Original Paper

Identification of Circular RNAs Altered in Mouse Jejuna After Radiation

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Key Words

Radiation-induced intestinal injury • CircRNAs • Sequencing

Abstract

Background/Aims: Circular RNAs (circRNAs) make up a large class of non-coding RNAs and play important roles in a variety of diseases, including nervous system diseases and cancers. The intestinal epithelium is sensitive to ionizing radiation, radiotherapy of abdominal or pelvic tumors or nuclear accident exposure can lead to high radiation toxicity, which can result in radiation-induced intestinal injury. The goal of this present study was to analyze the potential roles of circRNAs in radiation-induced intestinal injury. **Methods:** Mice were divided into two groups: control group and irradiated group. Irradiated group was 3.5 days after 14Gy abdominal irradiation (ABI) group. We started with RNA-seq of circRNA changes in mouse jejuna after radiation and validated by RT-PCR in the following experimental. miRNAs targeted mRNAs were predicted using proprietary software based on target scan and Miranda. The network of circRNA-miRNA-mRNA was illustrated by cytoscape software. **Results:** 2751 circRNAs were detected in the two groups. At day 3.5 post-radiation, 42 and 48 circRNAs were found to be significantly upregulated and downregulated, respectively, compared to the control ($p \leq 0.05$, Fold Change ≥ 2). Further, the altered expression of 10 circRNAs (chr18:35610871-35613502+, chr15:95864225-95894541+, chr3:96041338-96042928-, chr5:64096979-64108263+, chr19:16705875-16710941-, chr5:134491893-134500149-, chr19:42562552-42564341+, chr5:32640331-32664400+, chr3:72958113-72960367- and chr8:79343654-79372364-) were verified by RT-PCR. Compared the miRNA-targeted mRNAs with our mRNAs sequencing data, we found 14 upregulated circRNA-targeted mRNAs were also unregulated and 22 downregulated circRNAs-targeted mRNAs were also downregulated. Gene ontology and KEGG pathway analyses indicated the predicted genes were mainly involved in the MAPK signaling pathway. **Conclusions:** This study reveals that expression of circRNAs was altered in the jejuna of mice post-irradiation and provides a resource for the study of circRNAs in radiation-induced intestinal injury and repair.

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Introduction

Circular RNAs (circRNAs) are a large class of non-coding RNAs. Unlike traditional linear RNAs that contain 5' and 3' ends, circRNAs arise from 3'–5' ligations of the ends of linear RNA molecules [1]. Due to the absence of 5' and 3' ends, circRNAs are more stable than linear RNAs and resistant to RNase R [2, 3]. A significant majority of circRNAs are exonic circRNAs [4]. Models of formation for these circRNAs include lariat-driven circularization [5] and intron-pairing driven circularization, the latter of which also generates exonic-intronic circRNAs (EicRNAs). The generation of intronic circRNAs (ciRNAs) usually involves a consensus motif containing a 7-nt GU-rich element near the 5' splice site and an 11-nt C-rich element near the branchpoint [6]. In addition, some circRNAs also form through RNA-binding protein or trans-factor driven circularization [7].

These circRNAs have a wide distribution and are found in many species, including humans [8], mice [2], *Drosophila* [9], and fish [10]. Biological functions have been reported for circRNAs, where functions have been most widely studied for microRNAs (miRNAs) sponges. The circRNAs and messenger RNAs (mRNAs) contain shared miRNA response elements, where the circRNAs regulate gene expression through miRNA binding. For example, human CDR1as, which is mainly located in the brain, has over 70 miR-7 binding sites [11] and has been proposed to function as a sponge for miR-7 by reducing the amount of free miR-7 molecules and, consequently, influence brain functions associated with neuropsychiatric disorders. Certain ciRNAs [6] and EicRNAs [12] are abundant in nuclei, where ciRNAs interact with the polymerase II machinery, EicRNAs interact with U1 small nuclear ribonucleoproteins, and both modulate the expression of parental gene in a cis-acting manner. A subset of circRNAs can combine with proteins and regulate protein function and a few circRNAs can be translated.

Recent work has suggested that circRNAs play important roles in disease development. The circRNA CDR1as is highly expressed in the brain and has been implicated in nervous system diseases, including Parkinson's and Alzheimer's disease [13], and brain development. In some cancers, such as colon [14], liver [15], and stomach cancers [16], circRNA expression differs from expression in corresponding normal tissue, indicating a role for these circRNAs in tumor development. Radiotherapy is used to treat more than half of cancer patients and plays a critical role in curing 25% of cancers [17]. While radiotherapy does play an important role in cancer treatment, normal tissue surrounding the cancerous tissue also suffers from radiation damage [18]. The intestinal epithelium is sensitive to ionizing radiation and the intestine is one of the most sensitive organs to radiation toxicity [19]. Radiotherapy of abdominal and pelvic tumors and exposure to nuclear accidents can result in high radiation toxicity that can lead to radiation-induced intestinal injury. Radiation-induced bone marrow injury can be alleviated using bone marrow transplantation, however, there are no effective means of preventing or treating radiation-induced intestinal injury [20]. Radiation-induced intestinal toxicity is typically defined as clonogenic cell death and apoptosis of crypt cells, insufficient replacement of villus epithelium, breakdown of the mucosal barrier, and significant inhibition of compensatory proliferation [21]. The effects of toxicity on the intestines are collectively referred to as gastrointestinal acute radiation syndrome [22], which clinically manifests with symptoms including anorexia, vomiting, diarrhea, dehydration, systemic infection, and, in extreme cases, septic shock and death [23].

Researchers have reported on approaches to preventing radiation-induced intestinal injury. Gong [24] et al. found that mesenchymal stem cells (MSCs) can improve the rate of survival of mice suffering from radiation poisoning. After irradiating the abdomen with 14 Gy, administration of mesenchymal stem cells increases the rate of survival to 20% compared to 0% survival in the control PBS group. Liang [25] found that 12-O-tetradecanoylphorbol-13-acetate can improve survival after radiation by increasing intestinal crypt stem cell survival via acting through the PKC signaling pathway. Others have also found that improving differentiation of goblet cells [26] or preventing endothelial cell dysfunction [27] can

also alleviate radiation-induced intestinal injury. However, as mentioned above, there are currently no recognized means of preventing or treating radiation-induced intestinal injury.

Due to the high tissue and developmental stage specificity of circRNAs, these molecules are regarded as potential disease biomarkers. As circRNAs are widely expressed *in vivo*, we believe that there are circRNAs expressed in the intestine that play an important role in radiation-induced intestinal injury and repair. According to a previous report, mice suffer the most severe intestinal injury at 3.5 days post-radiation and recover by day 5 through intestinal repair. Therefore, sequencing of mouse jejunum was performed at 3.5 days post-radiation, in order to identify circRNAs associated with radiation-induced injury and protection.

Materials and Methods

Mouse model of radiation-induced intestinal injury

Male C57BL/6 mice, aged 8–10 weeks and weighing 20–22 g, were purchased from Huafukang Biotechnology Co. Ltd (Beijing, China). All mice were housed in a temperature-controlled and specific-pathogen-free environment with a 12 h light/dark cycle and fed standard chow and water *ad libitum*. Abdominal irradiation was performed using a Cs137 γ -ray irradiator (Atomic Energy of Canada, Chalk River, Ontario, Canada), where lead shielding was used to protect other parts of the body from irradiation. Mice were exposed to 14 Gy at a rate of 1 Gy/min at room temperature. Day 3.5 post-radiation, the mice were sacrificed and their jejunum harvested and frozen at -80°C . All experimental procedures and protocols were conducted in accordance with the guidelines of the local animal care and use committee.

Samples and RNA isolation

The mice were divided into control group and irradiated group. The control group contained three non-irradiated mice and the irradiated group contained three mice exposed to 14 Gy abdominal irradiation. RNA was isolated from the jejunum of the control group and irradiation group at 3.5 days post-radiation using TRIzol reagent (Ambion) according to the manufacturer's instructions. The sample RNA concentrations were measured at an OD_{260/280} using a NanoDrop ND-2000 instrument (Thermo, Waltham, MA, USA). The integrity of the RNA in the samples was assessed using denaturing agarose gel electrophoresis.

High-throughput sequencing

High-throughput whole transcriptome sequencing and subsequent bioinformatics analysis were performed by Cloud-Seq Biotech (Shanghai, China) as follows. First, rRNA was removed from the total RNA using a Ribo-Zero rRNA Removal Kit (Illumina, USA) according to the manufacturer's instructions. Library Construction RNA libraries were constructed using a TruSeq Stranded Total RNA Library Prep Kit (Illumina, USA) from the rRNA-depleted RNA according to the manufacturer's instructions. RNA quality and quantity in the libraries were controlled for using the BioAnalyzer 2100 system (Agilent Technologies, USA). Libraries were denatured into single-stranded DNA molecules, captured on Illumina flow cells, amplified *in situ* as clusters, and sequenced for 150 cycles on an Illumina HiSeq Sequencer according to the manufacturer's instructions.

Sequencing analysis of circRNA

Paired-end reads were obtained from the Illumina HiSeq 4000 sequencer. After 3' adaptor-trimming and removal of low quality reads using cutadapt [28] software (v1.9.3). The resulting high-quality trimmed reads were used to analyze circRNA. The high-quality reads were aligned to the reference genome/transcriptome using STAR [29] software and circRNAs were identified using DCC [30] software and annotated from the circBase [31] database.

Identification of differentially expressed circRNAs

Edger [32] software was used to normalized the data and analyze circRNAs for differential expression. Any circRNAs exhibiting fold-changes ≥ 2.0 with P-values ≤ 0.05 were considered significantly differentially expressed.

Experimental validation of circRNAs

Quantitative real-time PCR (qRT-PCR) was used to validate circRNA expression. Of the circRNAs identified, 5 upregulated and 5 downregulated circRNAs were selected for validation. The housekeeping gene GAPDH was used as a reference for normalization. The primers were designed using the “out-facing” strategy, where circle template was amplified (Table 1). Total RNA was reverse-transcribed

into complementary DNA using a PrimeScript RT Reagent Kit (Perfect Real Time; TaKaRa, Osaka, Japan) and subjected to qRT-PCR on an Applied Biosystems 7500 Fast Real-Time PCR System. Three independent assays were performed for all samples, where samples were assessed in triplicate. The relative expression of the circRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method.

Gene ontology and KEGG pathway analysis of selected circRNAs

Gene ontology (GO) and KEGG analyses were performed for differentially expressed circRNA-associated genes. GO analysis assessed molecular functions, biological processes, and cellular components. The top 10 enriched GO terms among the two groups were highlighted as differentially expressed. KEGG pathway analysis was performed to identify pathways associated with circRNAs targeted mRNAs.

Analysis of circRNA-miRNA-mRNA network

Recent studies have reported that circRNAs can precisely regulate miRNA levels by absorbing miRNA and, thus, acting as “miRNA sponge.” To explore the functions of circRNAs, as well as deduce which circRNAs function as miRNA sponges, predictions of interactions of differentially expressed circRNAs with miRNAs were made using popular miRNA target gene prediction software.

The miRNA binding sites and target mRNAs were predicted using proprietary software based on targets can and Miranda. Using each circRNA with its miRNA-binding and predicted miRNA sites as data, cytoscape software was used to construct a circRNA-miRNA-mRNA network map and, thus, visualize interactions between these molecules.

Results

Expression pattern of circRNAs in mouse jejuna after exposure to radiation

A total of 2751 circRNAs were identified in mice jejuna at baseline and 3.5 days post-radiation based on at least one read spanning a head-to-tail splice junction. Of these, 1809 circRNAs were already included in the circBase or had been identified in previous studies, while 942 circRNA were first observed in this present study.

The 2751 circRNAs were found to be located in genes across all chromosomes (Fig. 1A). Chromosomes 1-19 contained more than 80 circRNAs each and X had 42 circRNAs, while the Y and mitochondrial chromosomes contained less than 10 circRNAs. There were 2418 exonic circRNAs, which ranged in size from 80 nt to more than 2000 nt (3.34%) and most (14.36%) were 301-400 nt long (Fig. 1B), the average length was 732.58 nt.

Although most of the genes (67.70%) generated a single circRNA, 553 genes generated two or more circRNAs. Among these, 222 (13.97%) of genes generated more than two circRNAs and some genes (1.93%) generated more than 6 circRNAs (Fig. 1C). As presented in Fig. 1D, these 2751 circRNA were from a variety of regions, including exonic (87.90%),

Table 1. Primers for validation of circRNA expression

Gene Name	Primer Type	Primer Sequence	Product Length
chr18:35610871-35613502+	Forward primer	ACGCTGTTTCCAAGAAATGC	195
	Reverse primer	ACTGCTGCCACTTGGATCTT	
chr15:95864225-95894541+	Forward primer	CAGAGGCGAATCGACTTCAT	188
	Reverse primer	GGGCTTCCCGTTAAATCTT	
chr3:96041338-96042928-	Forward primer	CAAAGCACGTGACAGTGGTC	197
	Reverse primer	GATTCTGGCACTCTGGAAA	
chr5:64096979-64108263+	Forward primer	GAAACAGAGCCCAACAGCTC	204
	Reverse primer	CATTCTGAGAAATCCCTGCTC	
chr19:16705875-16710941-	Forward primer	TCACGACCTGGATAACACA	169
	Reverse primer	TTGAGGACACTCTCCAAGG	
chr5:134491893-134500149-	Forward primer	CCACAAACAGGAGGTGATCG	126
	Reverse primer	TGGGATGTCAAGGCTGTAC	
chr19:42562552-42564341+	Forward primer	GGAAGTAGTGTCTGGAGGAA	95
	Reverse primer	GGCACCCTTCAATCTCTAGT	
chr5:32640331-32664400+	Forward primer	TGAGGCTGCTCTGTATGGTC	113
	Reverse primer	CCATTATCAAATCTGGATATGGCAC	
chr3:72958113-72960367-	Forward primer	GAATCCAGCAGCTGACACA	103
	Reverse primer	TGGCTTTAAGTCAAGACTTTGTTGT	
chr8:79343654-79372364-	Forward primer	TACTGGGCGAGTCTGTGAAC	201
	Reverse primer	CGCAGAGGCAACAGAGATT	
Gapdh	Forward primer	AGGTCGGTGTGAACGGATTTC	95
	Reverse primer	GGGGTCGTTGATGGCAACA	

Fig. 1. Expression of circRNAs in jejuna of mice after exposure to radiation. (A) Chromosomal distribution of circRNAs. “ChrM” represents mitochondrial genome. (B) Length distribution of 2418 exonic circRNAs. The majority of circRNAs ranged from 301–400 nt (16.34%) in size. (C) Distribution of circRNAs per gene, where most genes contained one circRNA. (D) Host genes from which these 2751 circRNAs derived.

sense overlapping (9.16%), and intronic regions (2.51%), based on their location in their host genes.

Identification of differentially expressed circRNAs after exposure to radiation

Among the 2751 circRNAs, 1970 circRNAs were detected in the control group, 1845 circRNAs were detected at day 3.5 post-radiation, and 1064 circRNAs were detected in both groups (Fig. 2A). Of these, 90 circRNAs differentially expressed at 3.5 days post-radiation were selected, where 42 were upregulated and 48 downregulated (Fig. 2B). Based on the data presented in Table 2, the miRNAs and miRNA-target mRNAs associated with these circRNAs were identified using our mRNA sequencing results. As shown in Fig. 2C, 14 upregulated circRNA-targeted upregulated mRNAs and 22 downregulated circRNA-targeted downregulated mRNAs (Table 3) were subjected to GO and KEGG pathway analyses to characterize the circRNA-miRNA-mRNA network.

Validation of circRNA expression by qRT-PCR

To validate the expression levels of differentially expressed circRNAs, 5 upregulated and 5 downregulated circRNAs from day 3.5 post-radiation were selected and expression levels were confirmed by qRT-PCR (Fig. 3). The qRT-PCR results for the upregulated and downregulated circRNAs were consistent with the

Table 2. 90 differentially expressed circRNAs at 3.5 days post-radiation

CircRNAID	logFC	logCPM	P Value	Regulation	circRNA Type	Gene Symbol
chr15:99794409-99807743-	4.4561204	10.160646	0.0100157	Up	Exonic	Lima1
chr6:88355483-88358642-	2.5512206	10.365014	0.0449825	Up	Exonic	Eefsec
chr5:10039347-100408113-	3.6381245	10.191793	0.0335328	Up	Exonic	Sec31a
chr4:132857300-132871657-	3.8963318	10.003852	0.0442012	Up	Exonic	Stx12
chr14:55659200-55660098-	3.9533835	10.191841	0.0200185	Up	Exonic	Mdm1
chr14:27438599-27442620+	3.6243888	10.273047	0.028087	Up	Exonic	Fam120a
chr19:43810471-43814814+	3.2565184	10.382725	0.0480682	Up	Exonic	Abc2
chr6:134541652-134542045+	3.9583763	10.715693	0.0099943	Up	Exonic	Lrp6
chr11:58040391-58044050+	3.6411373	10.053388	0.0465824	Up	Exonic	Larp1
chr6:22132229-22145687+	3.6634433	10.151658	0.03783	Up	Exonic	Cpep1
chr19:41545875-41555701+	2.7124662	10.361739	0.0307496	Up	Exonic	Lcor
chrY:90793296-90793680+	2.8010458	10.619317	0.0050547	Up	Exonic	Erd1r
chr6:86944357-86967373+	3.6637903	10.184692	0.0332887	Up	Exonic	Aak1
chr15:95864225-95894541+	3.9069187	10.094897	0.0300941	Up	Exonic	Ano6
chrY:90793325-90793680+	4.1790799	10.101849	0.0204181	Up	Sense overlapping	Erd1r
chrY:90793325-90793680+	4.7300929	10.358742	0.0018511	Up	Sense overlapping	Erd1r
chr3:116618256-116621508+	4.1544319	10.145074	0.0183458	Up	Exonic	Sass6
chr4:58486477-58487224-	3.6635617	10.149518	0.0381737	Up	Exonic	Lpar1
chr18:35610871-35613502+	2.0825754	10.667326	0.0465572	Up	Exonic	Paip2
chr9:62912750-62923655-	3.1612465	10.05098	0.0484927	Up	Exonic	Pias1
chr19:38620443-38652111+	3.9124401	10.222612	0.0187912	Up	Exonic	Pice1
chr4:103319022-103353651+	2.697894	10.275169	0.0383703	Up	Exonic	Okazaki
chr12:85028770-85034034+	3.3020582	10.391282	0.0454927	Up	Exonic	Vp1m1
chr19:16705875-16710941-	3.670584	10.055549	0.0445849	Up	Exonic	Vps13a
chr3:96041338-96042928-	3.6635886	10.149032	0.0382528	Up	Exonic	Vps45
chr7:131074200-131081212-	3.6705236	10.056243	0.0443952	Up	Exonic	Dm1t1
chr8:76908270-76910028+	3.6949691	10.054757	0.0436874	Up	Exonic	Nr3c2
chr3:133320936-13339349+	4.0277757	10.066357	0.026111	Up	Exonic	Ppa2
chr5:64069799-64108263+	4.2291415	10.236954	0.0098858	Up	Exonic	Pgm1
chr6:28668558-28724981+	3.903782	10.004567	0.0438149	Up	Exonic	Snd1
chr1:143667625-143677855-	3.9271951	10.052548	0.0322657	Up	Exonic	Cd73
chr2:125108956-125116873-	3.629488	10.39052	0.024781	Up	Exonic	Myo2
chr7:131040887-131046588+	2.7077757	10.28224	0.0367382	Up	Exonic	Dm1t1
chr8:109876803-109895641+	3.6595546	10.234915	0.0290034	Up	Exonic	Philp2
chr13:74671308-74675906+	3.9207004	10.345085	0.014267	Up	Exonic	Erap1
chr16:14138343-14140372-	3.9823021	10.103344	0.0275104	Up	Exonic	Marf1
chr5:3755594-3772787-	4.4134059	10.244003	0.0063616	Up	Exonic	Ank1b1
chr9:121175735-121190130-	3.6655657	10.050021	0.0449695	Up	Exonic	Ekap1
chr6:83308933-83313546-	3.935621	10.00748	0.0422313	Up	Exonic	Mittd2
chr6:40819346-40829462+	3.6057348	10.049968	0.0493249	Up	Antisense	Mir1950
chr2:168705123-168710976-	1.6193747	10.082478	0.0368852	Up	Exonic	Atp9a
chr15:7299972-73031643-	3.3007505	10.425214	0.0449857	Up	Exonic	Trappc9
chr14:17963061-17963321+	4.085269	10.267883	0.0135292	Down	Exonic	Thrb
chr1:91384565-91385346-	4.084685	10.452431	0.0104339	Down	Exonic	Ilkap
chr9:66540062-66566903-	1.487613	12.017972	0.0085747	Down	Exonic	Usp3
chr10:75684292-75700095-	4.08553	10.269043	0.013477	Down	Exonic	Cabin1
chr17:64710704-64714712+	2.509476	10.390823	0.0463646	Down	Exonic	Man2a1
chr6:8537817-8579739+	3.839478	10.311776	0.0200598	Down	Exonic	Glic1
chr1:13904000-139062971+	2.826497	10.98719	0.0105501	Down	Exonic	Denn1b
chr3:142303943-142352885-	3.838912	10.092093	0.036973	Down	Exonic	Pdlim5
chr2:5973469-6010428+	4.294453	10.232286	0.0103388	Down	Exonic	Usp2
chr8:22535019-22545600+	4.294953	10.424847	0.0064924	Down	Exonic	Slc20a2
chr9:3441055-3477962+	1.717789	11.035597	0.0354392	Down	Sense overlapping	Cwfl1912
chr11:12365105-12378359-	3.542214	10.342831	0.0336192	Down	Exonic	Coat1
chr5:32640331-32664400+	2.170243	10.836196	0.0285077	Down	Exonic	Yes1
chr1:118419440-118419918+	4.295033	10.268226	0.0087054	Down	Exonic	Clasp1
chr13:93341756-93356079+	3.839227	10.348148	0.012922	Down	Exonic	Homer1
chr3:72958113-72960367-	1.159965	11.770768	0.0496387	Down	Exonic	Sis
chr13:48932297-48934078-	3.839929	10.135036	0.0344207	Down	Exonic	Fam120a
chr4:59606215-56912130-	1.746685	11.136284	0.0197419	Down	Exonic	Tmem245
chr2:12355277-12386679-	4.084959	10.232928	0.0151356	Down	Exonic	Fam188a
chr5:134491893-134500149-	4.642202	10.594748	0.0020114	Down	Exonic	Clp2
chr4:150406196-150406664+	2.262748	11.160516	0.0065991	Down	Exonic	Rere
chr6:35064898-35080226-	3.542212	10.336244	0.033781	Down	Exonic	Cnot4
chr16:93849130-93853898+	2.675834	10.604373	0.0247297	Down	Exonic	Morc3
chr18:36611800-36614817+	3.839683	10.19022	0.0273035	Down	Exonic	Ankhd1
chr8:85875634-85901957+	2.529703	10.418224	0.0433242	Down	Exonic	Phkb
chr3:72900418-72914691-	2.393849	10.93989	0.0120684	Down	Exonic	Sis
chr4:58861525-58885498-	4.085262	10.270121	0.0134399	Down	Exonic	Al314180
chr9:7151069-7155737-	3.839329	10.050721	0.0406776	Down	Exonic	Myap
chr19:5751630-5755618+	2.158643	10.630729	0.0378571	Down	Exonic	Parp9
chr6:143180587-143197565+	4.085772	10.307383	0.0122575	Down	Exonic	Etnk1
chr7:13025713-133081860-	4.085894	10.097627	0.0263189	Down	Exonic	Ctbp2
chr5:135121506-135124017+	1.136824	12.119923	0.0237733	Down	Exonic	Mxipl
chr17:50047297-50055627-	3.840355	10.095551	0.0369104	Down	Exonic	Rtn1
chr14:54758439-54759745-	1.740645	11.325961	0.0179354	Down	Exonic	Slc7a8
chr16:35963975-35964364+	3.838528	10.141791	0.0340012	Down	Exonic	Nr1h4
chr13:43412411-43425214-	4.086319	10.269478	0.0134425	Down	Exonic	Rarb9
chr19:42562552-42564341+	2.506461	10.914739	0.0080846	Down	Exonic	R3hcc11
chr11:26397746-26434500+	3.542513	10.186554	0.0435861	Down	Exonic	Fanc1
chr18:31988512-32001419-	4.295118	10.349589	0.0071641	Down	Exonic	Myo7b
chr5:23979488-24000019-	2.839174	10.39375	0.0210191	Down	Exonic	Fam127a
chr10:89473752-89483524-	1.772513	11.067811	0.0454736	Down	Exonic	Nr1h4
chr10:25220611-25289730-	4.085945	10.684208	0.0085185	Down	Exonic	Ataf1
chr10:115386962-115394036+	3.839686	10.186561	0.0276962	Down	Intronic	Zfch1
chr17:2626283-26267358+	3.839973	10.050553	0.0406993	Down	Exonic	Luc7l
chr13:5245518-55248077+	3.541814	10.229497	0.0391764	Down	Exonic	Nsd1
chr9:60850130-60854503+	3.839332	10.095441	0.0369529	Down	Exonic	Uaca
chr6:149068050-149084936-	2.690389	10.496555	0.0267503	Down	Sense overlapping	Denn5b
chr8:79343654-79372364-	4.084385	10.050006	0.0298367	Down	Exonic	Sma1

Fig. 2. Identification of circRNAs differentially expressed after exposure to radiation. (A) Expression of circRNAs in the control and irradiated group. (B) At day 3.5 post-radiation, 42 and 48 circRNAs were significantly upregulated and downregulated ($p \leq 0.05$, fold change ≥ 2), respectively. (C) Overlapping number of targeted mRNAs of differentially expressed circRNAs and mRNAs.

Fig. 3. Validation of circRNA expression by RT-PCR. Changes in expression were confirmed using RT-PCR for select circRNAs in the control and irradiated group. Bars represent mean $\pm$ SEM ($n=3$), ** $P < 0.05$.

trends observed in the sequencing data.

Functional analyses of miRNA target genes

To understand the functions of genes associated with the differentially expressed circRNAs, GO analysis was performed. GO analysis included identification of molecular functions (Fig. 4A), biological processes (Fig. 4B), and cellular components (Fig. 4C). Of the molecular functions, the two main functions identified were binding and catalytic activity. The primary biological process identified was secretion, such as cytokine and cell secretion. When classified according to cellular component, the largest proportion of target genes was in the cell part and cell categories.

The functions of the predicted circRNA-target mRNAs were characterized by KEGG pathway analysis

Table 3. mRNA for GO and KEGG signaling pathway analyses

Gene	Locus	D3.5 FPKM	Ctrl FPKM	log2(FC)	P Value	Regulation
Celsr2	chr3:108390850-108415552	0.798272	0.238657	1.74194	0.0006	Up
Trim6	chr7:104218792-104235152	2.40461	0.989552	1.28095	0.00015	Up
Zfp37	chr4:62189539-62208446	1.80806	0.883302	1.03346	0.00145	Up
Pex5l	chr3:32939313-33143247	0.649164	0.253207	1.35827	0.00455	Up
Tbc1d2	chr4:46604389-46650209	2.87155	1.39098	1.04573	0.0001	Up
Steap2	chr5:5664830-5694578	4.68727	2.28191	1.0385	0.00015	Up
Sfxn5	chr6:85213050-85333422	0.945922	0.382691	1.30554	0.019	Up
Polr1e	chr4:45018582-45084604	4.29142	1.8369	1.22418	0.0023	Up
Lrp8	chr4:107802258-107876840	1.18463	0.453724	1.38455	0.00175	Up
Pgap1	chr1:54472999-54557684	9.3491	3.84237	1.28283	0.00005	Up
Gm4724	chr2:175372027-175435807	1.23671	0.011015	6.81095	0.00545	Up
Slc1a2	chr2:102658658-102790784	0.967178	0.223213	2.11536	0.00355	Up
Ephb2	chr4:136647538-136835988	12.4825	5.80317	1.10499	0.00005	Up
Pappa	chr4:65124173-65357509	1.41139	0.703406	1.00469	0.00005	Up
Clec9a	chr6:129397296-129424764	0.573743	1.39147	-1.27813	0.01185	Down
Serpinb8	chr6:107590005-107608909	0.406235	0.984013	-1.27636	0.01965	Down
Hpgds	chr6:65116828-65144908	0.976493	2.09466	-1.10104	0.023	Down
Cml2	chr6:85865421-85869158	1.03316	2.21287	-1.09885	0.00005	Down
Gata3	chr2:9857077-9890034	0.250508	0.720621	-1.52439	0.0107	Down
Ilkzf2	chr1:69513931-69685960	0.451392	1.81571	-2.00808	0.00005	Down
Zfp831	chr2:174643533-174710832	0.188597	0.531756	-1.49545	0.00005	Down
Heyl	chr4:123233555-123249875	0.208397	0.940518	-2.17412	0.00005	Down
Cd53	chr3:106758860-106790149	1.7224	4.4461	-1.36812	0.00005	Down
Igj	chr5:88520511-88527891	127.153	421.247	-1.7281	0.00005	Down
Spr2a1	chr3:92215834-92257298	13.981	38.726	-1.46984	0.00005	Down
Srgap3	chr6:112717970-112947266	0.428386	1.16302	-1.44089	0.0004	Down
Cytip	chr2:58129136-58195532	4.25494	12.044	-1.5011	0.00005	Down
Itga4	chr2:79255425-79461107	2.67814	6.40145	-1.25717	0.00005	Down
Laptn5	chr4:130913124-130936141	6.88928	15.8094	-1.19835	0.00005	Down
Mras	chr9:99385421-99437376	0.753355	1.51864	-1.01138	0.02225	Down
Cacna1e	chr1:154392518-154884037	0.39532	0.869295	-1.13683	0.0002	Down
Kctd14	chr7:97451333-97459553	3.52574	7.56646	-1.10169	0.0078	Down
Prkcb	chr7:122288750-122634402	0.916544	3.11893	-1.76677	0.00005	Down
Ptpn7	chr1:135132724-135145326	0.471384	1.33024	-1.49671	0.00005	Down
Stg6alac3	chr3:153205404-153725062	0.412624	1.12331	-1.44486	0.009	Down
Cd84	chr1:171839696-171890718	1.22365	2.52975	-1.0478	0.0017	Down

Table 4. mRNA involved signaling pathway

Pathway ID	Definition	Fisher-P Value	Enrichment Score	GeneRatio	Genes
mmu04010	MAPK signaling pathway - Mus musculus (mouse)	0.000887403	3.051879	0.285714	Cacna1e/Mras/Prkcb/Ptpn7
mmu05140	Leishmaniasis - Mus musculus (mouse)	0.005967734	2.224191	0.142857	Itga4/Prkcb
mmu04724	Glutamatergic synapse - Mus musculus (mouse)	0.0181527	1.741059	0.142857	Prkcb/Slc1a2
mmu04670	Leukocyte transendothelial migration - Mus musculus (mouse)	0.01965651	1.706494	0.142857	Itga4/Prkcb
mmu04360	Axon guidance - Mus musculus (mouse)	0.02216906	1.654253	0.142857	Ephb2/Srgap3
mmu04530	Tight junction - Mus musculus (mouse)	0.02583172	1.587847	0.142857	Mras/Prkcb
mmu04020	Calcium signaling pathway - Mus musculus (mouse)	0.04145532	1.36242	0.142857	Cacna1e/Prkcb

Fig. 4. Gene ontology analysis and KEGG signaling pathway analysis of selected mRNAs. Gene ontology analysis identified (A) molecular functions, (B) biological processes, and (C) cellular components. (D) Relevant pathways were identified for selected mRNAs.

(Fig. 4D). The differentially expressed circRNA-target mRNAs were associated with the MAPK signaling pathway, leishmaniasis, glutamatergic synapses, leukocyte transendothelial migration, etc. For the MAPK signaling pathway (Table 4), four predicted mRNAs, *Cacna1e*, *Mras*, *Prkcb*, *Ptpn7* were found to be involved in this pathway. *Prkcb* was also found to be involved in the calcium signaling pathway, tight junction leukocyte transendothelial migration, glutamatergic synapses, and leishmaniasis. It has been reported that EGF-mediated activation of the Ras/Raf/Mek/Erk/MAPK signaling pathway is involved in intestinal stem cell proliferation [33] and more than half of mitosis by intestinal stem cells and transit amplifying progenitors relies on high levels of EGF within the crypt-domains [34]. Mihaescu [35] reported p38 MAPK is an important signaling pathway in radiation-induced colitis. Irradiation induces an increase in leukocyte and platelet recruitment, myeloperoxidase activity, CXCL chemokine levels, and intestinal leakage. Inhibition of p38 MAPK decreases radiation-induced leukocyte and platelet recruitment.

Fig. 5. Network of circRNA-miRNA-mRNA. Circles represent circRNAs, arrowheads represent miRNAs, hexagons represent mRNAs, red represents upregulated, and green represents downregulated.

Prediction of miRNA binding sites and circRNA-miRNA-mRNA networks

The selected 42 upregulated circRNAs with 14 associated targeted mRNAs and 48 downregulated circRNAs with 22 associated targeted mRNAs were used to construct a circRNA-miRNA-mRNA network (Fig. 5). The resulting network is very complicated, where one circRNA can interact with multiple miRNAs and one miRNA can inhibit multiple mRNAs. The miRNA mmu-miR-7092-3p interacted with the most circRNAs and mRNAs. Its target gene, *Ephb2*, is an intestinal stem cell marker and has been studied extensively in colorectal cancer [36]. The functions of circRNAs and their target genes in the intestine require additional studies in the future.

Discussion

In this present study, we performed a basic analysis of the functions of circRNAs associated with radiation-induced intestinal injury. Using sequencing technology, 2751

circRNAs were identified from control and irradiated mice, where 1809 circRNAs had been previously identified and 942 were first observed in this study. Among the 2751 circRNAs, 1845 circRNAs were detected at 3.5 days post-radiation, which is the time point where the most severe intestinal injury is observed. The 2751 circRNAs were located across all the chromosomes, including the sex and mitochondrial chromosomes. These circRNAs were derived from a variety of regions, including exonic, sense overlapping, and intronic regions.

We selected 90 significantly regulated circRNAs and predicted their interacting miRNAs and miRNA-targeted mRNAs. For certain circRNAs, both the circRNA and their target mRNA(s) were upregulated or downregulated, as circRNAs function as competing endogenous RNAs and can interact with microRNAs through microRNA-response elements to cause miRNA-induced gene silencing. By comparing the prediction results with the mRNA sequencing results, 14 upregulated circRNA-targeted mRNAs and 22 downregulated circRNA-targeted mRNAs were identified. Based on their consistency of regulation, they may have greater chances of interacting.

Using the selected mRNAs, GO analysis was performed. It was found these mRNAs function mainly in the biological processes of cytokine and cell secretion, which may be associated with radiation-induced enteritis [37]. Some researchers have compared inflammatory bowel disease and normal segments of colon from patients with radiation proctitis against normal controls and found that radiation-induced tissue damage is associated with elevated levels of interleukins, especially interleukin-1 β [38]. Perhaps these secretion processes are associated with increasing levels of interleukins.

KEGG signaling pathway analysis revealed the differentially expressed circRNA-target mRNAs are involved in the MAPK signaling pathway, leishmaniasis, glutamatergic synapses, and leukocyte transendothelial migration. As mentioned above, the MAPK signaling pathway is involved in intestinal stem cell proliferation [34] and radiation-induced colitis [35]. The third related pathway is leukocyte transendothelial migration, which is a key step in the inflammatory response. The KEGG signaling pathway analysis indicates that the differentially expressed circRNA-target mRNAs are mainly involved in the radiation-induced intestinal inflammatory response and intestinal stem cell regeneration.

The circRNA-miRNA-mRNA network is very complex, where one circRNA may have multiple miRNA binding sites and interact with multiple miRNAs. For example, CDR1as contains more than 70 binding sites for miR-7 and can also interact with miR-671 [3]. One miRNA can also interact with multiple circRNAs or one miRNA may play different roles by inhibiting different mRNAs. For example, in colon cancer, miR-7 inhibits the proliferation and migration of colon cancer cells by regulating FAK [39]. However, in the human colonic epithelial cell line LS174T, miR-7 binds to the 3' UTR of TFF3 to inhibit proliferation and migration via the PI3K-AKT signaling pathway [40]. Our comparison of circRNA-target mRNAs with sequencing mRNA results may facilitate screening for downstream targets.

More than 70% of cancer patients suffer from radiation therapy as the intestine is sensitive to radiation and is hard to shield. Treatments, such as hematopoietic stem cell transplantation and abdominal/pelvic tumor radiotherapy, injure the intestine to different degrees. Radiation-induced intestinal injury leads to intestinal mucosal injury, microbial translocation, inflammation, and, in severe cases, even death. Some researchers have reported methods of reducing structural damage to the intestine or promoting recovery of the intestinal structure after irradiation. However, there are currently no recognized approaches for the prevention and/or treatment of radiation-induced intestinal injury. The circRNA sequencing results in this study predicted that circRNAs are differentially expressed after irradiation and the miRNA and mRNA prediction results suggest that some mRNAs are involved in signaling pathways and biological functions with roles in the radiation-induced intestinal inflammatory response and intestinal stem cell regeneration. It is reasonable to conclude these differentially expressed circRNAs may play important roles in radiation-induced intestinal injury and/or repair.

In conclusion, our study is the first to delineate the expression profiles of circRNAs in mouse intestines after radiation. Abundant circRNAs were found in the jejuna of mice, where

some circRNAs were differentially expressed post-radiation. Interacting miRNAs were predicted for the differentially expressed circRNAs by analyzing the mRNA sequencing results, potential gene ontologies, and identifying involved signaling pathways. The sequencing results and predictions provide a resource for further mechanistic studies. However, the sponge function of circRNAs and their target mRNAs was predicted, this function needs to be experimentally verified. As there are currently no recognized methods of preventing and/or treating radiation-induced intestinal injury, the circRNA analysis performed in this present study may provide novel avenues for radiation-induced intestinal injury studies. We hope to identify circRNAs that help protect the intestine from irradiation that can be used or targeted to lessen intestinal injury from radiation therapy in the clinic.

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Disclosure Statement

The authors declare to have no competing interests.

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