

Changes in miRNA expressions in the injured small intestine of mice following high-dose radiation exposure

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Abstract. The small intestine is one of the most highly regenerative and radiosensitive tissues in mammals, including humans. Exposure to high doses of ionizing radiation causes serious intestinal damage. Recently, several investigations have been conducted using radioprotective agents to determine ways for reducing intestinal damage caused by radiation exposure. However, a thorough understanding of functional changes occurring in the small intestine of mice exposed to high-dose radiation is necessary for developing novel and more potent radioprotective agents. In this study, we examined changes in microRNA (miRNA/miR) expressions in the small intestine of mice at 72 h after X-ray exposure (10 Gy). We identified seven upregulated miRNAs and six downregulated miRNAs in the small intestine of mice following radiation exposure using miRNA microarray analysis. Particularly, miR-34a-5p was highly expressed, which was confirmed by reverse transcription-quantitative PCR. Forkhead box P1 (*Foxp1*) was predicted to be a target of the mRNA of miR-34a-5p using OmicsNet. Decreased *Foxp1* expression in the small intestine following radiation exposure was confirmed, suggesting that *Foxp1* expression recovery may induce the suppression of radiation-induced enteritis. Therefore, miR-34a-5p is a potential target molecule for developing novel radioprotective agents.

Introduction

The small intestine of mammals is one of the most sensitive organs to ionizing radiation (1). Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5)-positive stem cells present in the crypts of the small intestine are crucial for radiation-induced

intestinal regeneration (2). The death of Lgr5-positive stem cells due to high-dose radiation causes the disruption of intestinal homeostasis. The loss of the proliferative capacity of cells in the crypts of the small intestine results in the depletion of many cells in the intestinal mucosa, leading to intestinal tract destruction. Exposure to radiation doses of ≥ 10 Gy results in the development of classic gastrointestinal acute radiation syndrome (GI-ARS) in humans, usually within 5-10 days, if not appropriately treated (3). Other symptoms include the loss of appetite, diarrhea, infections, the loss of fluid and electrolytes, weight loss, malabsorption, and significant leukopenia (4,5). In 1999, three individuals involved in the Tokai-mura JCO accident experienced severe acute radiation syndrome during; of these, two died owing to severe GI-ARS (6-8). The development of radioprotective agents that can mitigate the effects of high-dose radiation exposure is necessary and has been highlighted by many researchers. Verginadis *et al*, have demonstrated the radioprotective action of curcumin on the intestinal tract (9). Yamamoto *et al*, have reported that treatment with ascorbic acid prior to radiation exposure prevents fatal GI-ARS in mice (10). To develop effective radioprotective agents, thoroughly investigating the functional changes in the small intestine due to radiation exposure is essential.

MicroRNAs (miRNAs) are short single-stranded RNAs comprising approximately 20 bases. They are involved in regulating gene expression via the post-transcriptional regulation of messenger RNA (mRNA) and the inhibition of protein translation (11). Previously, we reported altered gene expression in cells exposed to radiation (12,13). Other studies have also reported changes in mRNA expression in the small intestine following high-dose radiation exposure (14,15). However, information on gene expression changes in miRNAs following such exposure is limited.

In this study, we analyzed the effects of changes in miRNA gene expression in the small intestine of mice following whole body irradiation with 10 Gy of X-rays.

Materials and methods

Mice and X-ray irradiation. All experiments were performed in accordance with The Guidelines for Animal Experimentation of the Hirosaki University. The procedures were approved and monitored by The Animal Research Committee of Hirosaki University (approval nos. G12003 and G19005).

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Male C57BL/6Njcl mice were purchased from CLEA, Japan. All mice were housed in a conventional animal room with 12-h light/dark cycles; all mice were provided food and water *ad libitum*. Eight-week-old mice were exposed to X-rays (MBR-1520R-3 X-ray machine, Hitachi Medical Corporation) at a rate of 1.0 Gy/min (150 kVp, 20 mA, 0.5 mm aluminum, and 0.3 mm copper filters). Mice were observed for up to 30 days after irradiation. Before dissection, mice were anesthetized with 2% isoflurane (Pfizer). To minimize distress as a humane end point, we performed cervical dislocation before death of the mice. Mice with severe diarrhea were sacrificed.

Fluorescent TdT-mediated dUTP nick end labeling (TUNEL) assay. The small intestine from mice at 72 h after non-irradiation and 10 Gy irradiation of X-ray was directly excised under anesthesia. For tissue analysis, the small intestine was fixed with 4% paraformaldehyde solution in Dulbecco's phosphate-buffered saline (-) [D-PBS (-), pH 7.2]. The fixed small intestine was embedded in paraffin. Sections were cut at a thickness of 4 μ m and placed on glass slides. Paraffin-embedded sections of small intestine isolated from mice exposed to 10 Gy of X-rays on glass slides were deparaffinized with xylene and ethanol, followed by washing with D-PBS (-). Cell death analysis was performed using the DeadEnd™ Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. Nuclei were stained using the ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (Thermo Fisher Scientific, Inc.). The stained tissues were examined using a confocal laser scanning microscope LSM710 (Carl Zeiss).

Total RNA extraction. The non-irradiated and irradiated mice were sacrificed after 72 h for collection of small intestine. Total RNA was extracted from the small intestine using the Isogen II reagent (Nippon Gene) according to the manufacturer's instructions. RNA content in the small intestine was assessed using a NanoDrop spectrophotometer (NanoDrop Technologies). RNA samples had 260/280-nm absorbance ratios of 1.8-2.0. Total RNA quality was confirmed using the Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico kit (both Agilent Technologies, Inc.) as per the manufacturer's instructions.

Microarray analysis. Cyanine 3 (Cy3)-labeled miRNA was synthesized from total RNA obtained from the small intestines of irradiated (10 Gy, n=4) and non-irradiated (0 Gy, n=4) mice using the miRNA Complete Labeling Reagent and Hyb kit (Agilent Technologies). SurePrint G3 mouse miRNA microarray slides (8x60 K, v.21.0) were hybridized with the Cy3-labeled miRNA in a hybridization solution, which was prepared using a Gene Expression Hybridization kit (Agilent Technologies) as per the manufacturer's instructions. The images of Cy3 fluorescence signals on slides were obtained using the SureScan microarray scanner (Agilent Technologies) and were processed using the Feature Extraction software (v.10.7) based on the manufacturer's instructions. Expression data obtained were processed using the GeneSpring GX14.5 software (Agilent Technologies) to normalize all values to 90% percentile shift on the respective microarrays, followed by the normalization of the median expression level of all samples. miRNAs that were upregulated and downregulated by more than 2.000-fold in irradiated small intestine samples (10 Gy) compared with that in non-irradiated

small intestine samples (0 Gy) were analyzed ($P < 0.05$, unpaired t-test). The Benjamini Hochberg FDR was used for multiple testing correction. Moreover, OmicsNet (<https://www.omicsnet.ca/faces/home.xhtml>) was used to predict target miRNA genes. To predict target genes of miRNAs and their pathways, TargetScan Mouse (http://www.targetscan.org/mmu_72/) analysis and WikiPathways (<https://www.wikipathways.org/index.php/WikiPathways>) analysis were performed using the GeneSpring 14.5 software (Agilent Technologies). Pathway data of *Mus musculus* were downloaded from WikiPathways.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was used for synthesizing complementary DNAs (cDNAs) for miRNA expressions using the TaqMan™ miRNA RT kit and the prescribed 5xRT primer (both from Thermo Fisher Scientific, Inc.). Real-time PCR for miRNA expression was performed using a FastStart TaqMan probe master (Roche Diagnostics), 20x probe and the StepOne Plus Real-Time PCR system (Thermo Fisher Scientific, Inc.) under the following conditions: 10 min at 95°C, followed by 45 cycles at 95°C for 15 sec and 60°C for 60 sec. The comparative Cq method was used to assess miRNA expression levels. U6 small nuclear RNA was used as an internal control.

Western blotting. Western blotting was performed in the same protocol as before (16). In this study, the primary antibodies used were as follows: Anti-Forkhead box P1 (FoxP1) (D35D10) rabbit monoclonal antibody (no. 4402; Cell Signaling Technology, Inc.) and anti-Gapdh (D16H11) rabbit monoclonal antibody (no. 5174; Cell Signaling Technology, Inc.).

Statistical analysis. A Kaplan-Meier plot was used for assessing the survival of irradiated and non-irradiated mice. Statistical differences between irradiated and non-irradiated mice were determined using the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference. The Student's t-test was used to compare the results of the two groups.

Results

Exposure to 10 Gy X-rays in mice is fatal. We examined changes in body weight and survival time after exposure to 10 Gy X-rays. Weight loss was observed several days after irradiation (Fig. 1A). The 50% survival time was approximately 9 days following radiation exposure (Fig. 1B), indicating that exposure to 10 Gy X-rays in mice is fatal. The TUNEL assay confirmed DNA damage in the intestinal epithelial cells following radiation exposure. At 72 h after radiation exposure, the green fluorescent TUNEL labeling increased, showing many positive images of the small intestinal pit site, particularly rich in small intestinal epithelial stem cells (Fig. 1C). These results demonstrate that cell death is induced in the small intestine of mice exposed to 10-Gy X-rays.

Identification of upregulated and downregulated miRNAs in the small intestine of mice following radiation exposure (10 Gy X-rays). The small intestines of 8-week-old mice exposed to 10 Gy X-rays were extracted at 72 h after irradiation. Total RNA appeared to have an RNA integrity number of > 9.0 (Fig. 2A). Change in miRNA expressions in the small intestine

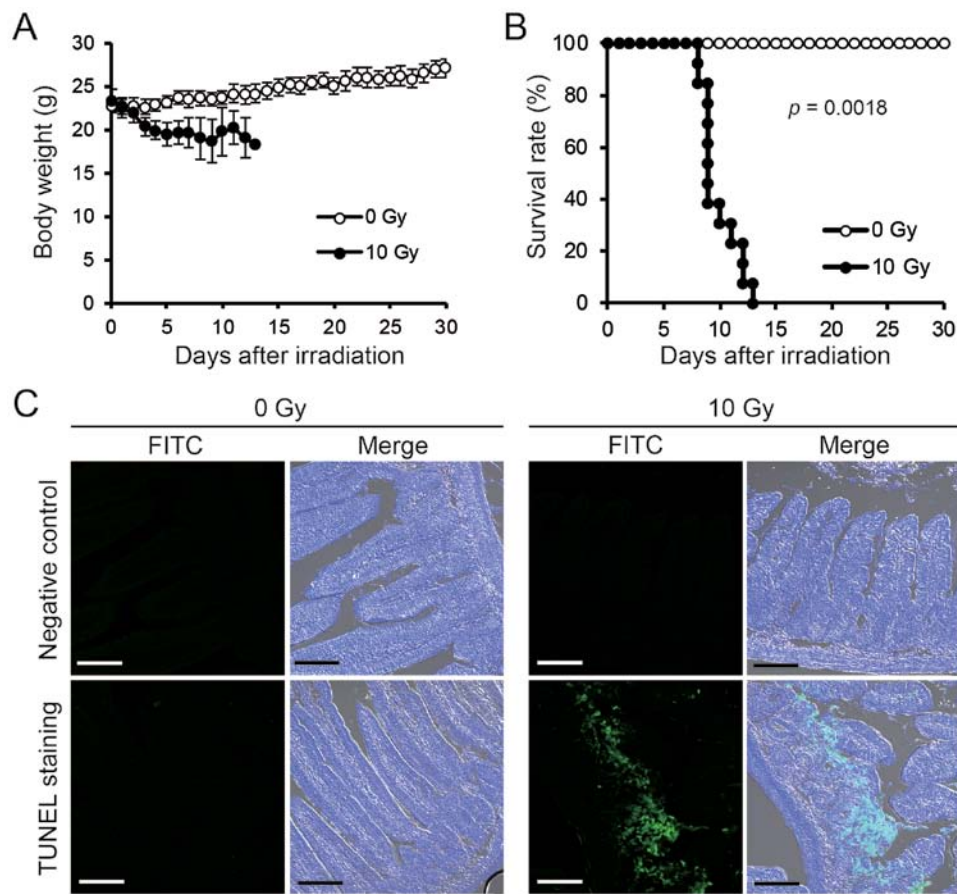


Figure 1. Effects of radiation exposure (10 Gy X-rays) on and injury in the small intestine. (A) Daily changes in body weight of irradiated C57BL/6 N male mice (10 Gy X-rays; n=13) and non-irradiated mice (0 Gy; n=5) up to 30 days after irradiation. The data are presented as the means \pm SD. (B) Kaplan-Meier plot for the survival of irradiated and non-irradiated mice. The statistical difference between irradiated and non-irradiated mice was determined using the log-rank test with $P < 0.05$ considered as statistically significant. (C) TUNEL assay of samples of small intestine following radiation exposure. Scale bar, 100 μ m.

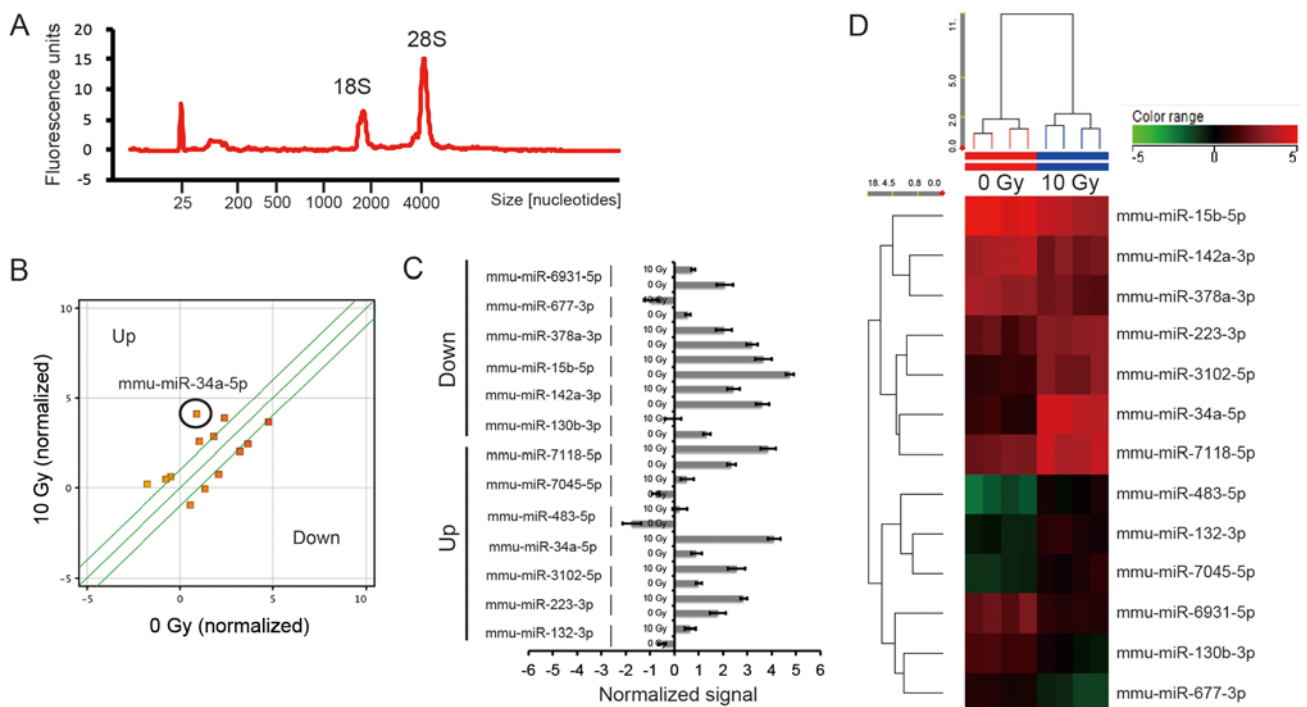


Figure 2. Changes in gene expressions in the small intestine of mice following radiation exposure (10 Gy X-rays). (A) Quality check of total RNA from small intestinal tissues using Agilent bioanalyzer. Gene expression of seven upregulated and six downregulated miRNAs in the small intestine of mice following radiation exposure using the SurePrint G3 mouse miRNA microarray. (B) Scatter plot analysis. (C) Variance of microarray data. Values are presented as the normalized signal mean \pm SD. (D) Hierarchical clustering analysis. miRNA/miR, microRNA.

Table I. Upregulated and downregulated miRNAs (>2.000-fold) in mouse small intestine at 72 h after exposure to 10 Gy of X-ray irradiation.

Systematic_name	Mirbase accession no.	Fold change (10 Gy vs. 0 Gy)	P-value
mmu-miR-132-3p	MIMAT0000144	2.209±0.131	0.000332
mmu-miR-223-3p	MIMAT0000665	2.081±0.536	0.001907
mmu-miR-3102-5p	MIMAT0014933	2.952±0.836	0.000360
mmu-miR-34a-5p	MIMAT0000542	9.137±0.624	0.000004
mmu-miR-483-5p	MIMAT0004782	3.911±1.901	0.000393
mmu-miR-7045-5p	MIMAT0027994	2.447±0.836	0.000407
mmu-miR-7118-5p	MIMAT0028133	2.826±0.642	0.000346
mmu-miR-130b-3p	MIMAT0000387	-2.626±0.314	0.000525
mmu-miR-142a-3p	MIMAT0000155	-2.261±0.771	0.001618
mmu-miR-15b-5p	MIMAT0000124	-2.117±0.331	0.002784
mmu-miR-378a-3p	MIMAT0003151	-2.241±0.283	0.002295
mmu-miR-677-3p	MIMAT0017246	-2.866±0.234	0.000189
mmu-miR-6931-5p	MIMAT0027762	-2.473±0.828	0.000583

miRNA, miR, microRNA; no, number.

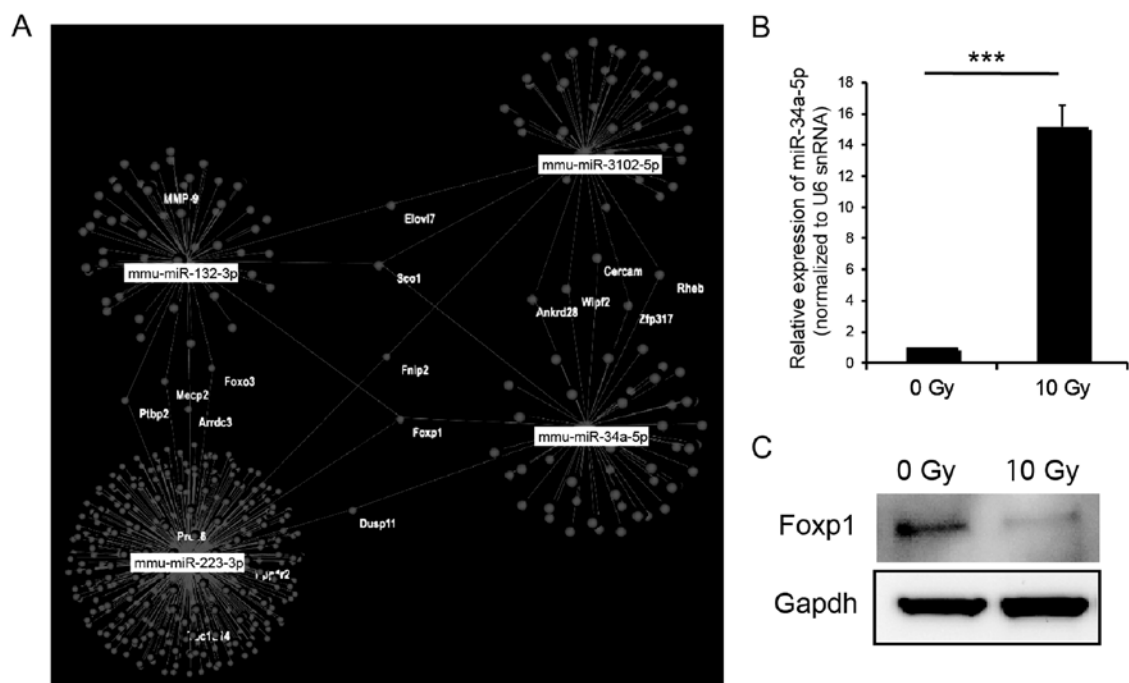


Figure 3. Search for target genes in upregulated and downregulated miRNAs in mouse small intestine following radiation exposure (10 Gy X-rays). (A) Network analysis of upregulated miRNAs and target genes using OmicsNet. (B) Gene expression changes of miR-34a-5p in small intestine of irradiated mice (10 Gy X-rays). Reverse transcription-quantitative PCR was performed using total RNA from mouse small intestine following radiation exposure. U6 small nuclear RNA was used as an internal control. *** $P < 0.001$. (C) Expression changes of Foxp1 in small intestine of irradiated mice (10 Gy X-rays). Western blotting was performed using protein lysate from mouse small intestine following radiation exposure. GAPDH was used as the housekeeping gene. miRNA/miR, microRNA; snRNA, small nuclear RNA; Foxp1, Forkhead box P1.

were investigated using microarray analysis. We selected changes in miRNA expressions of over 2.000-fold ($P < 0.05$) and identified seven upregulated and six downregulated miRNA, respectively, (Fig. 2 and Table I).

Prediction of target genes in upregulated and downregulated miRNAs. To predict the target mRNA of the upregulated

and downregulated miRNAs, we used OmicsNet. *Foxp1* was predicted to be a target of the mRNAs of upregulated miR-34a-5p, miR-132-3p and miR-223-3p via analysis using OmicsNet (Fig. 3A). In particular, miR-34a-5p was highly expressed, which was also validated using real-time PCR (Fig. 3B). Whereas, decreased Foxp1 expression in the small intestine following radiation exposure was confirmed using

Table II. Top 10 pathways predicted to be regulated by miRNAs up- and downregulated >2.000-folds in the small intestine of mice exposed to 10 Gy of X-ray irradiation.

A, Upregulated miRNAs

Pathway name	P-value
Mm_PluriNetWork_WP1763_89515	1.63x10 ⁻¹⁸
Mm_mRNA_processing_WP310_78419	7.06x10 ⁻¹¹
Mm_Non-odorant_GPCRs_WP1396_69993	2.45x10 ⁻⁸
Mm_Metapathway_biotransformation_WP1251_94721	2.45x10 ⁻⁸
Mm_Myometrial_Relaxation_and_Contraction_Pathways_WP385_95806	2.45x10 ⁻⁸
Mm_IL-6_signaling_Pathway_WP387_72091	2.45x10 ⁻⁸
Mm_Focal_Adhesion-PI3K-Akt-mTOR-signaling_pathway_WP2841_94308	2.45x10 ⁻⁸
Mm_Focal_Adhesion_WP85_94410	2.45x10 ⁻⁸
Mm_Chemokine_signaling_pathway_WP2292_97515	2.45x10 ⁻⁸
Mm_Cytoplasmic_Ribosomal_Proteins_WP163_78425	8.49x10 ⁻⁶

B, Downregulated miRNAs

Pathway name	P-value
Mm_mRNA_processing_WP310_78419	9.54x10 ⁻⁴³
Mm_PluriNetWork_WP1763_89515	3.56x10 ⁻³³
Mm_Chemokine_signaling_pathway_WP2292_97515	2.91x10 ⁻³¹
Mm_MAPK_signaling_pathway_WP493_78412	2.38x10 ⁻²⁹
Mm_TNF-alpha_NF-kB_Signaling_Pathway_WP246_69201	1.94x10 ⁻²⁷
Mm_Insulin_Signaling_WP65_88446	1.94x10 ⁻²⁷
Mm_Non-odorant_GPCRs_WP1396_69993	1.58x10 ⁻²⁵
Mm_EGFR1_Signaling_Pathway_WP572_82883	1.58x10 ⁻²⁵
Mm_Regulation_of_Actin_Cytoskeleton_WP523_71326	1.05x10 ⁻²¹
Mm_G_Protein_Signaling_Pathways_WP232_89955	8.51x10 ⁻²⁰

miRNA, microRNA.

western blotting (Fig. 3C). These results suggest that *Foxp1* may be the target gene of miR-34a-5p and be involved in response to high-dose radiation exposure in the small intestine.

Prediction of pathways involved in target genes of miRNAs. To predict target genes of miRNAs and their pathways, WikiPathways analysis were performed. Using TargetScan, 201 genes were predicted as targets of the upregulated miRNAs (Table SI) and 857 of the downregulated miRNAs (Table SII). Under a p-value of 0.05, 65 and 122 pathways were predicted as target genes and pathways of the upregulated and downregulated miRNAs, respectively (Tables SIII and SIV). The top 10 pathways involved in up- and downregulated miRNAs are presented in Table II. Among these, the 'TNF- α NF- κ B Signaling Pathway' and 'Regulation of Actin Cytoskeleton' are presented in Fig. S1.

Discussion

This study revealed changes in the expression of various miRNA in the small intestine of mice following radiation exposure (10 Gy X-rays). In particular, miR-34a-5p was highly

expressed at 72 h after radiation exposure, suggesting that this miRNA was the high-dose radio-responsive miRNA in the small intestine.

The body weight of irradiated mice decreased after several days of radiation exposure; in these mice, the 50% survival period was approximately 9 days (Fig. 1A and B). Fluorescent TUNEL assay confirmed that the small intestine was damaged by radiation exposure and TUNEL-positive cells were detected in the irradiation group and were not detected in the non-irradiated group. DNA damage was observed in the cells of the small intestine, particularly in stem cells, as the fluorescence of the small intestinal pit region strongly increased (Fig. 1C). Liu *et al* reported that intestinal Lgr5-positive stem cells underwent apoptosis following exposure to high-dose X-rays (17). Lgr5 is a marker of intestinal epithelial stem cells (18), and the death of Lgr5-positive cells indicates intestinal injury that is potentially lethal owing to poor regeneration ability of these cells. It will be interesting to examine if Lgr5-positive cells co-localize with TUNEL-positive cells in a future study.

We identified seven and six upregulated and downregulated miRNAs, respectively, in the small intestine of mice exposed to 10 Gy X-rays using miRNA microarray analysis. Particularly,

miR-34a-5p overexpression by more than nine fold was observed in irradiated mice compared with that in non-irradiated mice. This suggests that miR-34a-5p is a radiation-responsive miRNA in the small intestine. Reportedly, miR-34a-5p regulates various target mRNAs involved in the cell cycle, cell proliferation, senescence, migration, and invasion, including cyclin-dependent kinase 4/6, E2F transcription factor 3, cyclin E2, hepatocyte growth factor receptor, B-cell lymphoma 2 (Bcl-2), NAD-dependent deacetylase sirtuin-1, Myc, Notch, and CD44 (19,20). Furthermore, miR-34a-5p primarily induces p53-mediated apoptosis and cell cycle arrest in the G1 phase and during senescence (21,22). Radiation exposure has been reported to increase miR-34a-5p expression in different types of cells such as breast cancer cells (23), lung cancer cells (24), prostate cancer cells (25), lymphocytes (26), spleen cells (21), and colorectal cancer cells (27). However, the present study is the first to report on the increased miR-34a-5p expression in the small intestine following high-dose radiation exposure.

Reportedly, elevated miR-34a-5p expression in the small intestine suppresses Bcl-2 and Notch expressions, thus promoting apoptosis and resulting in the deterioration of the small intestine. Conversely, it is expected that increased miR-34a-5p expression suppresses RAD51, reduces double-strand break repair, and enhances radiosensitivity. Interestingly, miR-34a-5p has been reported to an indicator of radiation exposure (28); this report revealed that the serum miR-34a-5p level increases following radiotherapy in patients with breast cancer. In the future, it will necessary to examine the possibility of using serum miR-34a-5p as a biomarker for determining intestinal high-dose radiation exposure.

Foxp1 belongs to subfamily P of the forkhead box transcription factor family and plays important roles in organ development (29). Moreover, it is a valuable prognostic biomarker in lymphoma (30). However, the role of *Foxp1* in the intestinal tissue is slightly unclear. De Smedt *et al*, suggested that the loss of FOXP1 was associated with decreased survival of patients with colorectal cancer (CRC) and affected CRC proliferation and inflammatory responses (31). Presumably, *Foxp1* is a target of miR-34a-5p; however, the relationship between high-dose radiation and *Foxp1* expression has not been reported till date. The maintenance of *Foxp1* expression with the use of an miR-34a-5p inhibitor may suppress radiation-induced enteritis; thus, an miR-34a-5p inhibitor may be used as a novel radioprotective agent.

Gene expression is predicted to be upregulated and down-regulated by radiation exposure, and pathway analysis showed the enhancement of 'TNF- α NF- κ B Signaling Pathway' (Table II). TNF- α stimulation and ROS generation following radiation cause NF- κ B activation via I κ B kinase signaling and induce enteritis (32,33). The predicted enhancement of the 'TNF- α NF- κ B Signaling Pathway' suggests radiation-induced enteritis (Table II). Recently, radioprotective agents, such as α -lipoic acid and L-carnitine, have been studied, which alleviate radiation-induced enteritis as a side effect during radiotherapy (34,35). Understanding the underlying molecular mechanisms may lead to the development of novel effective radioprotective agents.

In this study, we examined miRNA expression in the small intestine after 72 h of radiation exposure using 8 week-old mice. In a previous study, we examined miRNA expression at

various time-course with a high-dose (7 Gy) of irradiation and observed a significant difference at 72 h compared with that at baseline (36). Based on these findings, changes after 72 h were examined in this study; however, there may be a possibility that the results of these two studies differ owing to the use of two radiation doses. In the future, analyzing miR-34a-5p expression at various time-course with 10 Gy irradiation will be necessary. In addition, the effects of radiation on the small intestine may vary depending on the age of the mouse. However, since it is difficult to use mice of all ages, 8 weeks of age was used as a representative of mice that reached adulthood. We plan to investigate the effects on the intestinal tract of children and older mice in the future.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

MC was a major contributor in performing the experiments and writing the manuscript. HU, IN, HK and SM helped conduct the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were performed in accordance with The Guidelines for Animal Experimentation of the Hirosaki University. The procedures were approved and monitored by The Animal Research Committee of Hirosaki University (approval nos. G12003 and G19005).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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