest did not occur until the gastrula stage, despite the presence of major DNA repair-related gene transcripts, passed on as maternal factors. Interestingly, P21/WAF1 accumulation began ∼6 h post-fertilization, although *p21* mRNA was upregulated by irradiation at 2 or 4 h post-fertilization. These results suggest that the cellular responses of zebrafish embryos at 2 or 4 h post-fertilization to radiation failed to overcome P21 protein accumulation and further signaling. Regulation of P21/WAF1 protein stabilization appears to be a key factor in the response to genotoxins during early embryogenesis.

Keywords: early development; radiation; zebrafish

Introduction

All organisms are surrounded by environmental stresses that affect their reproduction and development. Early-stage embryos are often extremely sensitive to the effects of ionizing radiation (Heyer et al., 2000; Valentin, 2003). Such high sensitivity can be partly explained by the fact that transcription and adaptive responses are inoperative during early development (Pelegri, 2003). However, the precise mechanisms by which living cells respond to environmental radiation during early development remain unclear.

The most frequently reported cellular responses to ionizing radiation in somatic cells begin with recruitment of the Mre11-Rad50-Nbs1 complex and ataxia-telangiectasia mutated kinase (ATM) at the site of double-strand breaks (DSBs) in DNA, followed by autoactivation of ATM (Price and D'Andrea, 2013; Smeenk and van Attikum, 2013; Watts, 2016). Autoactivated ATM phosphorylates histone H2AX, a variant of the H2A family of proteins, and forms γH2AX foci at the DSBs. Activated ATM also phosphorylates TP53 and activates p53 signaling, leading to upregulation of *p21* mRNA and P21/WAF1 protein levels (Hirao et al., 2000; Lossaint et al., 2011). Accumulation of the P21/WAF1 causes cell cycle arrest and promotes DNA repair (Gartel et al., 1996; Brugarolas et al., 1999; Wang et al., 1997; Roque et al., 2012). These signaling pathways are crucial for orchestrating DNA repair and maintaining genome stability in cells that survive irradiation (Wang et al., 1997; Hirao et al., 2000).

Cellular responses to irradiation during early development have been examined in various animal species. For example, irradiation of mice embryos before implantation either tends to be lethal or has no effects: this phenomenon is known as an all-or none stage (Russell and Russell, 1954; De Santis et al., 2007). During mouse development, γH2AX foci formation is delayed in embryos at the one-cell or two-cell stage, but occurs normally in six–eight-cell stage embryos (Adiga et al., 2007). At the one- or two-cell stage, mouse embryos have the ability to undergo G2/M cell cycle arrest (Yukawa et al., 2007); however, unlike two-cell stage embryos, one-cell stage embryos do not show an apoptotic response to irradiation (Adiga et al., 2007). In *Xenopus* embryos, irradiation during early developmental stages leads

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Abbreviations: ATM, ataxia-telangiectasia mutated kinase; DSBs, double-strand breaks; hpf, hours post fertilization; ATR, ataxia telangiectasia and Rad3-related protein

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to apoptosis of all embryonic cells (Anderson et al., 1997). In later stages (after mid-blastula transition), cells develop the ability to induce cell cycle arrest, which prevents apoptosis in the embryos (Anderson et al., 1997).

The zebrafish is a powerful model organism; its transparent body provides a distinct advantage for imaging studies, particularly during early development (Driever and Fishman, 1996; Brownlie et al., 1998; Dooley and Zon, 2000; Ward and Lieschke, 2002). Recently, this teleost fish has increasingly been used to study cellular responses to chemicals and environmental stresses (Hill et al., 2005; Hwang et al., 2007; MacRae and Peterson, 2015). Unlike *Xenopus*, zebrafish embryos do not undergo apoptosis until the mid-gastrulation stage (Ikegami et al., 1997; Negron and Lockshin, 2004). As the cellular machinery that responds to irradiation differs among organisms, further studies are required to reveal the underlying mechanisms that prevent radiation-induced apoptosis in zebrafish embryos in their early developmental stage. Previous studies showed that zebrafish embryos exposed to more than 4 Gy of radiation at 1 hour post-fertilization (hpf) are especially sensitive and unable to survive for longer than 24 hpf (McAleer et al., 2005). Increased cell death in retina and brain has been observed in zebrafish embryos irradiated with 5, 10, and 20 Gy at several timepoints in early developmental period, whereas such phenomenon can be ameliorated by a radioprotective agent, amifostine (Geiger et al., 2006). Continuous exposure to gamma irradiation to zebrafish embryos leads to their morphological abnormalities accompanied by altered gene expression profiles (Gagnaire et al., 2015; Hurem et al., 2017). It is of note here that the mRNA levels of *tp 53* and *sox19a*, a crucial factor in embryogenesis, in zebrafish embryos irradiated at 3 hpf were reported to show continuous upregulation from 24 to 72 h after radiation exposure (Praveen Kumar et al., 2017). However, at least to our knowledge, there have been very few studies that clarify the underlying cellular mechanisms by which zebrafish embryos differentially regulate their susceptibility to ionizing irradiation during early developmental stages.

In this study, we examined the changes in cellular responses over time and investigated cells in zebrafish embryos in early developmental stages respond to ionizing radiation.

Materials and methods

Animals

This study was approved by the Institutional Animal Care and Use Committee of Hiroshima University and carried out according to the Hiroshima University Animal Experimentation Regulations. Embryos were obtained from natural spawning events of wild-type colonies (SAT or WIK). Fish were maintained at the Research Institute for Radiation Biology and Medicine, Hiroshima University, on a 14-h light/10-h dark cycle at 28.5°C. Embryos were staged according to Kimmel et al. (1995) by the number of cells, somites, or hpf at 28.5°C.

Irradiation

Embryos at 2, 4, or 6 hpf were irradiated using a GammaCell 40 Exactor (^{137}Cs; Best Theratronics, Inc., Ontario, Canada). The dose rate was ∼0.8 Gy/min. Embryos were placed in 25-cm^2 flasks with ∼10 mL embryo medium. To examine time-dependent effects, 12–15 embryos per flask at 2, 4, or 6 hpf were irradiated with a total 1, 5, or 10 Gy. Embryos were irradiated at 1 Gy in experiments to examine the effect on cell cycle checkpoint, γH2AX foci formation, and *p21* mRNA and P21/WAF1 protein expression. For cell cycle checkpoint and γH2AX detection, 60–80 embryos per flask of 2, 4, or 6 hpf and 20–30 embryos per flask for *p21* real-time PCR were irradiated each time. All experiments were conducted in three biological replicates.

Immunohistochemistry

For γH2AX detection, embryos were fixed at intervals of 15 min after irradiation for 2 h and at intervals of 30 min for cell cycle checkpoint analysis.

Whole-mount immunostaining was performed as previously reported with slight modifications (Honjo et al., 2008). For anti-γH2AX antibody staining, embryos were fixed with methanol at −20°C and were incubated in acetone at −20°C for 7 min, and then incubated in blocking solution (2% normal goat serum, 1% bovine serum albumin, 1% Triton-X100, 1% dimethyl sulfoxide in phosphate-buffered saline [PBS]) after brief washing with PBSTx (1% Triton-X100 in PBS). For anti-phospho-HH3 antibody staining, embryos were fixed with 4% paraformaldehyde and were washed briefly in PBSTx, rinsed with water, and then washed again in PBSTx, after which they were incubated in blocking solution. For both antibody staining, the embryos were incubated overnight in primary antibody solution at 4°C after blocking. The embryos were then incubated in secondary antibody solution at room temperature (RT) for 4–5 h. An anti-γH2AX antibody (Gentex, Zeeland, MI, USA, GTX127342) was used at a dilution of 1:200, anti-phospho-HH3 antibody (Millipore, Billerica, MA, USA, #06-570) was used at a dilution of 1:100, and either Alexa Fluor 488- or 596-conjugated goat anti-rabbit polyclonal antibodies were used as secondary antibodies. Nuclei were stained with Hoechst 33342 (Sigma, St. Louis, MO, USA). Fluorescence was visualized with an Opera Phenix system (PerkinElmer) and analyzed using Hermony image analysis software (PerkinElmer). Positive foci number (γH2AX) or cell number (pHH3) per cell were calculated as the total

antibody-positive number among the total Hoechst positive number. The median number of total Hoechst-positive cells in average of nine embryos at each time point were 18,853 (range, 2,118–42,029) for γH2AX and 5,387.5 (range, 3,328–9,267) for pHH3 staining.

Real-time PCR

A total of 10 irradiated or normal embryos were pooled for each time point and all experiments were repeated three times. Total RNA was extracted using TRIzol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). cDNA was generated using the PrimeScript RT reagent kit (Takara). Each reaction contained 500 ng RNA in a total volume of 10 μL. cDNA was used as a template for real-time PCR using a SYBR Premix Ex Taq II kit (Takara) with a 7500 Real-time PCR system (Bio-Rad). The reaction involved an initial holding step at 95°C (30 s), followed by 40 cycles at 95°C (5 s) and 60°C (34 s). We quantified mRNA expression levels of DNA repair genes relevant for the following mechanisms: (i) homologous recombination (*rad51b*, *rad51c*, *rad 54*, *braca2*, and *mre11a*); (ii) non-homologous end joining (*ku70*, *ku80*, *xrcc4*, and *ligase 4*); (iii) base excision repair (*xrcc1* and *ligase 3*); (iv) DNA mismatch repair (*msh2* and *msh6*); (v) nucleotide excision repair (*xpc*, *ccnh*, and *xab2*). Also evaluated were the levels of mRNA expression of genes relevant for the p53 signaling pathway:*tp53*, *p21*/*waf1*, *ATM*, and ataxia telangiectasia and Rad3-related protein (*ATR*). The primers used are listed in Table 1. Real-time PCR results were analyzed using the comparative ΔΔCt method and normalized against 18S ribosomal RNA as a reference gene (McCurley and Callard, 2008). As a negative control, real-time PCR was carried out without a template. For repair-related gene expression, the expression of each gene was normalized to the reference gene expression level at each embryo stage. *p21* gene expression was normalized to the expression levels of control embryos at each timepoint after normalization to reference gene expression.

Generation of anti-P21/WAF1 antibody

Rabbit polyclonal antibody against zebrafish P21/WAF1 peptides was generated by Cosmo Bio, Inc. P21 peptides (pos:140–154; CQAKKRLVATPRKSGQ) were also designed and generated by Cosmo Bio, Inc. based on the cDNA sequence of zebrafish *cdkn1a*. Immunization and purification of the anti-P21/WAF1 rabbit serum was conducted by Cosmo Bio, Inc.

Western blotting

Irradiated or control embryos at 2, 4, and 6 hpf were collected in SDS sample buffer after 1 or 2 h of irradiation. Samples were separated on SuperSep Ace 5–20% gels (Wako Pure Chemicals, Osaka, Japan) at 20 mA and transferred onto polyvinylidene fluoride membranes (Bio-Rad). Blotted membranes were blocked with blocking solution (4% skim milk, 1% Tween 20 in PBS)

Table 1 Primer sequences used for real-time qPCR.

Gene name	Forward primer	Reverse primer
rad51B	TCTTCAACCTTCCTCAGGCT	TGAGCGTTTAGCTGACATCC
rad51C	TTCAACACTGCACCCTCCTA	GGTAAACCTCCGCTAACAGC
rad54	GGTGTCGTCTTGATGGACAG	GGTTTAGTCCCACTCCTCCA
brca2	GGCCTATCAGGCTTCTACTTTC	CTATTGGGCTTCTTCTCCATCC
mre11a	TGTGACCTTCGACGAGATTATG	TCTTGCGGGACGGTTTATT
Ku70	AAGGATGAGGTGGATGAGATAAAG	TGAAGAGAGCAGGTCGTAAATG
Ku80	GGCTGAGATTACAGAGGGAAAC	GCACGCTTGATCGAAAGAAAC
xrcc4	GATGGAGCGTGATCGGTATG	GGTGTCAGCTGGAAGGTAAA
lig4	TCGCATTGGATCAGGCTATAC	GGCTGCGGGAGGATTATTT
xrcc1	GAGGATAAACCCATCCCTGAAC	CGATGATGTAGCGCAGAAGTAG
lig3	GGAATCACAGGAGCAGAGTTT	CGTCTTCCAGTCCTTGTCATC
msh2	ACGGTGCGAGTGTTTGATAG	CTGTTCCCTGAACCCAGATTT
msh6	GGGAGGCAAATCCACTCTAATG	GACTCGATCCACAGGAGTAAGA
xpc	TGTTGCCCATAGGATGTGTT	TCACTGCTAAAGCGCAGTCT
ccnh	CCCATTGTTAGAGAACCCAGAG	GCAATCTGAGATGGAGAGAAGAG
xab2	GACGATGCTCGCACTATCTT	CTCAGCTCCATCTCTCCATATTC
tp53	GTACAAGTCCCTCCTGGAAATC	GGCAAATGCGTGTAAACAGTAA
p21	CCAGAGACGACACCGTTTATT	GGAAGACTGAGGAATGGATCTTT
atm	CAGTGGAGGAGTGAGAAATGAG	CCACGTCTCCCAGAAGAATATC
atr	GCTGTGTCAAAGTCCTCCTATC	GTCTGTTGGCATCTCCGATAAA

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for 1 h, followed by incubation with the primary antibody in blocking solution at RT for 1 h or at 4°C overnight. The membranes were then washed three times with 1% Tween 20 in PBS (PBST) for 5 min, followed by incubation in secondary antibody solution (horseradish peroxidase-conjugated goat anti-rabbit IgG antibody at a dilution of 1:3,000; Zymed Laboratories, South San Francisco, CA, USA, 81-6120 in blocking solution) for 30 min. After washing three times for 5 min with PBST, the membranes were developed using ECL Prime Western blotting detection reagent (Amersham Biosciences, Amersham, UK). Anti-gamma tubulin (at a dilution of 1:3,000; GeneTex, GTX113286) and anti-P21 (at a dilution of 1:2,000) were used as primary antibodies. Images were acquired using an Image Quant LAS 4000mini (GE Healthcare Japan Inc.).

Morpholino antisense oligo knockdown

The sequence of the *p21* Morpholino antisense oligo was used as described by Sidi et al. (2008). Morpholino oligo (MO) was purchased from Gene Tools, Inc. (Philomath, OR, USA). The p21 morpholino oligo was used at 0.01 M and approximately 5 nL of MO was injected into the embryos. Injected embryos were collected for western blot analysis at 6 hpf.

Statistical analysis

Statistical data were analyzed with Prism 6 software (GraphPad, Inc., Chicago, IL, USA). Data shown are presented as the mean ± SD. * denotes a significant p value of < 0.05, ** denotes P-value of <0.01, *** denotes P-value of <0.001. The P values were determined by a two-tailed unpaired student's t-test with Welch's correction or one-way analysis of variance (ANOVA) with Tukey's post-test where appropriate. The range of expression levels in qPCR was determined from six independent experiments with three biological replicates by calculating the standard deviation of the ΔCt (Pfaffl, 2001).

Results

Effects of radiation on embryos differed according to developmental stages

First, to examine time-dependent changes induced by irradiation of early-stage embryos, we irradiated zebrafish embryos at 2 (64-cell stage), 4 (blastula stage), and 6 (gastrula stage) hpf with 1, 5, and 10 Gy, respectively. At 24 hpf, the phenotypes of embryos irradiated at different times were variable. The most sensitive time-point was found to be 2 hpf, as previously reported (Figure 1, Table 2; Supplementary materials, Figure S1 and Table S1) (McAleer et al., 2005; Geiger et al., 2006). When exposed to 10 Gy of radiation at 2 hpf, no embryos survived until 24 hpf ($P < 0.001$), whereas more than 84% of embryos survived in control. At an exposure level of 5 Gy of radiation, the survival rate was more than 51.8%, although most embryos exhibited the shorter axis phenotype compare to control ($P < 0.001$). With 1 Gy exposures, no morphological abnormalities were detected, except that the brain exhibited a gray color, which is the possibility of indicator of TUNEL positive cell death as was previously demonstrated (Bladen et al., 2005, 2007; Geiger et al., 2006). The severity of malformation in irradiated embryos was milder when the embryos were irradiated at later stages of development. For example, approximately 94% embryos exposed to 10 Gy of radiation at 4 or 6 hpf survived until 24 hpf (Praveen Kumar et al., 2017). These results suggest that the ability of cells to respond and survive following radiation exposure differs and changes quickly according to the developmental stage in zebrafish embryos.

Maternal transcripts include abundant varieties and quantities of DNA repair genes

Early embryos use mRNAs derived from the oocyte rather than producing mRNA through zygotic transcription (Lee et al., 2014; Paranjpe and Veenstra, 2015; Svoboda, 2018). A few hours after fertilization, these maternal mRNAs, also known as maternal factors, begin to degrade and the transcription of zygotic genes is initiated. This maternal–zygotic transition occurs at approximately 3 to 4 hpf in zebrafish. DNA repair may not function if DNA repair-related genes are not present in the maternal transcripts. To evaluate whether DNA repair gene transcripts are found in zebrafish maternal factors, we examined the mRNA levels of various repair-related genes at 0, 2, 4 hpf and 6 hpf compared them to reference gene. For double strand break repair mechanisms, we examined *rad51b*, *rad51c*, *rad 54*, *braca2*, and *mre11a* for homologous recombination and *ku70*, *ku80*, *xrcc4*, and *ligase 4* for non-homologous end joining. For single-strand break repair mechanisms, we examined *xrcc1* and *ligase 3* for base excision repair, *msh2* and *msh6* for DNA mismatch repair, *xpc*, *ccnh*, and *xab2* for nucleotide excision repair and *tp53*, *p21/waf1*, *ATM*, and ataxia telangiectasia and Rad3-related protein (*ATR*) for the p53 signaling pathway.

Nearly all repair-related genes, including that of ATM or ATR, were abundant at all stages in the developing embryos (Figure 2; for ATM, also see Imamura and Kishi, 2005). The only exceptions were *p21/waf1* and *p21*, which were expressed at 4 and 6 hpf, although their mRNAs did not appear to be included in the maternal factors.

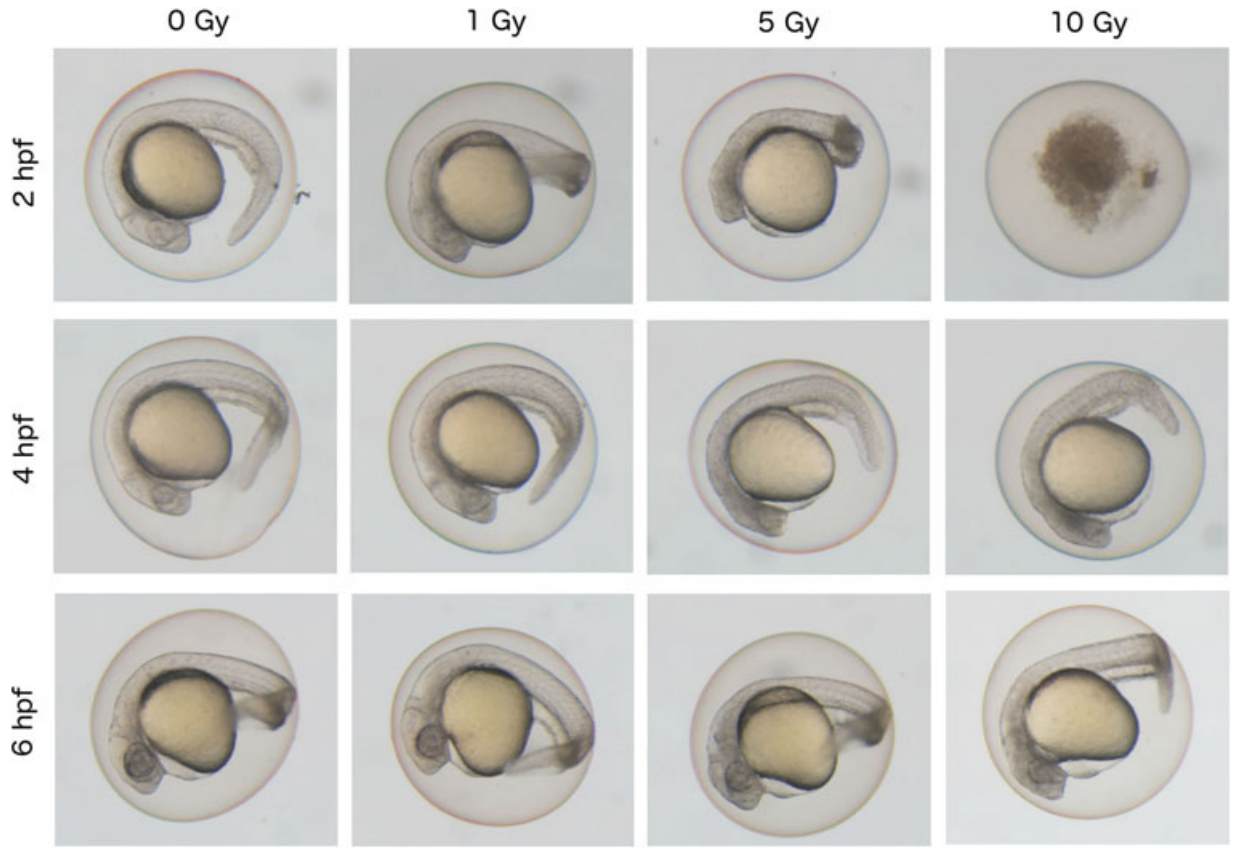

Figure 1 Differential effect of ionizing radiation on phenotypes of zebrafish embryos according to irradiated time-points during early developmental stages. Zebrafish embryos at 24 hpf after irradiation at 2, 4, or 6 hpf with doses of 0, 1, 5, and 10 Gy of radiation. Non-irradiated control embryos were shown in the left end panels (0 Gy). Whole embryos irradiated at 2 hpf (64-cell stage) showed the most severe phenotype. At a dose of 10 Gy, embryos irradiated at 2 hpf were dead, whereas those at 4 hpf (blastula stage) or 6 hpf (gastrula stage) showed normal body shape.

Table 2 Summary of effects of different doses of ionizing radiation on zebrafish embryos at 24 hpf after exposure to radiation at 2, 4, or 6 hpf.

Irradiated stages	Doses (Gy)	Survival at 24 hpf (%)	Body shape abnormality at 24 hpf (%)
2 hpf (minimum no. of embryos = 12)	0	84.9	12.5
	1	89.7	12.2
	5	51.8	100*** ($P = 0.0005$)
	10	0*** ($P = 0.0008$)	NC
4 hpf (minimum no. of embryos = 9)	0	100	0
	1	96.9	3.3
	5	88.8	31.6
	10	94.8	100*** ($P = 0.0005$)
6 hpf (minimum no. of embryos = 11)	0	90.4	3.3
	1	79.1	13.8
	5	95.5	2.7
	10	93.5	57.2* ($P = 0.0498$)

These results indicate that DNA repair-related gene transcripts are present in zebrafish maternal factors.

Detection of double-strand breaks in DNA using anti-H2AX antibodies in very early-stage embryos is not effective

The formation of γH2AX foci are among the very early changes observed in cells following DSBs in DNA. The number of γH2AX foci reflects the number of DSBs in the nuclei, which decrease as damage is repaired. When zebrafish embryos were irradiated at 6 hpf, the number of γH2AX foci peaked approximately at 30 min after irradiation and quickly decreased to background levels (Figure 3). In contrast, the number of γH2AX foci never exceeded 1 focus/nucleus when zebrafish embryos were irradiated at earlier timepoints (2 and 4 hpf). These observations suggest that the machinery for detecting damaged DNA may not be very efficient until developmental stages later than 4 hpf.

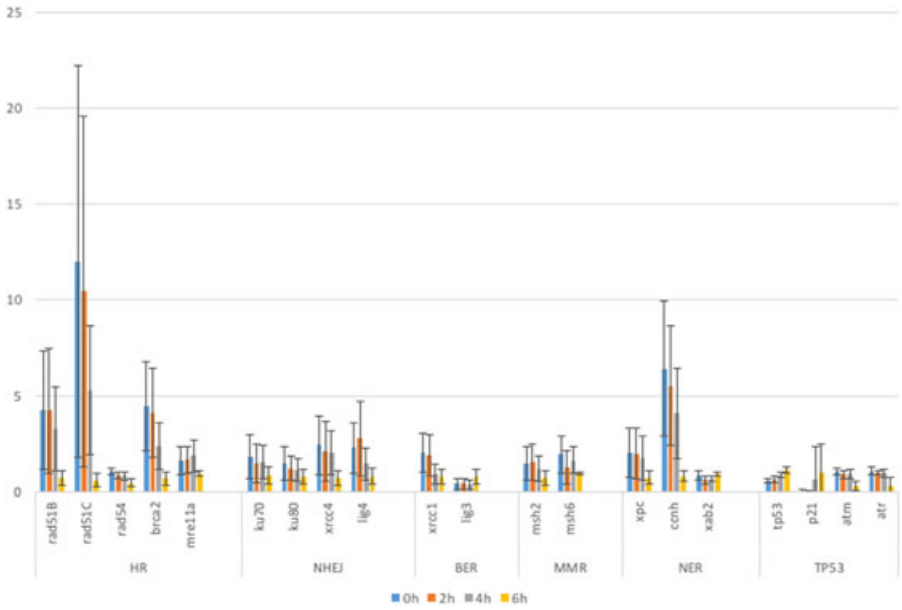

Figure 2 Expression levels of DNA repair-related genes in zebrafish embryos during early developmental stages. mRNA expression levels of various DNA repair genes in zebrafish embryos were quantified by real-time RT-PCR and their relative-fold expression at 0 hpf (blue bars), 2 hpf (orange bars), 4 hpf (gray bars), and 6 hpf (bright yellow bars) are shown in *Y*-axis compared with the expression level of reference gene. Types of genes denoted in *X*-axis are categorized as below: homologous recombination (HR)-related genes (*rad51B*, *rad51C*, *rad54*, *brca2*, *mre11a*), non-homologous end-joining (NHEJ) repair-related genes (*ku70*, *ku80*, *xrcc4*, *lig4*)base excision repair (BER)-related genes (*xrcc1*, *lig3*), nucleotide excision repair (NER)-related genes (*xpc*, *ccnh*, *xab2*), and p53 signaling (TP53)-related genes (*tp53*, *p21*, *atm*, *atr*). The columns and error bars represent the mean and SD, respectively.

Cell cycle arrest does not occur in early zebrafish embryos

Irradiation triggers cell cycle arrest to promote successful DNA repair in normal cells. We counted cells in M-phase at 0, 1, 2, and 3 h after irradiation using anti-phosphorylated histone H3 antibodies. Staining for phosphorylated histone H3 revealed that embryos irradiated at 6 hpf, but not at 4 hpf, show a reduction in M-phase cells (Figure 4).

This result, taken together with the temporal patterns of γH2AX foci formation, indicates that normal cellular responses to irradiation and DNA repair occur in zebrafish embryos at developmental timepoints of 6 hpf and later.

Early zebrafish embryos exhibit *p21* mRNA responses to irradiation

Collectively, our results raised the question of whether cells in early zebrafish embryos (2 or 4 hpf) contain the molecular machinery necessary for inducing adaptive responses to irradiation. P21/WAF1 is a key downstream target of the proto-oncogene product TP53, and upregulation of P21/WAF1 expression is a hallmark of activation of the p53 signaling pathway in response to irradiation (Langheinrich et al., 2002).

Unexpectedly, *p21* mRNA levels were upregulated when the embryos were irradiated at 2 hpf compared to those in embryos irradiated at 4 or 6 hpf (Figure 5). In embryonic stem cells, it has been reported that *p21* mRNA is upregulated when the expression levels of P21/WAF1 fail to increase after TP53 activation (Filion et al., 2009; Solozobova et al., 2009; Lee et al., 2010; Itahana et al., 2016). We first raised antibodies against the P21/WAF1 protein. This antibody recognized a protein of approximately 21 kD and the level of this protein was decreased in P21/WAF1 morpholino antisense oligo (P21 MO)-injected embryos (Figure 6A), suggesting that this antibody recognized the zebrafish P21/WAF1 protein. We examined the levels of P21 protein expression in zebrafish embryos irradiated at 2, 4, and 6 hpf at timepoints of 1 and 2 h after irradiation using this antibody. Although these embryos constantly expressed P21/WAF1, the levels of this protein remained unaffected after irradiation at the 2 hpf developmental stage. In contrast, embryos irradiated at later developmental stages exhibited upregulated expression levels of P21/WAF1 (Figure 6B).

Discussion

In this study, we found that cellular responses to ionizing radiation change quickly during the early periods of zebrafish development. Notably, irradiation did not induce γH2AX foci formation in embryos either at the 64-cell stage

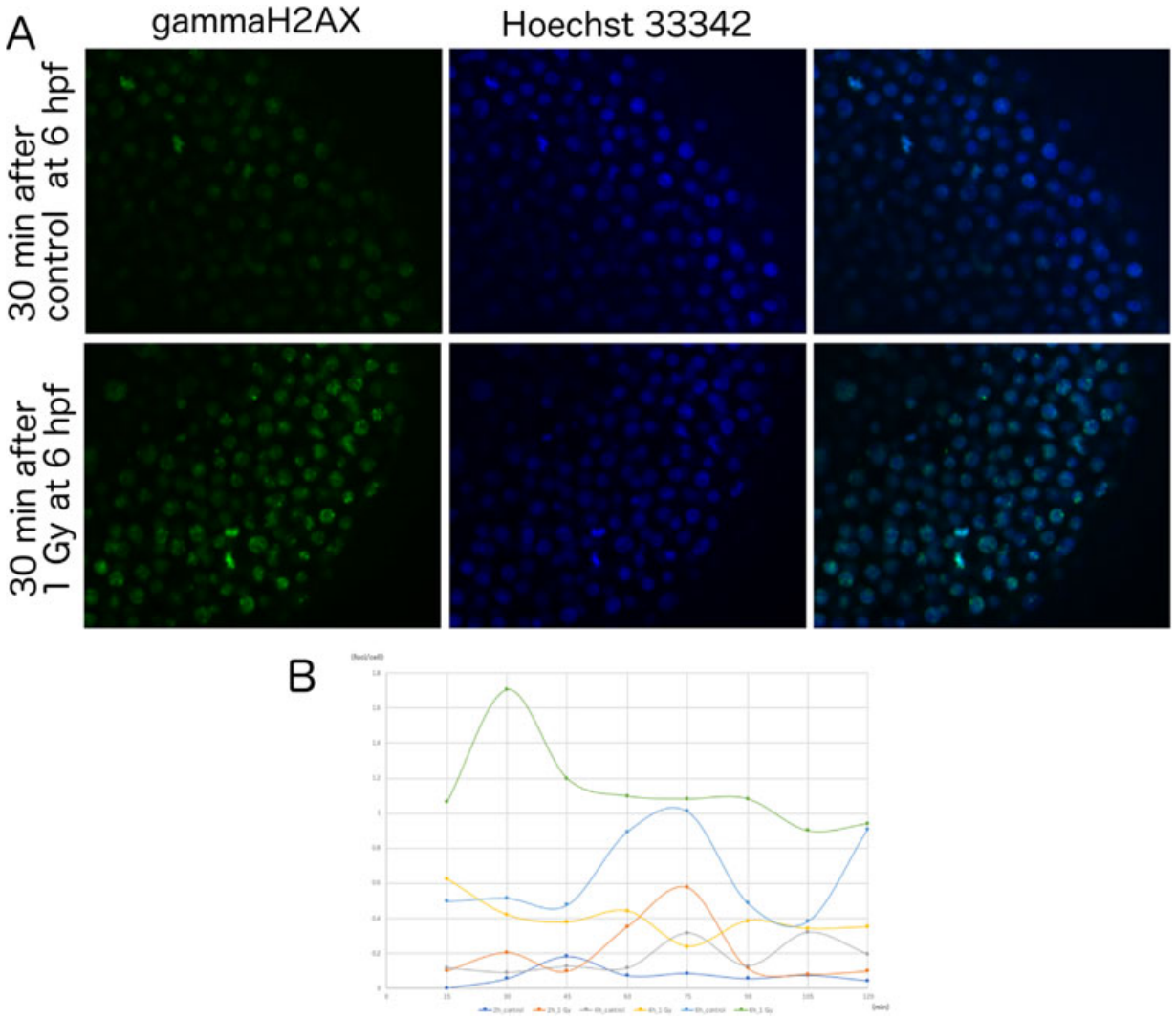

Figure 3 Formation of gamma-H2AX foci in zebrafish embryos after irradiation at early developmental stages. (A) Fluorescein immunostaining images of anti-γH2AX antibody (green, left panels), Hoechst 33342 (blue, middle panels) and merged images (right panels) in control embryos (upper panels) and 1 Gy-irradiated embryos (lower panels) at 6 hpf. Irradiated samples showed γH2AX foci within nuclei, whereas controls have almost no γH2AX foci. (B) Embryos irradiated with 1 Gy at 2, 4, or 6 hpf were fixed at 15-min intervals and stained with an anti-γH2AX antibody. The numbers of foci per nuclei at each timepoint were plotted as: 2 hpf control (light blue) and 1 Gy irradiated (orange), 4 hpf control (yellow), and 1 Gy irradiated (blue) or 6 hpf control (dark blue) and 1 Gy irradiated (brown). Only the 6 hpf embryos irradiated at 1 Gy showed γH2AX foci formation. Foci numbers peaked at 30 min after irradiation.

(2 hpf) and blastula stage (4 hpf). Staining for phosphorylated histone H3 revealed that embryos irradiated at 6 hpf, but not at 4 hpf, show a reduction in M-phase cells, consistent with previous findings that the cell number did not change significantly when zebrafish embryos at 6 hpf (after mid-blastula transition) were treated with a DNA polymerase inhibitor (Ikegami et al., 1997).

However, the levels of *p21* mRNA were upregulated in embryos irradiated at these stages, suggesting that the p53 signaling pathway was pre-activated. In embryos exposed to radiation at the gastrula stages (6 hpf), the expression levels of P21/WAF1 protein were observed to be upregulated after irradiation. The increased expression of P21/WAF1 likely accounts for the induction of both γH2AX foci formation and cell cycle arrest at the gastrula stage. Importantly, increased P21/WAF1 expression levels were observed at only after 2 h after irradiation of the blastula. This result indicates the lack of induction of γH2AX foci formation or cell cycle arrest.

The morphological effects of radiation exposure were most prominent in embryos irradiated at 2 hpf (64-cell stage). A likely explanation for this observation is that the

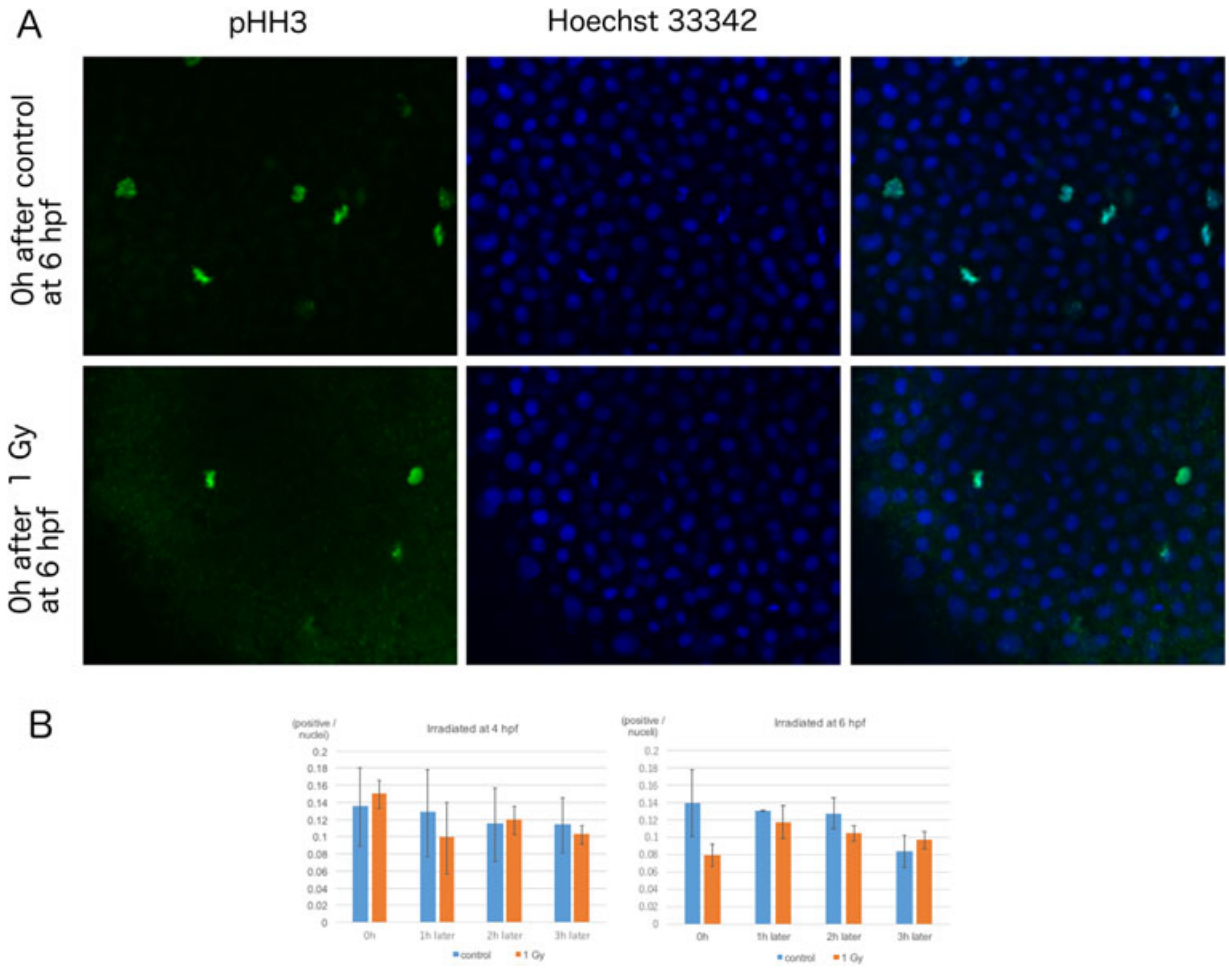

Figure 4 Effect of radiation exposure on M-phase cells in zebrafish embryos at early developmental stages. (A) Immunostaining images of anti-pHH3 antibody (green, left panels), Hoechst 33342 (blue, middle panels) and merged images (right panels) from embryos of 0 h after control (upper panels) or 1 Gy irradiation (lower panels) at 6 hpf. (B) Irradiated embryos were fixed at 1-h intervals. The M-phase marker, phospho-histone H3 (phospho-HH3) was detected by immunostaining; the ratio of positive cells/nuclei at each timepoint was calculated. In embryos irradiated at 4 hpf, the number of cells in M-phase did not change compared to those in the control embryos (blue bars). However, cells irradiated at 6 hpf (orange bars) showed a reduction in M-phase cells immediately after irradiation ($P = 0.06$). The columns and error bars represent the mean and SD, respectively.

activation of p53 signaling is inefficient because of the low accumulation of P21/WAF1 at this developmental stage, despite the upregulation of *p21* mRNA levels. The cellular inability to accumulate P21/WAF1 compromises the cell cycle arrest pathway and can result in inefficient DNA repair. A similar phenomenon was also reported in mice and human embryonic stem cells (Filion et al., 2009; Solozobova et al., 2009; Lee et al., 2010; Itahana et al., 2016). In these cells, although *p21* mRNA levels are upregulated, the synthesized P21/WAF1 protein degrades quickly, leading to insufficient accumulation of P21/WAF1, which may limit effective DNA repair responses to ionizing radiation in the early embryos of zebrafish.

Auto-activated ATM phosphorylates H2AX and TP53 to subsequently activate both γH2AX foci formation and the p53 signaling pathway. It is not yet clear why 2 hpf embryonic cells are incapable of forming γH2AX foci despite activation of the p53 signaling pathway. Although *ATM* and *ATR* mRNA are present in cells in the early phases of embryonic development, ATM may not function properly until the gastrula stage. If this holds true, the p53 signaling pathway is likely to be activated by pathways other than those linked to ATM or ATR. Alternatively, sufficient numbers of H2AX subunits may not be present in early zebrafish embryos, although this is not the case in mice (Adiga et al., 2007; Nashun et al., 2010). However, γH2AX foci formation was delayed in one-cell and two-cell mouse embryos, despite the abundance of H2AX subunits (Adiga et al., 2007; Nashun et al., 2010). More detailed analyses are required to understand these phenomena.

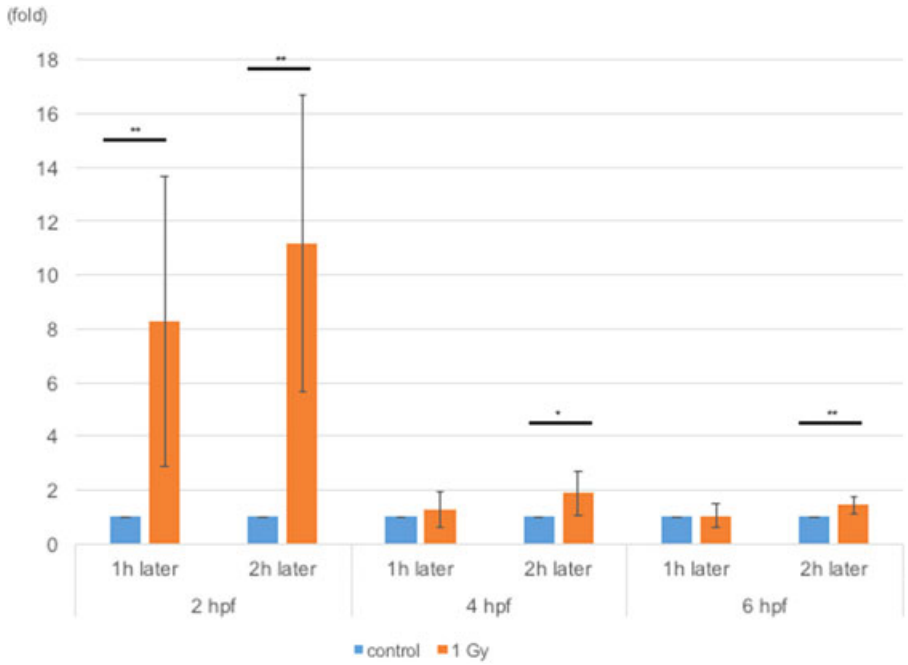

Figure 5 Levels of *p21* mRNA expression in irradiated zebrafish embryos. RNA samples were extracted 1 or 2 h after irradiating the embryos at 2, 4, or 6 hpf. Levels of *p21* mRNA were measured via qPCR and normalized to those in control embryos at each timepoint. At all timepoints, the levels of *p21* mRNA were upregulated in irradiated embryos. At all timepoints, 1 and 2 h after 2 hpf ($P = 0.008$ and $P = 0.001$), 2 h after 4 hpf ($P = 0.02$), and 2 h after 6 hpf ($P = 0.005$) were significantly different compared to the control at each timepoint. Note that upregulation of *p21* mRNA levels in embryos irradiated at 2 hpf were much higher than those irradiated at other time points. The columns and error bars represent the mean and SD, respectively.

It is known that in human or mice embryos, exposure to just 1 or 2 Gy of ionizing radiation is lethal. In contrast, zebrafish embryos are relatively resistant to radiation exposure, and the mechanisms underlying this resistance have been widely examined. Sussman (2007) has reported that the DNA repair capacity of zebrafish cells after exposure to UV radiation is much higher than that of human cells. *Ku70* and *Ku80* mRNAs are known to be present in the one-cell stage in zebrafish embryos, and injections of MO for both *Ku* genes, together with irradiation at 6 hpf, yielded more apoptotic cells at 24 hpf than control embryos (Bladen et al., 2005, 2007). These observations suggest that non-homologous end-joining DNA repair plays an important role in zebrafish embryos, at least in the gastrula stage. Further studies are needed to evaluate the DSB repair capabilities of zebrafish embryos at different developmental stages.

Conclusions

Our observations in this study suggest that the cellular responses of zebrafish embryos to ionizing radiation change quickly over time in a manner dependent on early developmental stages. Regulation of P21/WAF1 protein stabilization appears to be a key factor in the response to genotoxin during early embryogenesis.

The machinery for detecting damaged DNA does not appear to function in embryos prior to 4 hpf. Increased levels of *p21* mRNA in 0–2 hpf embryos after irradiation without a corresponding increase in P21 protein expression suggests the presence of other mechanisms that protect early embryos from TP53-mediated apoptosis. Further studies are required to identify the exact molecular pathways that regulate cellular responses to radiation exposure in zebrafish embryos at early developmental stages.

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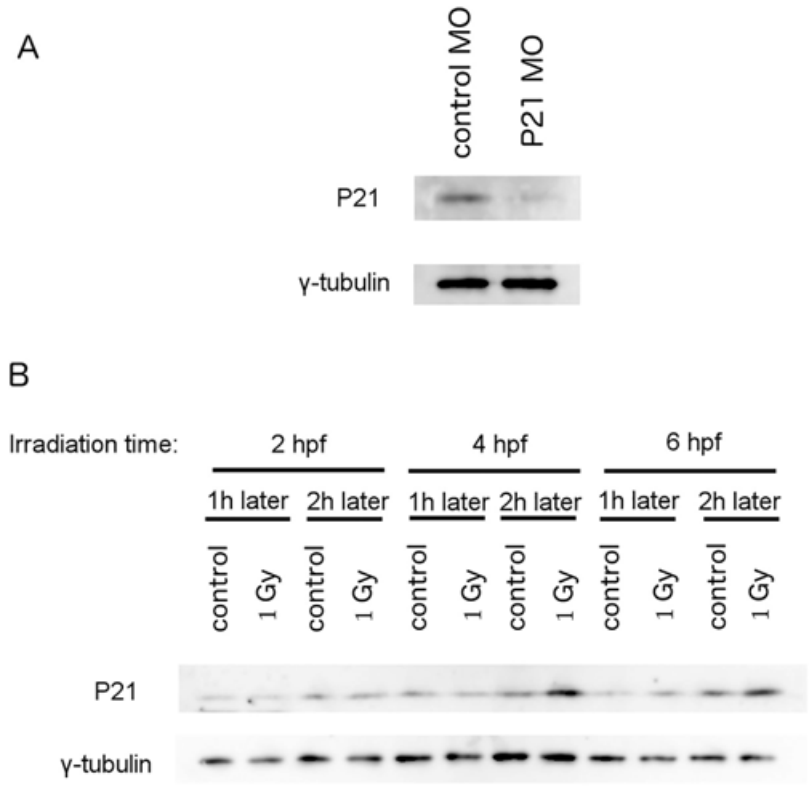

Figure 6 Levels of P21 protein expression in irradiated zebrafish embryos. A) Newly synthesized antibody recognized a protein between 20 and 25 kD in size. The amount of this protein decreased in the P21 antisense morpholino oligo (MO)-injected embryos compared to embryos injected with standard control MO. B) Protein samples were obtained 1 or 2 h after irradiating embryos at 2, 4, or 6 hpf. In embryos irradiated at 6 hpf, the levels of P21/WAF1 protein were upregulated at 1 h after irradiation, whereas in embryos irradiated at 4 hpf, P21/WAF1 protein levels were upregulated only after 2 h.

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Supporting Information

Additional Supporting Information should be found in the online version of this article at the publisher's web-site.

Figure S1. Images of pooled zebrafish embryos irradiated at 2, 4, or 6 hpf after irradiation with different doses (1, 5, and 10 Gy).

Figure S2. Repeated experiments of gamma-H2AX foci measurements in embryos exposed to irradiation at 2, 4, or 6 hpf.

Figure S3. Repeated western blotting analysis of P21/WAF1 protein expression in zebrafish embryos irradiated at different developmental stages.

Table S1. Summary of effects of different doses of ionizing radiation on zebrafish embryos at 4 dpf after exposure to radiation at 2, 4, or 6 hpf