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Mini-review

Embryos of the zebrafish *Danio rerio* in studies of non-targeted effects of ionizing radiation

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ABSTRACT

The use of embryos of the zebrafish *Danio rerio* as an *in vivo* tumor model for studying non-targeted effects of ionizing radiation was reviewed. The zebrafish embryo is an animal model, which enables convenient studies on non-targeted effects of both high-linear-energy-transfer (LET) and low-LET radiation by making use of both broad-beam and microbeam radiation. Zebrafish is also a convenient embryo model for studying radiobiological effects of ionizing radiation on tumors. The embryonic origin of tumors has been gaining ground in the past decades, and efforts to fight cancer from the perspective of developmental biology are underway. Evidence for the involvement of radiation-induced genomic instability (RIGI) and the radiation-induced bystander effect (RIBE) in zebrafish embryos were subsequently given. The results of RIGI were obtained for the irradiation of all two-cell stage cells, as well as 1.5 hpf zebrafish embryos by microbeam protons and broad-beam alpha particles, respectively. In contrast, the RIBE was observed through the radioadaptive response (RAR), which was developed against a subsequent challenging dose that was applied at 10 hpf when <0.2% and <0.3% of the cells of 5 hpf zebrafish embryos were exposed to a priming dose, which was provided by microbeam protons and broad-beam alpha particles, respectively. Finally, a perspective on the field, the need for future studies and the significance of such studies were discussed.

1. Introduction to non-targeted effects of ionizing radiation

In the conventional paradigm, the effects of ionizing radiation on cells occurred only because of direct energy deposition in the critical targets, i.e., the nuclear DNA in the irradiated cells themselves. Following the damage to DNA molecules, the affected cells might undergo repair, programmed cell death through apoptosis, cell cycle checkpoints and other outcomes. With the risk of misrepairing the damaged DNA molecules, ionizing radiation could ultimately lead to genomic mutation. However, studies in the past decades have demonstrated that damage from ionizing radiation does not necessarily occur only in the targeted cells. All the effects of ionizing radiation, which do not arise from direct energy deposition in the DNA of the irradiated cells, are classified as "non-targeted" effects. Such effects do not necessarily only occur on the level of cells but also on the levels of tissues or organisms. The radiation-induced bystander effect (RIBE) and radiation-induced genomic instability (RIGI), which were demonstrated *in vitro* and *in vivo*, are categorized as non-targeted effects of ionizing radiation.

1.1. Definition and history of the RIBE

Different definitions for the RIBE exist in the literature, which have been reviewed by Blyth and Sykes [1], wherein they described the bystander effects as "*any effect induced in a cell as a result of another cell(s) being exposed to radiation*".

Mothersill and Seymour [2] suggested another broad definition: "*The bystander effect in this context refers to the detection of responses in unirradiated cells that can reasonably be assumed to have occurred as a result of exposure of other cells to radiation.*" The RIBE describes the response in unirradiated cells that have not been exposed to ionizing radiation and is regarded as a non-targeted radiation effect. The unirradiated cells are commonly named as bystander cells. There has been no consensus on the spatial distribution of the classic RIBE, the location of unirradiated cells from irradiated cells or whether protective or adverse responses are induced in the unirradiated cells by the irradiated cells. In fact, this observation is because different effects may occur in different cells. Moreover, the magnitude of the effect may differ in the same cells under different conditions, e.g., at different levels of oxidative stress.

Although the mechanisms underlying the RIBE are not fully understood, gap-junction intercellular communication [3,4] and the secretion of soluble factors from irradiated cells to the

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bystander cells were found to play important roles [5,6]. Reactive oxygen species (ROS), which were generated from irradiated cells, were among the reported bystander signals, which could lead to oxidative stress and DNA damage in nearby unirradiated cells. In contrast, the ROS production in irradiated cells upon radiation exposure could also activate the synthesis of antioxidants [7], and an increase in the antioxidant level could stimulate a defense mechanism, and it is established that the defense mechanism plays a role in the radioadaptive response (RAR) [8]. The RAR could protect cells from a subsequent challenging radiation. Nevertheless, *in vitro* studies suggested that RAR might not necessarily share a mechanism with the toxic bystander effect [9]. Although the relation between the toxic bystander effect and the RAR remains under debate, the dual nature of the RIBE could be a result of differences in genetic and epigenetic factors [10].

The production of bystander factors is also known to be genetically dependent [11]. In particular, radiosensitive cell lines with small or no shoulders in their cell survival curves will generate larger bystander effects when compared with radioresistant cell lines with large shoulders in their survival curves.

Results from different studies on the dose dependence of the RIBE have also been equivocal. Although some studies reported that more than 10% of cells within a population should be irradiated to enable a full induction of the RIBE [12,13], there was another study that showed that the irradiation of less than 3% of cells within a population was enough to induce a saturated RIBE [14]. The RIBE was induced by both high and low LET radiation. Nonetheless, there were relatively few studies on the relation between the quality of radiation and the RIBE [15–21]. For a charged particle with a specific energy, the LET is defined for a medium as the quotient dE_L by dl, where dE_L is the average energy that is locally imparted to the medium by the charged particle while traversing a distance dl. A high-LET radiation produces a denser track of ionization and excitation along the particle trajectory when compared with a low-LET radiation. As such, a larger LET indicates that the radiation can increase the complexity of the induced DNA damage and, therefore, increase the amounts of unrepaired or misrepaired DNA damage. However, the results regarding the dependence of the RIBE on LET are equivocal. For example, bystander-induced chromosome aberrations and cell cycle-related bystander response were found to be independent of the LET of the radiation [17,18]. In contrast, considering the induction of p53 protein expression in rat epithelial cells, X-ray exposure led to a less pronounced RIBE than alpha-particle exposure [22]. Significant LET dependence was also reported in the study of bystander-mediated micronucleation and cell proliferation, with high-LET radiation being more effective in inducing the RIBE than low-LET radiation [16]. From the above equivocal results, it is difficult to draw conclusions on the dependence of the RIBE on the radiation dose and on the quality of radiation [23].

When referring to the long-distance bystander effect, the term 'abscopal effect' was used, which was first introduced by Mole in 1953 [24]. The abscopal effect describes the response of the unirradiated tissue, which is located outside the volume of the irradiated region and that of the scattered radiation [25,26]. Some literature also described the long-distance bystander effect within the individual as the "out of field" bystander effect [27].

Before the first demonstration of an *in vitro* RIBE by Nagasawa and Little in 1992 [14], there was already evidence indicating that animals or humans that are exposed to ionizing radiation could produce soluble factors in the blood plasma, which, when transferred to the cell cultures from non-irradiated individuals, could cause chromosome damage in the latter. This type of indirect damage to the non-irradiated cells, which was inflicted by the plasma that was harvested from irradiated animals or humans, was reported in the late 1950s. Parsons et al. [28] observed that the reatment of chronic granulocytic leukemia led to cell damage in the bone marrow of patients who received radiation to the spleen; however, the methodology to ensure the non-irradiation of the bone marrow during the irradiation of the spleen was not described.

The presence of clastogenic factors in the plasma from the irradiated individual was supported by the finding of Souto [29], who showed that mammary tumors were significantly induced in rats by the injection of plasma from irradiated animals. Subsequently, Hollowell and Littlefield studied chromosomal damage in normal unirradiated lymphocytes, which was inflicted by plasma that was harvested from X-ray irradiated patients [30] and from high-dose radiotherapy patients [30,31]. Their results confirmed that both chromosome and chromatid breakages were induced by the plasma that was harvested from an irradiated individual. This type of plasma clastogenic activity was also shown in alpha-particle irradiated animals. Poncy et al. [32] observed an increase in sister chromatid exchanges in rats that were previously exposed to alpha-particle emitting radon and suggested that clastogenic factors might have been translocated from the lungs to the bone marrow of rats that were exposed to alpha-particles. Although the plasma clastogenic factor (CF) has been extensively studied [33–36], its nature is not fully understood. The CF has a low molecular mass and its stability is changed by changes in temperature; however, the clastogenic activity can be conserved over a year under frozen conditions. Considering these properties, the CF has been suggested to contain several chromosome-damaging components instead of a single factor. In particular, superoxide dismutase (SOD) inhibited the formation of the CF, which indicated the involvement of superoxide radicals in the CF. In contrast, the cytotoxic cytokine tumor necrosis factor-α (TNF-α) and the formation of lipid peroxidation products, as well as the production of free radicals were proposed to be related to the clastogenic activity [37]. Notably, free radical scavengers have been reported to successfully reduce the clastogenic activity in many studies. As reviewed by Goldberg and Lehnert [38], the lipid peroxidation in fatty acids that were contained in blood or other extracellular fluids was initiated by the generation of free radicals, which would then form DNA-damaging lipid peroxidation products. As such, extracellular free radicals might play an important role in mediating the clastogenic effect between distant organs, e.g., from the lungs to bone marrow. Remarkably, there is currently a consensus that the soluble CF can reach different tissues within an organism through the circulation of blood cells from the area that is exposed to radiation [39,40].

In fact, studies that were performed on the Japanese atomic bomb survivors [41], and later on the people involved in the Chernobyl nuclear accident [42–44] as well as radiotherapy patients [45–48], also supported the presence of the RIBE. Studies on plasma clastogenic effect were later reviewed by Mothersill and Seymour [2], Goldberg and Lehnert [38], Morgan [49] and Lindholm et al. [37]. It would also be interesting and beneficial to study whether the CF can be transmitted at the inter-organismic level. Recently, there has been massive evidence of the RIBE occurring at the inter-organismic level, e.g., between mice [50,51], zebrafish [52] and medaka [53].

An *in vitro* RIBE was first successfully demonstrated by Nagasawa and Little [14] by irradiating Chinese hamster ovary cells with low-dose alpha particles (as low as 0.32 mGy). Nagasawa and Little observed a significant change in the induction of sister chromatid exchanges (SCE) in the unirradiated cells when the alpha particles traversed less than 1% of the cell nuclei directly using their experimental setup. Their results gave the first evidence of the induction of genetic damages on the non-targeted cells. The RIBE was subsequently demonstrated through a wide range of biological endpoints, including the induction of apoptosis [54–56], gene mutation [57,58], the production of reactive oxygen species [59],

micronucleus formation [60,61] and neoplastic transformation [62,63]. The RIBE has been extensively studied and reviewed [1,23,49,64–80].

The RIBE was more prominent in the low-dose radiation region [81]. There was a consensus that an increase in the dose to the irradiated cells did not further intensify the effect on the bystander cells [82]. For alpha-particle or heavy-ion traversals, the magnitude of the bystander effect did not depend on the dose [1]. For low-LET radiation, such as X-rays, an increase in the probability of induction of the bystander effect at doses as low as 50 mGy was reported by Schettino et al. [83]; however, the magnitude of the bystander effect did not depend on the applied dose. In contrast, a dose-dependent bystander effect was observed in a medium-transfer experiment using a low-LET radiation [84]. In fact, it would be necessary to discuss the dose dependence of the RIBE for different radiation types. For high-LET radiation, such as alpha particles, even the traversal of a single particle can deposit a substantial dose in the targeted cell and cause profound biological effects, such as clustered-type DNA damage, in contrast to the deposition of a small dose in the instance of low-LET radiation, such as X-rays. For example, the dose in the core of the track of HZE particles can reach the order of hundreds of Gy. As such, when discussing the dose dependence of the RIBE for radiation with different LET values, it is relevant to report the data as a function of the mean absorbed dose or as simply in fluence. The occurrence of the RIBE followed an on or off mechanism with the probability of an increased effect with the radiation dose to the target cell [67,83,85]. Not all cell types could produce or respond to bystander signals [63,86]. However, the RIBE could be transmitted to the progeny of the bystander cells [63,86,87]. A non-detrimental RIBE was also reported, including an increase in cell proliferation [88,89] or in the induction of the RAR [90,91]. In regards to the implications on radiotherapy, more tumor cells could be killed through the RIBE by the anti-tumor abscopal effect [92]. The present review will focus on the *in vivo* non-targeted effects of ionizing radiation, and the readers who are interested in more details regarding the *in vitro* studies on the RIBE are referred to the many excellent reviews [1,23,49,64–80].

1.2. Definition and history of RIGI

RIGI refers to the delayed lethal mutations or reproductive cell death in the progeny of irradiated cells that persist for many generations, and elevations in the rate of the *de novo* appearance of chromosomal aberrations and gene mutations, which appear in the progeny of irradiated cells. RIGI can be observed in progeny, which are many generations after the initial irradiated cells, whereas RIGI had not been detected in the irradiated cells themselves or their immediate progeny, which are unstable and mutation-prone but appear to be healthy [10,49,64,86,93–95]. RIGI in *in vitro* experiments has been extensively studied and reviewed [64,76,79,96–99]. Various biological endpoints were employed for RIGI studies, including chromosomal aberrations [94,95,100–105], micronucleus formation [106,107], gene mutation [108–112], reproductive cell death [84,113,114] and apoptosis [115–117].

When cells were irradiated *in vitro* and cultured, non-clonal cells with chromosomal aberrations could be found in the progeny many generations after the irradiated cells. RIGI, with respect to chromosomal aberration, was first described by Wright and colleagues in 1992, who found a high non-clonal frequency of chromosomal aberration in the progeny of hemopoietic stem cells that were irradiated *in vitro* with alpha particles [94]. Subsequently, extensive *in vitro* studies demonstrated the occurrence of RIGI in a variety of cell types [101,102,105,111,118–120]. In fact, RIGI was reported for both high- and low-LET radiation [121]. Some studies showed that radiation quality could cause differences in the yield of chromosome versus chromatid aberrations and that high-LET radiation was found to be more effective in inducing chromatid-type aberrations [121]. Furthermore, RIGI depended on the radiation dose. Interestingly, RIGI was apparent at low doses and, in some instances, was reduced at high doses of radiation [79]. The present review will focus on the *in vivo* non-targeted effects of ionizing radiation, and the readers who are interested in more details regarding the *in vitro* studies on RIGI are referred to the many excellent reviews [64,76,79,96–99].

The RIBE effectively enlarges the target for radiation effects through "horizontal transmission" to enlarge the affected population size, whereas RIGI enlarges the target for radiation effects through "vertical transmission" to draft the progeny into the affected population [10]. Both the RIBE and RIGI lead to damage in the affected population, which is similar to radiation damage. In fact, the RIBE and RIGI were linked to each other [98,122]. The inflammatory-type response to radiation-induced stress, which was observed in both the RIBE and RIGI, might suggest a common mechanism for the two non-targeted effects [123]. As anticipated, these non-targeted effects have far reaching implications, e.g., on cancer-risk assessment in the low-dose region [124] and on the induction of secondary cancer [77,80], etc.

As expected, the majority of research studies on the non-targeted effects of ionizing radiation have focused on *in vitro* studies. Whereas *in vitro* experiments are more convenient to perform, the extrapolation of the results back to the biology of the whole living organism might not always be straightforward. As such, *in vivo* studies are valuable to help better estimate the health risks of exposure to ionizing radiation, particularly following exposure to low doses/low fluences.

The present paper will present embryos of the zebrafish *Danio rerio* as the *in vivo* model for non-targeted effects of ionizing radiation on an organism or a tumor, which will be presented in Section 3. First, an introduction to the general use of embryos of zebrafish (*D. rerio*) as an *in vivo* model will be given in Section 2. Section 4 will give the conclusions. Table 1 summarizes the niche areas that are provided by the zebrafish embryo model for studying RIBE/RIGI and a summary of the current progress in these studies.

2. Popularity of embryos of zebrafish (*D. rerio*) as an *in vivo* model

Zebrafish (*D. rerio*) has emerged as a popular vertebrate model in many fields of research studies, such as developmental biology, physiology, toxicology, and environmental research as well as cancer research [125]. Such popularity has apparently stemmed from the many advantages behind its use as an *in vivo* model. In particular, the human and zebrafish genomes share considerable homology, including the conservation of most DNA repair-related genes [126,127]. Whole zebrafish genome sequencing, which was recently conducted, has further facilitated the comparison of the human genome with that of zebrafish genome. This comparison showed that approximately 70% of the human genes had at least one obvious zebrafish ortholog [127]. It is remarked that among different fish species, zebrafish has the most complete database on genomics, molecular genetics and embryology, which is available on the website of the Zebrafish Information Network (http://www.zfin.org/ZFIN). The high conservation of the human and zebrafish genomes, as well as the availability of genomic data, constitute the most important reasons for the zebrafish to be employed as a powerful animal model. The fact that zebrafish has vascular, hematopoietic, immune, and central nervous systems as well as organs, such as the heart, liver, and kidney, with some phenotypic

Table 1
Advantages of the zebrafish embryo model, and a summary of RIBE/RIGI studies using zebrafish embryo model. The numbers of sections for the corresponding discussion are indicated to help the reader navigate through the paper.

Advantages of the zebrafish embryo model	Studies prior to the zebrafish embryo model	Examples of potential usefulness	RIBE/RIGI studies using zebrafish embryo model
Allow studies on high-LET radiation (in addition to low-LET radiation)	Most *in vivo* studies on RIBE and RIGI made use of low-LET photons (Sections 3.1 and 3.2)	Useful for assessing non-targeted effects from exposures to environmental radon progeny, hadron therapy, targeted atomic nanogenerators or targeted alpha therapy (Section 4.1)	(1) Non-targeted effects of alpha particles, together with design of experimental setup and procedures (Section 5.1) (2) Dose response of zebrafish embryos irradiated with alpha particles at 1.5 hpf, incorporating RIGI, was revealed at 24 hpf (Section 5.2) (3) RIBE was demonstrated in zebrafish embryos through successful induction of radioadaptive response (RAR) when only <0.3% of the embryo cells on 5 hpf zebrafish embryos were hit by alpha particles during the priming exposure (Section 5.3)
Allow studies on microbeam radiation (in addition to broad-beam radiation)	Most *in vivo* studies on RIBE and RIGI made use of broad-beam photons (Sections 3.1 and 3.2)	Useful for assessing non-targeted effects from exposures to modulated radiation fields commonly used nowadays in radiotherapy (Section 4.2)	(1) Non-targeted effects of microbeam protons, together with the design of special experimental setup and procedures (Section 5.1) (2) Dose response of zebrafish embryos irradiated with microbeam protons at the 2-cell stage, incorporating RIGI, was revealed at 24 hpf (Section 5.2) (3) RIBE was demonstrated in zebrafish embryos through successful induction of radioadaptive response (RAR) when only <0.2% to <1% of the embryo cells on 5 hpf zebrafish embryos were hit by microbeam protons during the priming exposure, depending on the distribution of hit cells (Section 5.3)
Allow studies on embryo models	Restricted to bystander induction of cancers and studies on RIBE in tumors (Section 4.1)	Useful for attempts to fight cancer from the perspective of developmental biology (Section 4.3)	All the RIBE/RIGI studies using zebrafish embryo model described above can also be viewed as progress in this category (Sections 5.1 to 5.3)

features resembling those features in humans, has made zebrafish an animal model with a great potential for research studies on the molecular mechanisms of diverse human diseases [128].

In vitro mechanistic studies of non-targeted effects of ionizing radiation revealed the important role played by the DNA repair pathways [79,80]. Nonetheless, *in vivo* mechanistic studies of non-targeted effects of ionizing radiation were limited [87,129]. If *in vitro* mechanisms also apply to *in vivo* situations, then DNA repair mechanisms should also play important roles in *in vivo* situations. As such, there is an increasing demand for choosing an appropriate animal model for effective studies of DNA repair mechanisms. Pei and Strauss [130] remarked that the zebrafish genomic DNA contained orthologs of genes that are involved in all DNA repair pathways in higher eukaryotes, which include non-homologous end joining (NHEJ), and that the role of specific DNA damage response genes in each repair pathway could be studied by simple morpholino-based or shRNA knockdown experiments. As such, Pei and Strauss concluded that zebrafish formed an ideal model for studies on DNA damage and repair pathways.

There are also many practical advantages for using zebrafish embryos as a model for studying radiation-induced non-targeted effects: (1) zebrafish embryos are optically transparent, which enables the microscopic inspection of embryogenesis development; (2) zebrafish embryos develop rapidly, which leads to shorter experiments. The developmental stages of zebrafish embryos were described in detail by Kimmel et al. [131]: major organ systems were evident within 24 h post-fertilization (hpf), and the entire development was completed in approximately five days post-fertilization (dpf); (3) zebrafish embryos can be produced in great number daily due to the high fecundity of zebrafish, which facilitates a high throughput of experiments; (4) zebrafish embryos can take up drugs directly from the medium, which is much more convenient for therapeutic studies when compared with the need for drug injections into experimental models, such as mice. As such, the zebrafish embryo model has become increasingly popular in studies of toxicology [132], developmental biology [125] and carcinogenesis [133].

3. *In vivo* studies on non-targeted effects of ionizing radiation

3.1. In vivo studies on RIBE using animal models

In the present review, *in vivo* studies only refer to those studies using whole organism models and do not include those studies involving tissue models, which have also been regarded by some researchers as *in vivo* models. Ureter models [55,134], as well as commercially available skin reconstruct models containing both epidermis and dermis or the epidermal layer alone [135–137], are classified as tissue models here. Although tissue models can provide some valuable information regarding the non-targeted effects of ionizing radiation in the multicellular tissue environment, it remains necessary to extrapolate such results back to the biology of the whole living organism; thus, there remains a marked advantage to perform the relevant studies in whole organism models [138,139]. As remarked by Bertucci et al. [139], studies using whole organisms, which target specific cells, cell groups or organs, would be needed to realistically elucidate the mechanisms underlying the RIBE. We will hereafter focus on whole organism models when discussing *in vivo* models.

Most *in vivo* studies on the RIBE and RIGI made use of broad-beam low-LET photons. The rodent model has been commonly employed for *in vivo* studies on the RIBE within an organism. Khan

et al. [5] observed an increase in micronucleus formation in the unirradiated area of the rat lung and proved that the bystander effect propagated from the irradiated lung area to the non-irradiated lung area with the involvement of reactive oxygen species (ROS) and nitric oxide (NO) [6]. The sequel study by Khan et al. also confirmed the involvement of cytokines in the RIBE [140]. A series of cranial exposure experiments using a mouse model were performed by Koturbash et al. [141–143]. In their experiments, one group of mice received X-ray exposure to the skull only, whereas the rest of the animal's body was protected by a lead shield. Molecular changes in the distant shielded bystander tissues and organs were examined, and the results from cranial exposure were compared with those results that were obtained from whole body exposure. The RIBE induced in mice with cranial exposures was found to be sex specific [39,143] and tissue specific [40]. Additionally, the RIBE could persist for a long period, which was up to seven months post-irradiation [142]. Mancuso et al. [3,4] studied the role of gap junctional intercellular communication (GJIC) in the RIBE in mice with partial-body X-ray irradiation. Long-range radiation damage to the mouse brain was reduced when the expression of connexin 43 was inhibited. An *in vivo* RIBE was also reported in other model organisms, such as the plant *Arabidopsis thaliana* [144–148], the instar silkworm *Bombyx mori* larvae [149] and the nematode *Caenorhabditis elegans* [139,150]. In the studies abovementioned, the mechanisms that were responsible for the *in vivo* RIBE were found to be largely consistent with those mechanisms that were responsible for the *in vitro* RIBE, in that communication between bystander cells and irradiated cells could be achieved through either gap junctions [3,4] or the transmission of soluble factors [5,6]. Lipid peroxidation products, such as hydroperoxides and aldehydes, TNF-α, TGF-β, IL1β, NO and ROS, were potential soluble factors that were involved in the bystander intercellular signaling [6]. In contrast, the sex-specific bystander effect that was reported by Koturbash et al. [39,143] was observed together with DNA methylation changes, which is an important epigenetic phenomenon that is involved in the regulation of gene expression and genome stability. In fact, evidence indicated that epigenetic changes were involved in both direct and indirect radiation effects [151]. Until now, few studies on epigenetic changes that were involved in the *in vivo* bystander effect have been performed. More extensive epigenetic studies should be able to greater enlighten us regarding the mechanisms underlying *in vivo* RIBE.

Studies on the RIBE in animal models were also performed in relation to radiotherapeutic considerations. The RIBE in normal tissue and in tumors in mice was reported by Camphausen et al. [152]. These authors studied the radiation abscopal effect by monitoring the growth of a tumor, which was implanted at a distance from the irradiated normal leg of a mouse, and demonstrated that the RIBE induction in normal tissues and tumors was dependent on the function of p53. Mancuso et al. [3] revealed the relation between the bystander effect and cancer risk. Partial-body irradiation with a medium to high dose (3, 8.3 Gy) of X-rays on a Patched-1 (*Ptch1*) heterozygous mouse model with shielded brains gave rise to a drastic increase in medulloblastoma, which was the first demonstration of a bystander induction of cancer [3]. Xue et al. [153] investigated the damaging effect from radiolabeled tumor cells to unlabeled tumor cells in nude mice and concluded that unlabeled tumor cells were killed because of the bystander effect, which was created by *in vivo* bystander factor(s) that were present within and/or released from the radiolabeled tumor cells. The results of Xue et al. had important implications on the assessment of therapeutic responses to radionuclide therapy, as well as on the assessment of environmental radiation risks, such as the risks due to the inhalation of radon progeny. Dilmanian et al. [154] transaxially irradiated spinal cords of rats with a single microplanar beam with a thickness of 270 μm and a dose of 750 Gy, and found a loss of oligodendrocytes, astrocytes and myelin in 2 weeks. However, repopulation and remyelination were nearly complete by 3 months. The authors suggested that beneficial bystander effects were involved in the repair processes leading to tissue restoration. In particular, angiogenesis could have been promoted to replace damaged capillary blood vessels, whereas the proliferation, migration and differentiation of progenitor glial cells were promoted to produce new, mature and functional glial cells [154]. Recently, through bystander clonogenic reporter assays, Fernandez-Palomo et al. [155] demonstrated the presence of the RIBE in healthy and tumor-bearing Wistar rats, which had been exposed to a high dose of radiation (17.5, 35, 70 or 350 Gy) from synchrotron microbeam radiation therapy (MRT) and homogenous synchrotron radiation (HSR). The production of bystander signals was found to be higher in tumor-bearing tissues than in tumor-free tissues, which suggested that tumor and normal tissues might induce the RIBE through different mechanisms. As such, it is pertinent to explore the different mechanisms in the production and transmission of bystander signals in tumors and normal tissues. With such knowledge, one can separately control the RIBE in tumors and in normal tissues, and there is a possibility of optimizing radiotherapy through maximizing the RIBE on tumors while minimizing the RIBE on normal tissues.

3.2. In vivo studies on RIGI using animal models

In vivo RIGI was also primarily demonstrated in the rodent model (see the review by Morgan [49]). Wright and colleagues examined whether chromosomal instability that was induced *in vitro* could be transmitted *in vivo* by the transplantation of hemopoietic stem cells that were exposed to either high or low LET radiation [100,156]. The transplantation of *in vitro* alpha-particle irradiated bone marrow stem cells, which were obtained from male mice, to the female CBA/H mouse recipients induced the long-term persistence of chromosomal instability [156]. A similar study was later performed by Watson et al. [100], wherein the chromosomal instability in the hemopoietic system was induced by X-rays and neutrons. However, significant inter-animal variations in the expressions of both stable and unstable aberrations were reported. In contrast, a study regarding RIGI through the *in vivo* irradiation of mice, followed by *in vitro* analyses, was performed by Weissenborn and Streffer [157]. The neutron irradiation of one-cell mouse embryos was found to increase the aberration frequency in the third post-irradiation mitoses. Chromosomal aberration that lasted several cell generations was also reported by Ullrich and colleagues [158], who demonstrated cytogenetic instability in mammary epithelial cells *in vitro* after irradiation *in vivo*. On a different note, the *in vivo* irradiation of the rodent model followed by measuring the instability *in vivo* revealed large variability in the RIGI results, which depended on the mouse strain [156,159–161]. *In vivo* RIGI was also observed among the offspring of the irradiated parents. The persistently increased rate of mutation in the non-exposed offspring of the irradiated parents was called transgenerational genomic instability [162]. An increase in the mutation rate of the first- and second-generation offspring of irradiated male mice was reported in many studies [163–165]. In particular, Barber et al. [164] suggested that a genome-wide destabilization after fertilization was involved in the transgenerational RIGI. Whereas transgenerational RIGI was primarily researched using the rodent model, Huumonen et al. [166] recently provided the first evidence of transgenerational RIGI in *C. elegans*, where the progeny of irradiated *C. elegans* showed an increase in the frequency of delayed mutations.

Only a few studies have been performed using microbeam irradiation on whole organisms, including rats [154], the plant *A. thaliana* [144,146,147], the instar silkworm *B. mori* [149], and the nematode *C. elegans* [139,150].

4. Bridging the gaps in studies on non-targeted effects of ionizing radiation on an organism or a tumor

4.1. Studies on high-LET-radiation induced non-targeted effects

Broad-beam low-LET photons have been commonly used in most *in vivo* studies on the RIBE. Most studies on the RIBE within organisms *in vivo* adopted the "partial shielding" approach, where the partial-body irradiation of the whole organism was made possible using a shield. Regarding RIGI, except for those effects that were induced *in vitro* and that were transmitted *in vivo* by transplantation of irradiated cells, broad-beam low-LET photons also seemed to be most practical for RIGI experiments. It would be ideal to establish an animal model for convenient studies on non-targeted effects of both high-LET and low-LET radiations, and by making use of both broad-beam and microbeam radiations. Although the rodent model has been employed for studies regarding the non-targeted effect for many years, relatively few investigations have been conducted using high-LET radiation. The difficulties in deploying the rodent model for experiments with some high-LET radiations such as alpha particles were due to the short ranges.

From *in vitro* studies, it has been well established that the RIBE can be induced by both low-LET and high-LET radiation; thus, *in vivo* studies regarding the RIBE of high-LET radiation are interesting and pertinent. In fact, humans and other living organisms are inevitably exposed to naturally occurring alpha particles. For example, alpha particles, which have high LET values, are continuously emitted by members of the natural ^{238}U decay series, e.g., ^{238}U and ^{226}Ra, which are present in geological materials, including rocks and soils. Within this ^{238}U decay series, there is a member, ^{222}Rn, which is a natural radioactive gas called radon, which poses health hazard to living organisms. The risk arises when the short-lived progeny of ^{222}Rn, i.e., ^{218}Po, ^{214}Pb, ^{214}Bi and ^{214}Po, are inhaled and when the sensitive cells in the bronchial and bronchiolar regions of the lung are irradiated by the alpha particles that are emitted by ^{218}Po and ^{214}Po [167]. It has been estimated that about half of the total effective dose that is received by humans from environmental radiation is attributed to the short-lived radon progeny. Epidemiological studies have also provided reasonably firm estimates of the risk of radon-induced lung cancers. Brenner and Sachs [168] proposed the radon risk to be dominated by RIBE in the instance of domestic radon exposures.

In regards to medical applications, the number of hadron-therapy facilities has grown in recent years, along with the advancing beam-delivery and accelerator technologies. Hadron therapy includes the use of protons and heavy ion particles (mainly carbon ions). Additionally, hadron therapy makes use of the properties of the Bragg peaks to deliver extremely localized radiation doses to precise volumes. However, there were few studies on the role of the RIBE in the clinical setting [169]. Another advancement in the medical application is called targeted atomic nanogenerators [170] or targeted alpha therapy (TAT) [171]. This technique involves targeting molecular-sized generators of alpha-particle emitting radionuclides into cancer cells, by coupling carefully chosen parent radionuclides to internalize monoclonal antibodies to form the bioconjugates. The short ranges and the high LET values of alpha particles ensures maximum cytotoxicity to cancer cells and simultaneously ensures minimal damages to the surrounding non-targeted normal tissues, which cannot be achieved by low-LET ionizing radiation, such as beta particles. Other advantages of using high-LET alpha particles for the targeted therapy over the low-LET ionizing radiations include the independence of alpha-particle biological effectiveness on hypoxia or cell cycle considerations [172,173] and the relatively low gamma-ray components of most alpha-particle emitting radionuclides, which allow for outpatient treatments and impart lower radiation doses to the involved nuclear medicine staff [174]. Notably, radium-223 chloride was granted the Fast Track designation by the U.S. Food and Drug Administration for the treatment of hormone-refractory prostate cancer in patients with bone metastases [175]. Despite all these advantages, the RIBE in the non-targeted normal tissues surrounding the targeted cancer cells is not fully understood. In particular, if the alpha-particle emitting daughter radionuclides in the decay chain of the nanogenerators are not sequestered at the target site, then these daughter radionuclides can migrate and deliver a potentially toxic dose to non-targeted tissues as well [171]. The volume of normal tissues that are affected by RIBE from these runaway radionuclides will become even larger.

In fact, effects from high-LET and low-LET radiations can be extremely different. For example, Chauhan et al. [176] detected the expression of Fas and TNF-α in human monocytic THP-1 cells after the cells were irradiated with alpha particles and X-rays with mean absorbed doses from 0 to 1.5 Gy; however, the expression levels of TNF-α were significantly higher for α-particle irradiation. TNF-α could activate the NF-κB/COX-2 signal pathway to induce inflammation-type effect in bystander cells, whereas cyclooxygense-2 (COX-2) was identified as a "central component" of RIBE [74] To cite another example, Anzenberg et al. [177] found LET-dependent differences in the signal that was released from DU-145 human prostate carcinoma cells by irradiating these cells with alpha particles or 250 kVp X-rays. The authors showed that the X-rayed tumor cells succeeded, whereas alpha-particle-irradiated tumor cells failed to cause medium-mediated bystander effects in co-cultured unirradiated AG01522 human fibroblasts. As such, a model that enables comparisons between the effects that are induced by high-LET and low-LET radiation will be particularly useful.

4.2. Studies on non-targeted effects using microbeam radiations

Microbeams, including charged-particle and X-ray microbeams, are unique tools for studying the RIBE. As succinctly summarized by Prise et al. [138], the advantages included:

(1) the capability of delivering a predetermined precise dose to individual cells, particularly for charged-particle microbeams, which facilitates studies on the biological effects of individual radiation tracks;
(2) the possibility of irradiating a chosen site with a cell or a tissue, thereby elucidating the radiosensitive sites within cells and tissues; and
(3) the capacity to localize the exposure of cells or tissues to radiation to determine the roles and patterns of intracellular and intercellular signaling.

With such advantages, microbeam facilities have been widely used for studies on the RIBE, which have already enlightened us regarding different aspects of the RIBE, including the underlying mechanisms. In regards to medical applications, microbeam studies using animal models can help us understand the effects of spatially modulated radiation fields where non-uniform doses within the treatment fields are delivered, which cannot be achieved using simple broad-beam facilities. Modulated radiation fields are commonly used at present in radiotherapy to maximize the radiation dose to the tumor while minimizing the radiation dose to the normal tissues, e.g., in stereotactic radiotherapy and radiosurgery. Techniques, such as GRID therapy [178], microbeam radiotherapy [179] and X-ray microplanar beams [154,180], were also proposed and practiced with a view to exploit the enhanced cellular tolerance due to the dose modulation within the treatment fields.

Moreover, the detection of more active regions within tumors enabled by functional imaging, such as PET scanning, may also advocate for non-uniform delivered radiation doses. However, the biological effects of spatially modulated doses are not yet clear. Several studies revealed the significant differences in cell survival [181–185] and DNA damage responses [183] because of modulated field exposures. In particular, Mackonis et al. compared the survival of cells that were irradiated uniformly and those cells that were irradiated with spatially modulated doses using a radiotherapy beam that was modulated by a clinical multileaf collimator and discovered that cell survival was actually affected by the fate of neighboring cells through three distinct types of RIBEs [186]. More recently, Asur et al. examined the high-dose bystander effect in GRID irradiated regions and found expression in DNA damage and cellular stress response signaling genes in bystander cells [187]. As such, it is indeed interesting and pertinent to establish an *in vivo* animal model for studying the RIBE from microbeam radiation in addition to broad-beam radiation.

4.3. Embryogenesis and tumorigenesis

Whereas studies have provided valuable information on the RIBE within tumors, as well as on abscopal effects on tumors [3,153–155], we might be able to gain extra insight into non-targeted effects by studying radiobiological effects of ionizing radiation on tumors through developing an embryo model. The embryonic origin of tumors has been gaining ground in the past few decades (e.g., see review by Ma et al. [188]), and attempts have begun to fight cancer from the perspective of developmental biology [189,190]. In fact, tumorigenesis and embryonic development were found to be related to each other [191–195]. Significant similarities have been identified between early embryonic development and tumorigenesis in terms of biological behaviors and molecular basis, including migration and invasion [196], gene expression and protein profiles [197], signaling pathways [190,198–202], mechanisms of immune escape [203–206], activities of specific enzymes and their isozymes in the cells, cell metabolism, proliferation and differentiation [207,208]. In particular, both tumor cells and embryonic cells have high rate of proliferation [208]. The recognition of the embryonic origin of tumors has facilitated a two-way exchange of knowledge between the research fields of embryogenesis and tumorigenesis. On one hand, we hope to be able to apply the abundant cancer research theories and models to help us understand embryogenesis. On the other hand, we aspire to fight cancer from the perspective of developmental biology and to develop new diagnostic and therapeutic targets for cancers [189,190].

As such, it will indeed be advantageous to establish an *in vivo* animal embryo model for studying the RIBE and RIGI from both high-LET and low-LET radiation, as well as from both broad-beam and microbeam radiation. Such a model can also facilitate studies on the effects of non-uniform radiation doses that are delivered to the tumors, e.g., because of techniques, such as GRID therapy [178], microbeam radiotherapy [179] and X-ray microplanar beams [154,180], as described in the previous section.

5. Embryos of zebrafish (D. rerio) as an *in vivo* model for studies on non-targeted effects of ionizing radiation on an organism or a tumor

The benefits of using embryos of the zebrafish (*D. rerio*) for experiments can also be exploited in *in vivo* studies on targeted and non-targeted radiobiological effects. In fact, zebrafish embryos have been employed as an animal model for radiological research in recent years [209–212]. Moreover, zebrafish embryos can also bridge the gaps for more versatile studies on non-targeted effects of ionizing radiation on an organism or on a tumor.

5.1. Pioneer works on studies of the effect of high-LET ionizing radiation

Studies on non-targeted effects of high-LET ionizing radiation using zebrafish embryos as the *in vivo* model began with studies on the effects of alpha particles, together with the design of a special experimental setup and procedures [213–215]. The biggest challenge in these experiments was posed by the short ranges of alpha particles. The excessive or variable absorption of the energy of the alpha particles before these particles reach embryonic cells can be due to (1) the substrate holding the embryos, (2) the medium holding the embryos, and (3) the chorion and the fluid enclosed by the chorions of the embryos. To solve the first problem, it was important to employ a thin substrate. At the beginning, a polyallyldiglycol carbonate (PADC) polymer film [216] was chosen as the support substrate for the alpha-particle irradiation of dechorionated embryos. To solve the second problem, Yum et al. [214,215] designed the irradiation in such a way that alpha particles came from the bottom and passed through the substrate holding the embryos. To solve the third problem, Yum et al. [214,215] performed dechorionation by using a pair of sharp forceps to remove the chorions of the embryos before alpha-particle irradiation. Manual dechorionation could avoid the stress response that is induced during the enzymatic digestion of the chorions. Alpha-particle irradiation of the dechorionated embryos was performed using a planar ^{241}Am source (with an alpha-particle energy of 5.49 MeV under vacuum and an activity of 0.1151 μCi). This setup was adopted throughout later investigations by Yum et al. on non-targeted effects that are induced by alpha particles. The effects of alpha particles were assessed through the number of apoptotic signals on the entire zebrafish embryo at 24 hpf.

In contrast, studies on non-targeted effects of microbeam radiations using zebrafish embryos as the *in vivo* model began with studies on the effects of microbeam protons, together with the design of a special experimental setup and procedures [217,218]. Similar to the irradiation by alpha particles that was described in the last paragraph, the protons came from the bottom and passed through a thin mylar film substrate. The mylar film was used instead of a PADC film because the mylar film was also biocompatible and because the locations of proton traversals were predefined instead of having to be revealed through the alpha-particle tracks that were developed on the chemically etched SSNTD. The embryos were manually dechorionated as described above to expose the cells. Such a setup permitted the irradiation of chosen cells on the embryos and enabled the delivery of predetermined doses to individual cells. The effects of microbeam protons were also assessed through the number of apoptotic signals on the entire zebrafish embryo at 24 hpf.

5.2. RIGI in zebrafish embryos

One of the advantages for using zebrafish embryos as a model for studying radiation-induced non-targeted effects is that zebrafish embryos develop rapidly. For example, major organ systems are evident within 24 hpf and the entire development is completed in approximately 5 dpf [131]. In particular, this high cell proliferation rate is shared by embryonic cells and tumor cells [208], as described above in the discussion of the embryonic origin of tumors. Zebrafish embryos, with such a high cell proliferation rate, might provide valuable information on RIGI. It is understood that tumor cells often exhibit genomic instability, which contributes to the activation of oncogenes and/or the inactivation of tumor suppressor genes [219]. Given a sufficient lag time, the radiation dose

response of zebrafish embryos should be an integrated one reflecting both the RIBE and RIGI. As such, zebrafish embryos can be a good model for studying RIGI and the RIBE.

When all the cells of a zebrafish embryo are irradiated, the RIBE will be minimal, and the overall effect will effectively represent the ultimate outcome of RIGI. Yum et al. [220] irradiated 1.5 hpf zebrafish embryos (16-cell stage, with 4×4 array of cells) with alpha particles, which have a maximum incident energy of 3.49 MeV for 1, 2, 4 and 8 min, using a planar ^{241}Am source with an activity of 0.1151 μCi. The alpha-particle hit rate on the embryo for the irradiation set up could be estimated from the results that were obtained by Choi et al. [221] by assuming similar cross-sectional areas of zebrafish embryos at 1.5 and 5 hpf. By making use of a PADC film as the support substrate for zebrafish embryos during alpha-particle irradiation and through counting the number of alpha-particle tracks that were revealed on the PADC film upon subsequent chemical etching, Choi et al. [221] found that the number of alpha-particle hits on the embryos was ~60 for a maximum exposure time of 60 s by using the same ^{241}Am source. As such, the alpha-particle hit rate on an embryo was ~1 per s. With this alpha-particle hit rate, the Poisson probability of a cell on a 1.5 hpf zebrafish embryo receiving no alpha-particle hit for irradiation at 1, 2, 4 and 8 min was approximately 0.02, 6×10^{-4}, 3×10^{-7} and 9×10^{-14}, which were effectively zero. The overall effects were assessed through the number of apoptotic signals on the entire zebrafish embryo at 24 hpf. Incidentally, the occurrence of extra apoptotic signals at 24 hpf potentially hinted at the presence of RIGI because apparently some of the irradiated cells and their earlier progeny, which were unstable and mutation prone, had appeared to be healthy and were able to survive many generations before the apoptosis pathways were initiated. Interestingly, the results of Yum et al. [220] revealed a biphasic dose response, which was a characteristic for radiation hormesis. Hormetic responses are characterized as biphasic dose–response relations exhibiting low-dose stimulation and high-dose inhibition [222].

Along a similar line, Choi et al. [223] studied the 2-cell stage zebrafish embryos and irradiated both cells with the same number (between 10 and 2000) of microbeam protons, which each have an energy of 3.37 MeV and an LET of 11 keV/μm. This experiment ensured that precisely the same number of protons irradiated on each cell and avoided the need to consider the Poisson distribution of the number of proton hits on the cells. The overall effects were also assessed through the number of apoptotic signals on the entire zebrafish embryo at 24 hpf. Significantly, a triphasic dose response was revealed with three different zones, namely, (1) the subhormetic zone, with an increase in apoptotic signals for <200 protons; (2) the hormetic zone, with a reduction in apoptotic signals below the spontaneous number for 200–400 protons; and (3) the toxic zone, with an increase in apoptotic signals again for >600 protons.

5.3. RIBE in zebrafish embryos

The RIBE has been clearly demonstrated in zebrafish embryos by inducing the radioadaptive response (RAR) through a low-dose particulate radiation during the priming exposure. The adaptive response (AR) or radioadaptive response (RAR) occurs when a small preceding priming dose decreases the biological effectiveness of a subsequent large challenging dose. When only a minute proportion of the embryo cells are hit by the particulate radiation during the priming exposure, the RAR can only be successfully induced if the unhit cells acquire the adaptation against the subsequent challenging exposure through the RIBE.

Choi et al. [224] successfully induced the RAR against a subsequent challenging radiation dose by exposing 5 hpf zebrafish embryos to a priming dose, which was provided by alpha particles from a planar ^{241}Am source with an activity of 0.1151 μCi for 24 s. The alpha particle source was not rotating, and the absence of hot spots was confirmed by visually inspecting the nuclear tracks that formed on SSNTDs by the irradiation with the alpha particle source and the subsequent chemical etching. The alpha-particle hit rate on the embryo was estimated as ~1 per s; thus, approximately 24 cells were hit by alpha particles during the priming dose exposure. The 5 hpf zebrafish embryos were close to the 50%-epiboly stage (5.25 hpf), and many deep cells were in late cycle 14 [131]; thus, the total number of cells was roughly estimated as $\sim 8 \times 10^3$. As such, <3 of 1000 cells were hit by alpha particles under the priming exposure. The successful RAR induction strongly supported the action of the RIBE. Interestingly, in a related investigation, Yu et al. [225] successfully induced the AR against a subsequent challenging exposure to the heavy metal cadmium also by exposing 5 hpf zebrafish embryos to a priming dose from a planar ^{241}Am source with an activity of 0.1151 μCi for 24 s, which typified an antagonistic multiple stressor effect, that was provided from alpha particles having a maximum incident energy of 5.09 MeV after passing through the support substrate. In reality, living organisms are exposed to a mixture of environmental stressors, e.g., ionizing radiations, heavy metals, etc., and the resultant effects due to such exposures are called multiple stressor effects. The evidence showed that toxicity could be modified by simultaneous or sequential exposures to multiple environmental agents [225–227].

In a separate study, Choi et al. [217] reported the induction of the RAR in zebrafish embryos using microbeam protons for the priming exposure at 5 hpf and X-radiation for the challenging exposure at 10 hpf. The RAR was successfully induced when 5 protons were delivered to each of 10 separate irradiation positions on the zebrafish embryos, i.e., 50 protons were irradiated onto the embryos [217]. When the number of irradiation positions on the embryos was reduced to one, at least 200 protons were required as the priming exposure for a successful induction of the RAR [218]. The results intriguingly demonstrated that successful induction of the RAR depended on the distribution of hit cells, and thus, the distribution of cells that were affected by the RIBE, on the target organism. Incidentally, these results also showed cross adaptation between two different types of ionizing radiations with different LET values. Table 2 summarizes the above key findings of the non-targeted effects of ionizing radiation that were obtained using embryos of the zebrafish *D. rerio*.

6. Conclusions and discussion

A brief review on the two non-targeted effects of ionizing radiation, namely, the radiation induced bystander effect (RIBE) and radiation induced genomic instability (RIGI), was first given in the Introduction, including the history and background information. The general use of zebrafish (*D. rerio*) embryos as an *in vivo* model was then described. *D. rerio* has emerged as a popular vertebrate model in many fields of research studies, such as developmental biology, physiology, toxicology, and environmental research, as well as cancer research. In particular, the human and zebrafish genomes share considerable homology, including the conservation of most DNA repair-related genes. The practical advantages for using zebrafish embryos as a model for studying radiation-induced non-targeted effects were assessed.

Then, the use of zebrafish embryos as the *in vivo* model for non-targeted effects of ionizing radiation on an organism or on a tumor was described. An introduction to previous studies using other models was given, which primarily involved the rodent model. This introduction was followed by a summary of key points and advantages of using zebrafish embryos as an *in vivo* model for studying non-targeted effects of ionizing radiation. In particular, it is an animal model that enables convenient studies on non-targeted effects

Table 2
Key findings of non-targeted effects of ionizing radiation obtained using embryos of the zebrafish *Danio rerio*. In all these experiments, the overall effects were assessed through the number of apoptotic signals observed on the entire zebrafish embryos at 24 hpf.

Non-targeted effect	Irradiation conditions	Radiation used	Observations	References
RIGI	Irradiation of all 16 cells of 1.5 hpf zebrafish embryos	Broad-beam alpha particles	A biphasic dose response: low-dose stimulation and a high-dose inhibition	Yum et al. [220]
RIGI	Irradiation of all 2 cells of 2-cell stage zebrafish embryos	Microbeam protons	A triphasic dose response with three different zones, namely, (1) subhormetic zone at ultra low dose, (2) hormetic zone at low dose, and (3) toxic zone at high dose	Choi et al. [223]
RIBE	Exposing < 0.3% of the cells of 5 hpf zebrafish embryos to a priming dose	Broad-beam alpha particles	RAR against a subsequent challenging dose applied at 10 hpf	Choi et al. [224]
RIBE	Exposing < 0.2% of the cells of 5 hpf zebrafish embryos to a priming dose	Microbeam protons	RAR against a subsequent challenging dose applied at 10 hpf	Choi et al. [217]

of both high-LET and low-LET radiation and that makes use of both broad-beam and microbeam radiation. One major difficulty in deploying other animal models for experiments with some high-LET radiations such as alpha particles was due to the short ranges. Investigations on high-LET radiation-induced non-targeted effects will deepen our understanding on various medical applications, including hadron-therapy, targeted atomic nanogenerators or targeted alpha therapy. In contrast, investigations on microbeam (low- and high-LET) radiation-induced non-targeted effects can also further our understanding of medical applications employing spatially modulated radiation fields, such as stereotactic radiotherapy and radiosurgery, GRID therapy, microbeam radiotherapy and X-ray microplanar beam radiotherapy. Furthermore, the zebrafish embryo model is a convenient embryo model for studying radiobiological effects of ionizing radiation on tumors. The embryonic origin of tumors has been gaining ground in the past few decades, and attempts have begun to fight cancer from the perspective of developmental biology. In fact, tumorigenesis and embryonic development were found to be related to each other. Significant similarities have been identified between early embryo development and tumorigenesis in terms of biological behaviors and their molecular basis.

Evidence of the involvement of RIGI and the RIBE in zebrafish embryos were then given. The results of RIGI were obtained for (1) the irradiation of all 16 cells of 1.5 hpf zebrafish embryos by broad-beam alpha particles, which led to a biphasic dose response, namely, a low-dose stimulation and a high-dose inhibition [220], and (2) the irradiation of all 2 cells of 2-cell stage zebrafish embryos by microbeam protons, which led to a triphasic dose response with three different zones. These zones were as follows: (1) the subhormetic zone at an ultra-low dose, (2) the hormetic zone at a low dose, and (3) the toxic zone at a high dose [223]. In contrast, the RIBE was observed through the RAR, which was developed against a subsequent challenging dose that was applied at 10 hpf, when (1) <0.3% of the cells of 5 hpf zebrafish embryos were exposed to a priming dose that was provided by broad-beam alpha particles [224] and when (2) <0.2% of the cells of 5 hpf zebrafish embryos were exposed to a priming dose that was provided by microbeam protons [217].

With the development and establishment of zebrafish embryos as an *in vivo* model for studying non-targeted effects of ionizing radiation, we are now in a better position to perform more extensive studies on the molecular mechanisms underlying the RIBE and RIGI *in vivo*. As previously explained, *in vivo* experiments provide directly applicable results and avoid the extra step required by *in vitro* experiments to extrapolate the results back to the biology of the whole living organism. Furthermore, as previously explained, tumorigenesis and embryonic development were found to be related to each other. As such, we aspire to fight cancer from the perspective of developmental biology and to develop new diagnostic and therapeutic targets for cancers with the help of the zebrafish embryo model.

One particularly important and interesting area of research on non-targeted effects of ionizing radiation is the role of epigenetics. Epigenetic mechanisms, including DNA methylation, histone modifications and small RNA-mediated silencing, can contribute to non-targeted effects. As explained in the text, the sex-specific bystander effect that was reported by Koturbash et al. [39,143] was observed together with DNA methylation changes. Studies have also shown that epigenetic changes were involved in both direct and indirect radiation effects [151]. Ilnytskyy and Kovalchuk [228] recently reviewed the role of DNA methylation and small RNAs in directly irradiated and bystander tissues and in radiation-induced transgenerational effects. Ilnytskyy and Kovalchuk gave evidence that epigenetic mechanisms could lead to radiation-mediated effects. Until now, few studies on epigenetic changes that were involved in the *in vivo* non-targeted effect have been performed. The zebrafish embryo model is a convenient model to study epigenetic mechanisms, particularly those mechanisms that are involved in radiation-induced transgenerational effects because of the rapid development of zebrafish embryos and thus shorter experimental turnaround times.

Another interesting area that can be studied using the zebrafish embryo model is the interactions between multiple tumors in the body. Multiple tumors can arise from multiple independent primary cancers or because of metastases from one primary tumor. It is currently well-established that the RIBE not only exists through communication within an individual but also is found through signal communication outside individuals at the inter-organismic level. Yum et al. [215] reported the communication of alpha-particle-induced bystander signals between 1.5 hpf zebrafish embryos. Choi et al. [229,230] further demonstrated that the RIBE communication between zebrafish embryos *in vivo* could actually induce the RAR against a challenging dose and induce a hormetic effect, respectively, in partnered unirradiated zebrafish embryos sharing the same medium with irradiated embryos. Moreover, unirradiated bystander zebrafish embryos were found to release a feedback signal back to the irradiated zebrafish embryos, which lead to a mitigation of the effects of radiation in the irradiated embryos [231]. The chemical messengers that are responsible for communicating the RIBE between irradiated and bystander naïve embryos were not yet fully elucidated. Choi et al. [232] investigated the effect of carbon monoxide (CO) on the induction of the RIBE. More recently, the roles of NO and CO on the induction of the RIBE by high-dose X-ray irradiation in zebrafish embryos were also reported by Choi et al. [52]. Considering the zebrafish embryo model as a good tumor model, the above results might be able to provide valuable information for

understanding the interactions of multiple tumors in the body and for more effective treatments.

Conflict of Interest

The authors declare no conflicts of interest.

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