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Cellular responses and gene expression profiles of colonic Lgr5$^+$ stem cells after low-dose/low-dose-rate radiation exposure

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ABSTRACT

We previously found that high-dose-rate radiation induced a replenishment of the colonic Lgr5$^+$ stem cell pool, whereas low-dose-rate radiation did not. To identify key molecules that determine the dose-rate effects on this stem cell pool, we harvested colonic Lgr5$^+$ stem cells by cell sorting at 2 weeks after exposure to 1 Gy of high-dose-rate (30 Gy/h) or low-dose-rate (0.003 Gy/h) radiation and analyzed their gene expression profiles using RNA-Seq. We found that pathways related to DNA damage response, cell growth, cell differentiation and cell death were upregulated in Lgr5$^+$ stem cells irradiated with high dose rates, whereas pathways related to apical junctions and extracellular signaling were upregulated in low-dose-rate–irradiated colonic Lgr5$^+$ stem cells. Interestingly, biological events involving apical junctions are known to play an important role in the exclusion of transformed cells that are surrounded by normal epithelial cells through 'cell competition'. We speculated that cell competition, through apical junctions and extracellular ligands, might contribute to the dose-rate effect on Lgr5$^+$ cell replenishment. To understand this mechanism, we focused on 69 genes that were significantly upregulated in low-dose-rate–irradiated cells, which we named DREDGE (Dose-Rate Effect Determining GEnes). Based on these findings, we propose a possible mechanism underlying the dose-rate effect observed in the colonic stem cell pool.

Keywords: tissue stem cells; Lgr5; dose-rate effect; RNA-Seq

INTRODUCTION

Organs and tissues comprise different types of functional cells that play specific roles. These functional cells are replaced by cellular turnover; however, the rate of replacement differs among tissues. Since functional cells have a limited lifetime, they have to be replenished by parental progenitor cells, which are continuously regenerated by stem cells. According to the 'cell-of-origin in cancer' model, solid tumors are considered to originate from tissue stem cells that undergo genetic mutations [1]. Ionizing radiation, a physical mutagen, induces DNA damage and genetic mutations. The number of DNA double-strand breaks (DSBs) increases in a dose-dependent manner, and insufficient repair of DSBs leads to mutations, genomic instability, and chromosomal aberrations [2–4]. Therefore, cancer is considered to originate from tissue stem cells with accumulated DNA damage [5, 6]. We hypothesized that DNA damage accumulates in pools of tissue stem cells, as they have sufficient self-renewability to maintain a tissue throughout their lifetime [7–9]. Recent studies have suggested that cancer risk in humans is related to the total numbers of stem cell divisions, and that accumulation of mutations caused by steady-state cell division does not depend on environmental factors [10, 11]. This 'bad luck' hypothesis is a controversial issue in stem cell research and suggests that tissue stem cells are important as a target of cancer origin.

Cancer risk induced by ionizing radiation does not seem to be determined by the cumulative dose of exposure. An epidemiological study has shown that the incidence of solid cancers among

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atomic bomb survivors increased with the radiation dose, exhibiting a linear dose–response relationship [12]. On the other hand, another epidemiological study reported that cancer incidence among individuals living in high-background radiation areas (HBRAs), where they receive extremely low-dose-rate radiation throughout their life, did not increase with the cumulative dose of radiation [13]. Thus, the discrepancies between these studies may be attributed to the dose rate, rather than the overall dose. 'Dose-rate effects' are well-known responses in which the biological effect of low-dose rate radiation is lower than that of the same dose at high-dose-rate radiation [14]. However, there is no evidence of a dose-rate effect on tissue stem cells. Therefore, we addressed this gap in knowledge to understand the biological mechanisms behind dose rate–dependent carcinogenesis.

INTESTINAL STEM CELLS AS AN ORIGIN OF CARCINOGENESIS

Intestines are the major target organ in radiation-induced cancer [15]. The intestinal epithelium consists of epithelial monolayer cells, all of which are derived from intestinal stem cells, which are located at the bottom of the crypts. Driver mutations of genes such as *Apc* and *Ctnnb* (β-catenin) of the intestinal tissue stem cells can trigger carcinogenesis [16–18]. However, for progenitors and terminally differentiated cells, driver mutations are insufficient to trigger carcinogenesis; further stimulations such as severe inflammation are required for tumor development, in addition to the acquisition of driver mutations [19]. Intestinal crypts contain stem cells with different characteristics such as actively cycling and slow cycling, which can be distinguished by their molecular markers as shown in Fig. 1 [20]. For instance, intestinal stem cells expressing leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) are cycling stem cells, which are necessary for maintaining tissue in a steady state. Lgr5 was first identified as a molecular marker on stem cells that could develop into tumors as cells of origin in cancer; for example, adenomas were induced when the *Apc* gene was specifically depleted in Lgr5$^+$ stem cells [16]. Parts of both the small intestine, such as the duodenum, and the large intestine, such as the colon, contain Lgr5$^+$ stem cells in the bottom of crypts. Besides

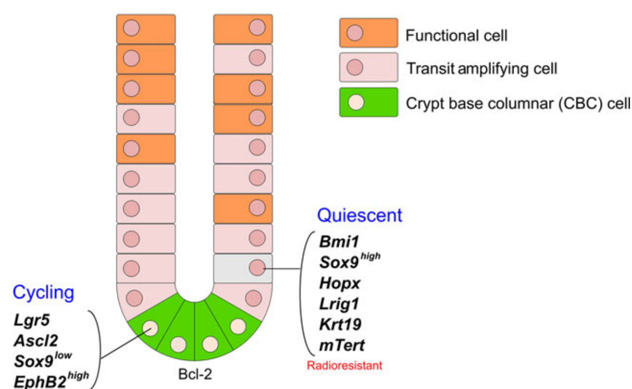

Fig. 1. Stem cell populations in colonic crypts. Bold gene names denote common stem cell markers. Functional cells include enteroendocrine cells, goblet cells, and enterocytes.

Lgr5, markers for actively cycling stem cells such as Ascl2 and Olfm4 are also expressed in crypt base columnar (CBC) cells [21, 22]. Quiescent stem cells, which express markers such as Bmi-1 and mTert, play an important role in the repopulation of actively cycling stem cells when the pool undergoes severe damage from stress, such as high-dose radiation exposure [17, 23].

THE DOSE-RATE EFFECT IN REPLENISHMENT OF COLONIC LGR5$^+$ STEM CELLS

We previously found that colonic Lgr5$^+$ stem cells were highly radiosensitive, compared with duodenal Lgr5$^+$ stem cells, because the number of colonic Lgr5$^+$ stem cells significantly decreased after exposure to 1 Gy of high-dose-rate (30 Gy/h) radiation [24]. As the dose-rate effect has not been evaluated in these cells, we studied the effect of radiation on Lgr5$^+$ stem cells using the Lgr5-lineage tracing technique. This is a common technique for understanding the stem cell fate by tagging specific stem cells and their daughter cells with a reporter gene such as *lacZ* or a gene for a fluorescent protein, based on tamoxifen-driven Cre–loxP recombination.

In this study, we compared the effects of high-dose-rate (30 Gy/h) and low-dose-rate (0.003 Gy/h) radiation on the replenishment of Lgr5$^+$ stem cells using *Lgr5-EGFP-IRES-Cre^{ERT2}* × *ROSA26-LSL-LacZ* mice. In these mice, Lgr5$^+$ stem cells constantly express Cre recombinase fused to a modified estrogen receptor (ERT2). As a ligand, tamoxifen (4-hydroxytamoxifen) binds to ERT2 and induces translocation of Cre recombinase to the nucleus, where Cre recombinase cuts out the translational stop sequence (LSL) and activates expression of the *lacZ* gene. A significant loss of LacZ$^+$ crypts was observed after high-dose-rate irradiation, suggesting the replenishment of the Lgr5$^+$ stem cell pool by quiescent stem cells [24]. However, no significant acceleration of stem cell replenishment was observed upon low-dose-rate irradiation [25]. We also studied the kinetics of DNA repair and tissue response by quantifying the number of 53BP1 foci in each cell, which is a surrogate marker for DSBs, and the number of cells expressing Ki-67 and phosphorylated histone H3 (PH3), which are markers of proliferating and mitotic cells, respectively. After high-dose rate irradiation, the number of 53BP1 foci immediately increased in colonic Lgr5$^+$ stem cells, but DSBs were efficiently repaired thereafter. High-dose-rate radiation also induced considerable reduction in cell numbers in the colonic crypts and dramatic increase in mitosis, which may stimulate the replenishment of the stem cell pool [26]. Therefore, the abnormal growth stimulation to replenish the Lgr5$^+$ stem cell pool may contribute to the accumulation of genetic mutations in tissue stem cells. Based on these findings, we speculated that the dose rate, rather than the cumulative dose, might contribute to the replenishment of tissue stem cells and the dose-rate effect on tissue stem cell turnover might affect cancer risk. High-dose-rate whole-body irradiation reduces the number of tissue stem cells by inducing cell death [27, 28]. Even if the cells do not die at the time of radiation, all tissue stem cells are simultaneously damaged by high-dose-rate irradiation. DNA damage can lead to aging and exhaustion of stem/progenitor cells in tissue stem cells [29, 30]. Quiescent (or slow-cycling) stem cells must rescue tissues after drastic loss of the actively cycling stem cells to repopulate the stem cell pool [31]. In fact, the

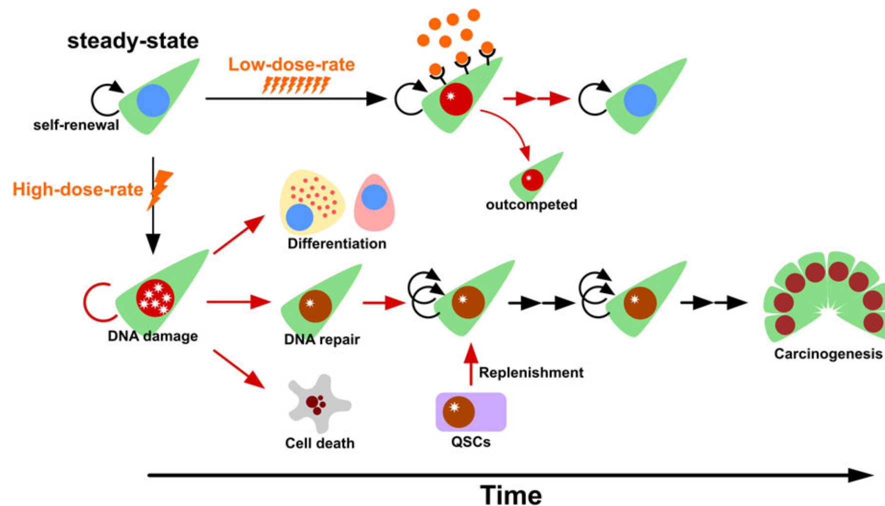

Fig. 2. A possible model of dose-rate effect in the replenishment of Lgr5$^+$ stem cells. After high-dose-rate irradiation, the cell cycle of colonic Lgr5$^+$ stem cells immediately stalls due to DNA damage, and some cells die or differentiate. As the number of colonic Lgr5$^+$ stem cells decrease after irradiation, the Lgr5$^+$ stem cell pool is replenished by the surviving Lgr5$^+$ stem cells and/or radioresistant, quiescent stem cells. This unscheduled growth stimulation may result in the accumulation of mutations. After low-dose-rate irradiation, responses related to cell competition are induced in the colonic Lgr5$^+$ stem cell pool, and some cells may be excluded from the pool to maintain the integrity of tissue stem cells.

proliferation of slow-cycling stem cells is induced by high-dose irradiation [23]. In the hematopoietic system, DSBs in quiescent stem cells have to be repaired by non-homologous end joining (NHEJ), although DSBs on cycling stem cells can be repaired by homologous recombination (HR), which is relatively error-free compared with NHEJ [32]. Therefore, quiescent stem cells are considered intrinsically vulnerable to genetic mutation. Growth stimulation of those quiescent stem cells after loss of cycling stem cells may trigger replenishment and expansion of mutated stem cells in the cycling stem cell pool. Thus, for tissue stem cells, preventing proliferation of slow-cycling stem cells is important for tissue integrity.

ISOLATION OF CANDIDATE GENES DETERMINING THE DOSE-RATE EFFECT

The dose-rate effect on the replenishment of Lgr5$^+$ stem cells is a possible mechanism that circumvents the accumulation of mutated cells in its pool. To confirm our hypothesis, we identified key molecules involved and the mechanism behind the induction of the dose-rate effect, by comparing gene expression profiles in colonic Lgr5$^+$ cells of mice exposed to high-dose-rate and low-dose-rate irradiation. We harvested colonic Lgr5$^+$ cells by cell sorting of the EGFP$^+$ population at 2 weeks after exposure to 1 Gy of high-dose-rate (30 Gy/h) or low-dose-rate (0.003 Gy/h) radiation. RNA-Seq was used to analyze gene expression profiles of the harvested cells. A gene set enrichment analysis revealed that pathways related to DNA damage responses, cell growth, cell differentiation, and cell death were upregulated in high-dose-rate–irradiated Lgr5$^+$ cells. Interestingly, pathways related to apical junctions and extracellular signaling were upregulated in low-dose-rate–irradiated Lgr5$^+$ cells (Otsuka *et al.*, manuscript in preparation). Biological events involving apical junctions are known to play an important role in the extrusion of transformed cells surrounded by normal epithelial cells through a process called 'cell competition' [33, 34]. We speculated that cell competition through apical junctions and extracellular ligands might contribute to dose-rate effects in Lgr5$^+$ cell replenishment. Based on our findings, we propose a possible mechanism behind the dose-rate effect on colonic stem cells (Fig. 2). To understand the molecular mechanism underlying the dose-rate effect, we hypothesized that genes specifically activated in low-dose-rate–irradiated Lgr5$^+$ stem cells determine the consequence of the dose-rate effect. We created a list of genes that are significantly upregulated in low-dose-rate–irradiated colonic Lgr5$^+$ stem cells compared with non-irradiated and high-dose-rate–irradiated colonic Lgr5$^+$ stem cells. We found 69 genes that were significantly upregulated in low-dose-rate–irradiated colonic Lgr5$^+$ stem cells, which we named DREDGEs (Dose-Rate Effect Determining GEnes). In future studies, we aim to identify the features of DREDGEs to uncover their functions in the dose-rate effect on colonic Lgr5$^+$ stem cell replenishment.

CONCLUSION

Although radiation-specific mutation spectra have been published previously [35–37], these reports were based on high-dose-rate radiation. Recent studies have demonstrated that dose-rate-specific mutation spectra are important for studying cancer risk during exposure to various dose-rate irradiations [38]. Although the importance of the dose-rate effect on tissue stem cells is considered in radiation protection [39], the mechanism behind the dose-rate effect is still unknown. Our approach for investigating this mechanism by studying the features of DREDGEs may help us understand

how tissue stem cells maintain their integrity during exposure to low-dose-rate radiation.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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REFERENCES

1. Visvader JE. Cells of origin in cancer. *Nature* 2011;469:314–22.
2. Morgan WF, Day JP, Kaplan MI et al. Genomic instability induced by ionizing radiation. *Radiat Res* 1996;146:247–58.
3. Rothkamm K, Lobrich M. Evidence for a lack of DNA double-strand break repair in human cells exposed to very low X-ray doses. *Proc Natl Acad Sci U S A* 2003;100:5057–62.
4. Pfeiffer P, Goedecke W, Obe G. Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. *Mutagenesis* 2000;15:289–302.
5. Huen MS, Chen J. The DNA damage response pathways: at the crossroad of protein modifications. *Cell Res* 2008;18:8–16.
6. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature* 1998;396:643–9.
7. Reya T, Morrison SJ, Clarke MF et al. Stem cells, cancer, and cancer stem cells. *Nature* 2001;414:105–11.
8. Niwa O. Roles of stem cells in tissue turnover and radiation carcinogenesis. *Radiat Res* 2010;174:833–9.
9. Rossi DJ, Jamieson CH, Weissman IL. Stems cells and the pathways to aging and cancer. *Cell* 2008;132:681–96.
10. Tomasetti C, Li L, Vogelstein B. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. *Science* 2017;355:1330–4.
11. Tomasetti C, Vogelstein B. Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science* 2015;347:78–81.
12. Preston DL, Ron E, Tokuoka S et al. Solid cancer incidence in atomic bomb survivors: 1958–1998. *Radiat Res* 2007;168:1–64.
13. Nair RR, Rajan B, Akiba S et al. Background radiation and cancer incidence in Kerala, India–Karanagappally cohort study. *Health Phys* 2009;96:55–66.
14. Ruhm W, Azizova TV, Bouffler SD et al. (9 March 2016) Dose-rate effects in radiation biology and radiation protection. *Ann ICRP*, 10.1177/0146645316629336.
15. ICRP. The 2007 Recommendations of the International Commission on Radiological Protection. ICRP Publication 103. *Ann ICRP* 2007;37:1–332.
16. Barker N, Ridgway RA, van Es JH et al. Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* 2009;457:608–11.
17. Sangiorgi E, Capecchi MR. *Bmi1* is expressed *in vivo* in intestinal stem cells. *Nat Genet* 2008;40:915–20.
18. Asfaha S, Hayakawa Y, Muley A et al. $Krt19^{+}/Lgr5^{-}$ cells are radioresistant cancer-initiating stem cells in the colon and intestine. *Cell Stem Cell* 2015;16:627–38.
19. Schwitalla S, Fingerle AA, Cammareri P et al. Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* 2013;152:25–38.
20. Hendry JH, Otsuka K. The role of gene mutations and gene products in intestinal tissue reactions from ionising radiation. *Mutat Res* 2016;770:328–39.
21. Liu W, Li H, Hong SH et al. Olfactomedin 4 deletion induces colon adenocarcinoma in ApcMin/+ mice. *Oncogene* 2016;35: 5237–47.
22. Schuijers J, Junker JP, Mokry M et al. Ascl2 acts as an R-spondin/Wnt–responsive switch to control stemness in intestinal crypts. *Cell Stem Cell* 2015;16:158–70.
23. Montgomery RK, Carlone DL, Richmond CA et al. Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells. *Proc Natl Acad Sci U S A* 2011;108: 179–84.
24. Otsuka K, Hamada N, Magae J et al. Ionizing radiation leads to the replacement and *de novo* production of colonic Lgr5 stem cells. *Radiat Res* 2013;179:637–46.
25. Otsuka K, Iwasaki T. Effects of dose rates on radiation-induced replenishment of intestinal stem cells determined by Lgr5 lineage tracing. *J Radiat Res* 2015;56:615–22.
26. Otsuka K, Suzuki K. Differences in radiation dose response between small and large intestinal crypts. *Radiat Res* 2016;186: 302–14.
27. Potten CS. Extreme sensitivity of some intestinal crypt cells to X and gamma irradiation. *Nature* 1977;269:518–21.
28. Hua G, Thin TH, Feldman R et al. Crypt base columnar stem cells in small intestines of mice are radioresistant. *Gastroenterology* 2012;143:1266–76.
29. Tumpel S, Rudolph KL. The role of telomere shortening in somatic stem cells and tissue aging: lessons from telomerase model systems. *Ann NY Acad Sci* 2012;1266:28–39.
30. Blasco MA. Telomere length, stem cells and aging. *Nat Chem Biol* 2007;3:640–9.
31. Tian H, Biehs B, Warming S et al. A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature* 2011;478:255–9.
32. Naka K, Hirao A. Maintenance of genomic integrity in hematopoietic stem cells. *Int J Hematol* 2011;93:434–9.
33. Hogan C, Dupre-Crochet S, Norman M et al. Characterization of the interface between normal and transformed epithelial cells. *Nat Cell Biol* 2009;11:460–7.
34. Kon S, Ishibashi K, Katoh H et al. Cell competition with normal epithelial cells promotes apical extrusion of transformed cells through metabolic changes. *Nat Cell Biol* 2017;19: 530–41.
35. Schwartz JL, Jordan R, Sun J et al. Dose-dependent changes in the spectrum of mutations induced by ionizing radiation. *Radiat Res* 2000;153:312–7.
36. Adewoye AB, Lindsay SJ, Dubrova YE et al. The genome-wide effects of ionizing radiation on mutation induction in the mammalian germline. *Nat Commun* 2015;6:6684.

37. Kakinuma S, Nishimura M, Amasaki Y et al. Combined exposure to X-irradiation followed by N-ethyl-N-nitrosourea treatment alters the frequency and spectrum of Ikaros point mutations in murine T-cell lymphoma. *Mutat Res* 2012;737:43–50.
38. Tsuruoka C, Blyth BJ, Morioka T et al. Sensitive detection of radiation-induced medulloblastomas after acute or protracted gamma-ray exposures in Ptch1 heterozygous mice using a radiation-specific molecular signature. *Radiat Res* 2016;186: 407–14.
39. Niwa O, Barcellos-Hoff MH, Globus RK et al. Stem cell biology with respect to carcinogenesis aspects of radiological protection. ICRP Publication 131. *Ann ICRP* 2015;44:7–357.