RNA Profiling Reveals a Common Mechanism of Histone Gene Downregulation and Complementary Effects for Radioprotectants in Response to Ionizing Radiation

Changhui Ge[1] , Fei Su[1], Hanjiang Fu[1], Yuan Wang[1], Baolei Tian[1], Bin Liu[1],
Jie Zhu[1], Yong Ding[2], and Xiaofei Zheng[1]

Abstract

High-dose ionizing radiation (IR) alters the expression levels of non-coding RNAs (ncRNAs). However, the roles of ncRNAs and mRNAs in mediating radiation protection by radioprotectants remain unknown. Microarrays were used to determine microRNA (miRNA), long ncRNA (lncRNA), and mRNA expression profiles in the bone marrow of irradiated mice pretreated with amifostine, CBLB502, and nilestriol. Differentially expressed mRNAs were functionally annotated by Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses. Some histone cluster genes were validated by real-time PCR, and the effects of radioprotectant combinations were monitored by survival analysis. We found that these radioprotectants increased the induction of lncRNAs and mRNAs. miRNA, lncRNA, and mRNA expression patterns were similar with amifostine and CBLB502, but not nilestriol. The radioprotectants exhibited mostly opposite effects against IR-induced miRNAs, lncRNAs, and mRNAs while inducing a common histone gene downregulation following IR, mainly via nucleosome assembly and related signaling pathways. Notably, the effects of nilestriol significantly complemented those of amifostine or CBLB502; low-dose drug combinations resulted in better radioprotective effects in pretreated mice. Thus, we present histone gene downregulation by radioprotectants, together with the biological functions of miRNA, lncRNA, and mRNA, to explain the mechanism underlying radioprotection.

Introduction

Exposure to high-dose ionizing radiation (IR) could lead to life-threatening injuries, primarily to the more radiosensitive, self-renewing tissues. IR primarily affects hematopoietic and gastrointestinal systems, resulting in acute radiation syndrome. The pre-administration of radioprotectants and radiomitigators, such as amifostine, CBLB502, and nilestriol reportedly protect against IR damage and promote recovery.[1,2]

Although the underlying protective mechanism of these radioprotectants against high-dose IR remains unclear, several biological and molecular mechanisms have been suggested.[2,3] Amifostine (*S*-2[3-aminopropylamino]-ethylphosphorothioic acid; WR-2721) is a thiol compound whose active metabolite is a free radical scavenger, which regulates cell cycle checkpoints and gene expression while facilitating DNA repair.[4]

[1] Department of Experimental Hematology and Biochemistry, Beijing Key Laboratory for Radiobiology, Beijing Institute of Radiation Medicine, Beijing, China
[2] 5th Medical Center, The General Hospital of Chinese People's Liberation Army, Beijing, China

Corresponding Authors:
Xiaofei Zheng, Department of Experimental Hematology and Biochemistry, Beijing Key Laboratory for Radiobiology, Beijing Institute of Radiation Medicine, #27 Taiping Rd., Haidian Dist., Beijing 100850, China.
Email: xfzheng100@126.com

Yong Ding, 5th. Medical Center, The General Hospital of Chinese People's Liberation Army, #8 East St., Fengtai Dist., Beijing 100071, China.
Email: dingyong307h@163.com

Table 1. Numbers of Differentially Expressed miRNAs, lncRNAs, and mRNAs in Mice Pretreated With Radioprotectant-Only (amifostine, CBLB502, and nilestriol), or Pretreated With Radioprotectant Before Ionizing Radiation (radioprotectant + IR).

Array	Treatment	Amifostine	CBLB502	Nilestriol
miRNA	Radioprotectant-only	113	80	136
	Radioprotectant+IR	105	127	71
LncRNA	Radioprotectant-only	2618	2390	2251
	Radioprotectant+IR	6360	6519	6241
mRNA	Radioprotectant-only	489	561	689
	Radioprotectant+IR	1788	1896	1636

Fold-changes were set to 2.0 (miRNAs) or 3.0 (lncRNAs and mRNAs).

CBLB502, a truncated derivative of the *Salmonella* flagellin protein, shows high radioprotective efficacy by binding to Toll-like receptor 5 of target cells and activating nuclear factor-κB signaling, which modulates the expression of numerous genes, including apoptosis inhibitors, scavengers of reactive oxygen species, and a spectrum of cytokines.[5-7] Nilestriol, an estrogen derivative, is a long-acting radiomitigator that acts systemically by accelerating the post-irradiation restoration of the radiosensitive tissues by activating pro-inflammatory signaling pathways and stimulating hematopoietic system regeneration.[3,8,9] The estrogenic activity of nilestriol is 230-fold and 510-fold greater than that of estriol when used intravenously and orally, respectively.[10]

Nevertheless, the complex mechanisms underlying the altered biological processes after irradiation and regulation by radioprotectants are not completely understood. In addition to the mRNAs, increasing evidence has pointed to significant changes in the expression levels of non-coding RNAs, such as microRNAs (miRNAs) or long non-coding RNAs (lncRNAs) upon IR and their critical roles in the cellular response to IR.

MiRNAs are endogenous small non-coding RNAs (21–23 nucleotides) that act as post-transcriptional modulators of gene expression by either inhibiting mRNA translation or inducing mRNA degradation.[11] A single miRNA can influence the expression of up to 1,000 genes and influence various cellular processes, such as cell differentiation, proliferation, and apoptosis.[12-15] IR can alter miRNA expression both *in vivo* and *in vitro*, depending on cell type, radiation dose, and exposure time.[16-21] Although several studies have suggested that miRNAs regulate the DNA damage response (DDR) at the transcriptional level[22,23] in a p53-dependent manner,[24,25] little is known about how miRNAs respond to IR.

Recently, lncRNAs have gained attention and been related to multiple cellular processes, including DDR.[26,27] Several lncRNAs play key roles in p53-dependent DDR and immune regulation.[28-30] Besides other lncRNAs reportedly regulate apoptosis and the cell cycle after DNA damage induction, DNA repair pathways, and DDR protein modification.[31] Despite significant progress in understanding the roles of lncRNAs in the last few years, their specific functions and regulation networks are unclear.

Thus, we aimed to compare the effects of radioprotectants (amifostine, CBLB502, and nilestriol) on whole-genome expression profiles of miRNAs, lncRNAs, and mRNAs in the bone marrow of mice after whole-body high-dose irradiation, using microarray analysis. Functional and pathway analyses of the differentially expressed RNAs before and after IR and between treatment groups were performed to determine the potential molecular mechanisms contributing to their radioprotective effects. Our findings provide fundamental insights into the mechanisms of radioprotection while highlighting the roles of non-coding and coding RNAs as potential targets for the treatment and prevention of IR-induced damage.

Material and Methods

Mice and Irradiation

Male C57BL/6 mice (6–8 weeks old) were purchased from SPF (Beijing) Biotechnology Co. Ltd. (Beijing, China) and randomly divided into 8 groups: phosphate-buffered saline (PBS) control, IR-only, radioprotectant-only (amifostine, CBLB502, or nilestriol), and radioprotectant + IR (3 mice per group). Animals were irradiated using a ^{60}Co γ-ray radiation source at the Beijing Institute of Radiation Medicine (Beijing, China) with a total dose of 8.0 Gy at a dose rate of 1.29 Gy/min. All animal experiments were approved by the Institutional Animal Care and Use Committee of Academy of Military Medical Sciences, Beijing, China (permit number: IACUC of AMMS-13-2015-011).

Drug Administration

Nilestriol (Beijing Institute of Radiation Medicine) was dissolved in PBS and administered orally at 10 mg/kg at 48 h pre-irradiation. Amifostine (Dalian Merro Pharmaceutical Factory, Dalian, China) and CBLB502 (Beijing Institute of Radiation Medicine) were dissolved in PBS and administered intraperitoneally at 0.5 h pre-irradiation at 150 and 0.2 mg/kg, respectively. The PBS was administered to the control animals.

RNA Isolation and Microarray Analysis

At 12 h post-irradiation, bone marrow cells were collected and pooled from 3 animals per group and single-cell suspensions were prepared. RNA isolation and microarray analysis were performed as described previously.[32] For miRNA analysis, the miRNA was labeled with the miRNA Complete Labeling and Hyb Kit (Agilent Technologies) and hybridized to the Agilent Mouse miRNA microarray (8 × 60 K) V19.0 (Agilent Technologies). For lncRNA and mRNA analyses, total RNA was amplified and labeled with a Low Input Quick Amp Labeling Kit (Agilent Technologies) and then hybridized to the Agilent Mouse lncRNA 4 × 180 K microarray (Agilent Technologies). Raw data were normalized by the Quantile algorithm Gene Spring Software 11.0 (Agilent Technologies).
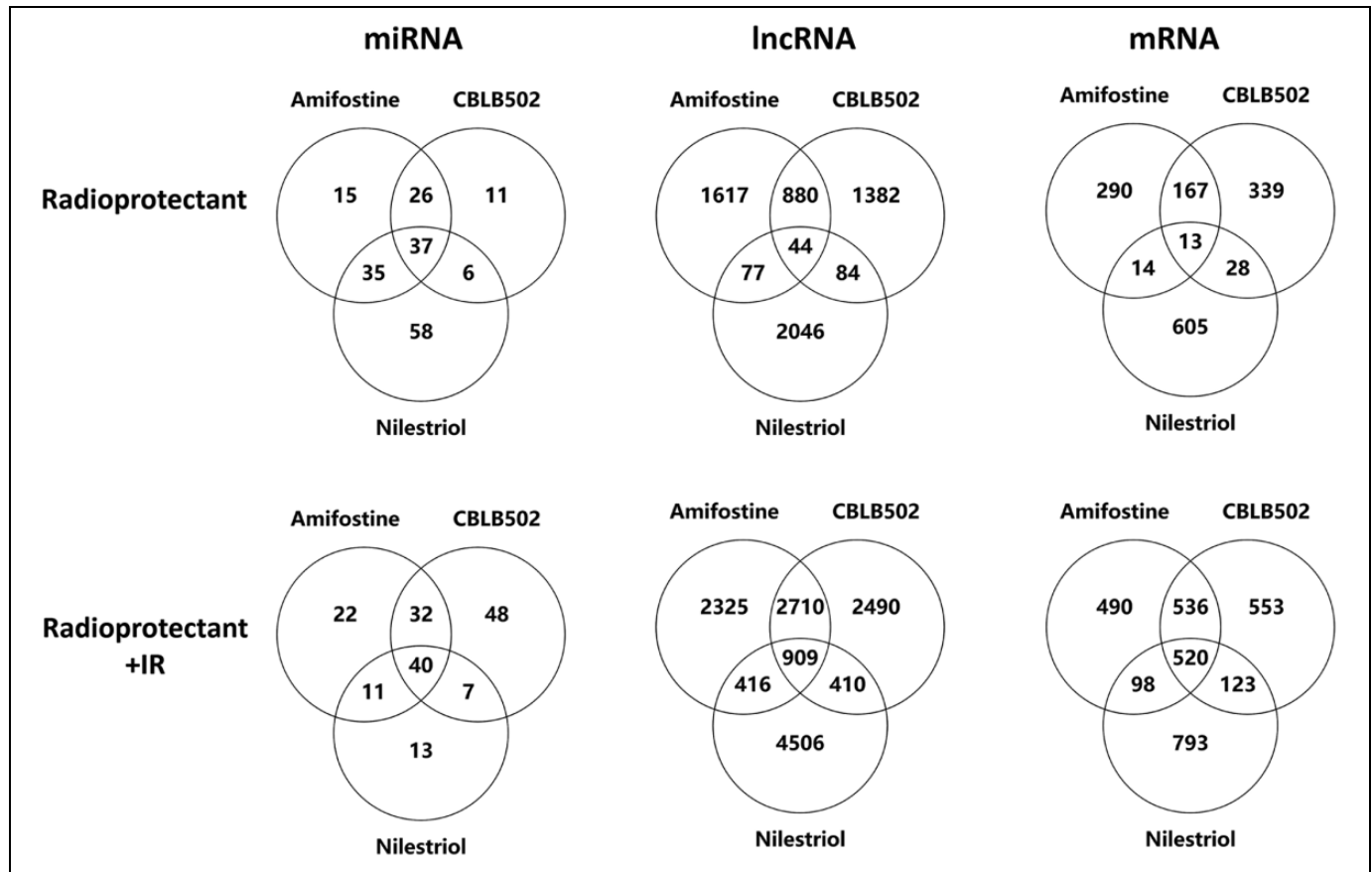
Figure 1. Venn diagram showing the common miRNAs, lncRNAs, and mRNAs induced by radioprotectant-only and pre-treatment with radioprotectants before ionizing radiation (radioprotectant + IR). The fold-change of miRNAs was set to 2.0; the fold-changes of lncRNAs and mRNAs were set to 3.0.

Data Processing

Data processing was performed using the SBC Analysis System from Shanghai Biotechnology Corporation (Shanghai, China). miRNAs showing a fold-change (FC) > 2.0 and lncRNAs or mRNAs showing an FC > 3.0 between IR and/or radioprotectant-treated and PBS-treated samples were considered significantly differentially expressed. The intersecting set of miRNAs, lncRNAs, and mRNAs between the different groups was analyzed using Venny 2.1.0 (http://bioinfogp.cnb. csic.es/tools/venny/). The microarray data were deposited in the NCBI Gene Expression Omnibus (GEO) database (accession no. GSE137013).

Functional Annotation

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of differentially expressed mRNAs were conducted using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) webtool.[33,34] Pathway enrichment and biological process analyses with GO-SLIM annotation for miRNAs were conducted in the radioprotectant treatment groups using DIANA Tools mirPath V3 (http://diana.imis.athena-innovatio

n.gr).[35] The GO terms and KEGG pathways with p values of <0.05 in the amifostine group were considered as significantly enriched compared to all radioprotectant groups.

Construction of the Protein-Protein Interaction Network

The protein-protein interaction (PPI) network for the treatment groups was constructed using STRING (http://string-db.org).[36] The representative subnetwork containing nodes with high levels of interconnection was further derived from the PPI network at a confidence score of 0.900. The network graphs were produced using Cytoscape 3.7.1 (The Cytoscape Consortium, San Diego, CA, USA).[37]

Real-Time PCR Analysis

Real-time PCR for histone cluster genes was performed on the Stratagene Mx3000P (Agilent) using the TB Green Premix Ex Taq[TM] kit (Takara, Shiga, Japan) as previously described.[21] The primers sequences are listed in Supplementary Table 1. The endogenous glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene was used as an internal control. Fold differences for each of the genes were calculated using the $2^{-\Delta\Delta CT}$ method.[38]
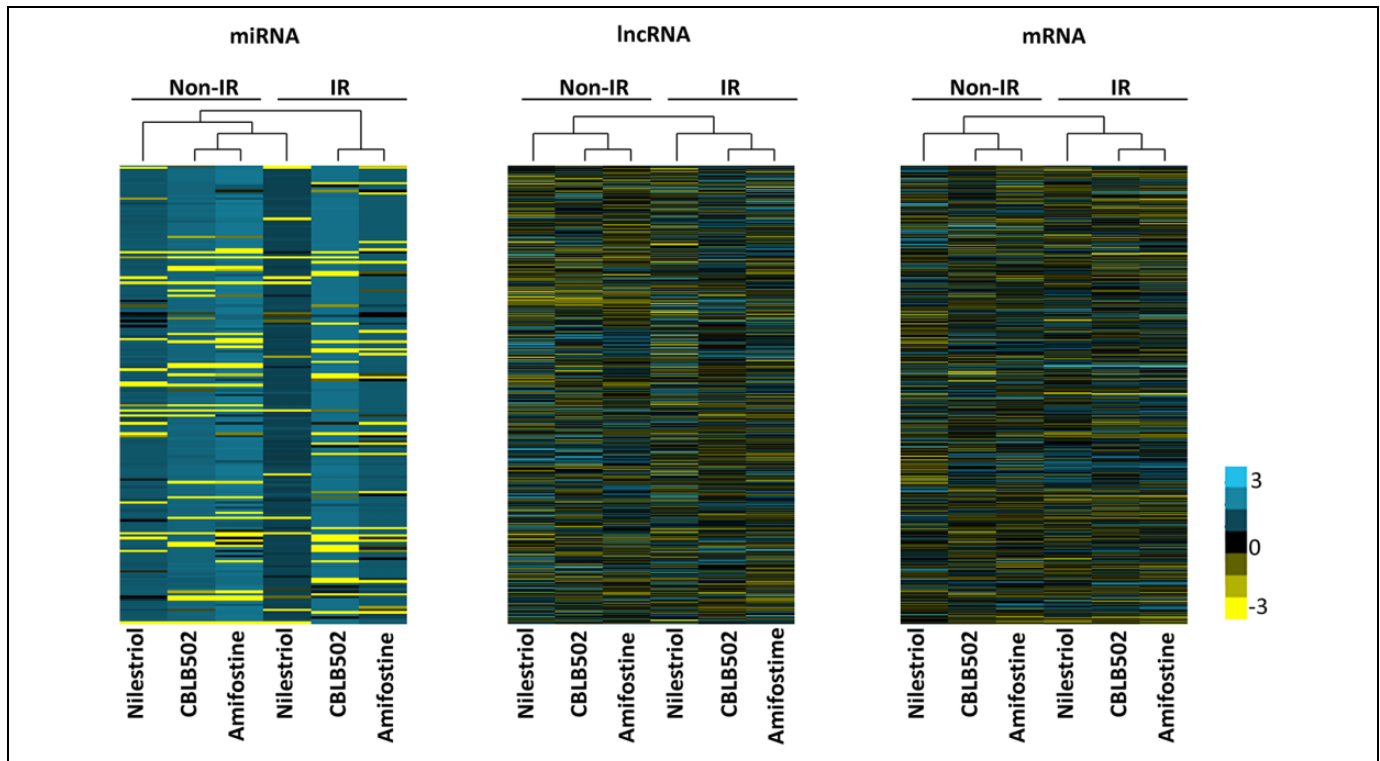
Figure 2. Hierarchical clustering of differentially expressed miRNAs, lncRNAs, and mRNAs in mouse bone marrow. Mice were pretreated with amifostine, CBLB502, and nilestriol or PBS, followed by whole-body irradiation at 8.0 Gy or non-irradiated sham treatment. Bone marrow was removed from the mice 12 h after radiation for miRNA and RNA microarray analysis.

Survival and Body Weight Analysis Following Radioprotectant Combination

Mice (30 per group) were administered 75 mg/kg amifostine and 0.02 mg/kg CBLB502[5] intraperitoneally at 0.5 h pre-irradiation, or 5 mg/kg nilestriol orally at 48 h pre-irradiation, or a combination of amifostine + nilestriol or CBLB502 + nilestriol. Mice were irradiated and survival time was recorded daily for 30 days, and body weights of surviving mice were recorded on day 30.

Statistical Analysis

Data are presented as means $\pm$ SEM. The statistical analyses were performed using Prism 7 software (GraphPad, San Diego, CA, USA). Real-time PCR results were analyzed by 1-way analysis of variance (ANOVA) followed by the Dunnett post hoc test. The Kaplan–Meier survival curves were compared using the log-rank test. The body weights were compared using 1-tailed Student's t-test. $p < 0.05$ was considered significant.

Results

Radioprotectants Induce Numerous Differentially Expressed RNAs After Irradiation

As shown in Table 1, the numbers of differentially expressed lncRNAs and mRNAs increased by 2.42–2.77- and 2.37–3.66-folds, respectively, in irradiated mice pre-administered amifostine, CBLB502, and nilestriol, compared to the numbers in radioprotectant-only groups. However, there were no differences in the numbers of differentially expressed miRNAs between the radioprotectant-only groups and radioprotectant + IR groups (Table 1). To understand the mechanism of radioprotectant-induced gene expression, we focused on RNAs commonly regulated by the drugs. We found that the intersecting sets of RNAs regulated by all 3 radioprotectants were increased by 20.66- and 40.00-fold in the numbers of altered lncRNAs and mRNAs in the radioprotectant + IR groups compared to those in the radioprotectant-only groups, respectively. However, there was only a 1.08-fold increase in the numbers of miRNAs altered in the radioprotectant + IR groups (Figure 1). These results support the essential roles of these commonly regulated radioprotectant-induced RNAs after irradiation.

Amifostine and CBLB502 Treatments Result in Similar RNA Profiles

Overall, there was a similar expression pattern of altered RNAs in mice pretreated with amifostine and CBLB502, regardless of irradiation exposure, which differed from the pattern of change in RNAs induced by nilestriol (Figure 2). Specifically, most altered miRNAs, lncRNAs, and mRNAs were either co-upregulated or co-downregulated (71, 3606, and 1051, respectively) between the CBLB502 and amifostine groups, with only a few showing opposite trends of change

Table 2. Comparison of Regulation of Differentially Expressed miRNAs, lncRNAs, and mRNAs Between Any 2 Radioprotectant Pretreatments Before IR.

Array	Groups	Total number	Both upregulated	Both downregulated	Opposite regulated
miRNA	Amifostine∩Nilestriol	51	24	22	5
	Amifostine∩CBLB502	72	35	36	1
	CBLB502∩Nilestriol	47	20	20	7
LncRNA	Amifostine∩Nilestriol	1325	477	630	218
	Amifostine∩CBLB502	3619	1906	1700	13
	CBLB502∩Nilestriol	1319	363	726	230
mRNA	Amifostine∩Nilestriol	620	217	374	29
	Amifostine∩CBLB502	1059	279	772	8
	CBLB502∩Nilestriol	646	198	413	35

Opposite regulation means upregulation in 1 group and downregulation in another group or vice versa. Fold-changes were set to 2.0 (miRNAs) or 3.0 (lncRNAs and mRNAs).
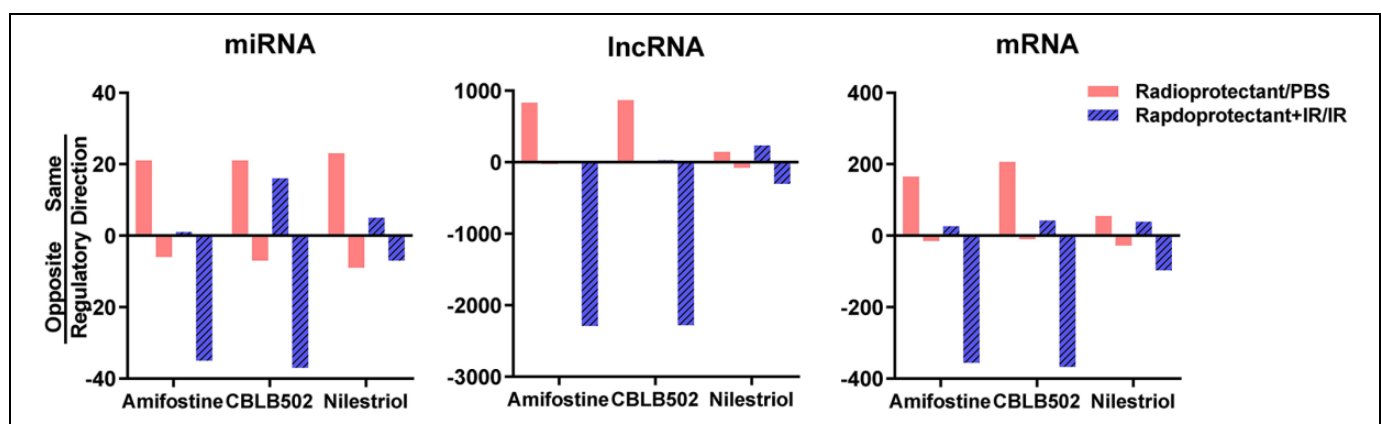

Figure 3. Differential regulation of IR-induced RNAs by radioprotectant-only and radioprotectant + IR treatment. First, the differentially expressed RNAs in the radioprotectant-only group were compared to those in the PBS group. The overlapping RNAs that were differentially expressed in the same or the opposite regulatory directions were assessed between the radioprotectant-only/PBS and IR-only groups. Similarly, the differentially expressed RNAs in the radioprotectant + IR group were compared to those in the IR-only group. The numbers of RNAs for each differential regulatory direction were assessed by overlapping the differentially expressed RNAs between the radioprotectant + IR/IR and IR-only groups. Fold-changes were set to 2.0 (miRNAs) and 3.0 (lncRNAs and mRNAs). Bars represent the sum of the gene count values shown in Supplementary Table 2.

(1.39%, 0.36%, and 0.76%, respectively) (Table 2). In contrast, although there were several co-regulated miRNAs, lncRNAs, and mRNAs between the amifostine and nilestriol groups (46, 1107, and 591, respectively), higher percentages of altered RNAs were found in the opposite regulatory direction (9.80%, 16.45%, and 4.68%, respectively), indicating the different regulatory patterns between these 2 radioprotectants. Similarly, 14.89%, 17.44%, and 5.42% of the respective RNAs in the opposite regulatory direction showed different patterns between the CBLB502 and nilestriol groups (Table 2). Taken together, these results suggested that amifostine and CBLB502 have a more similar regulation mechanism, which is distinct from that of nilestriol, during radioprotection.

RNA Protection Against Irradiation Occurs During Radioprotection But Not Before Irradiation

To further investigate the role of the radioprotectants in IR-induced damage, we comparatively analyzed the differentially expressed RNAs between radioprotectant + IR and the IR-only groups (radioprotectant + IR/IR, i.e. FC of RNA expression in the radioprotectant + IR group *vs.* that in the IR-only group > 2). Consequently, we identified the effects on RNA expression that were triggered by radioprotectant treatments but excluded those effects induced by IR. Then we analyzed the regulatory direction of the differentially expressed RNAs in radioprotectant + IR/IR and radioprotectant/PBS groups against that of differentially expressed RNAs in the IR-only group to reveal the effects of pre-treatment with radioprotectants on IR-induced RNAs in non-irradiated mice and irradiated mice without IR-induced RNA changes. As shown in Figure 3 and Supplementary Table 2, most differentially expressed RNAs in the radioprotectant/PBS groups showed the same regulatory direction in the IR-only group (i.e., IR-induced RNAs and radioprotectant-induced RNAs were either co-upregulated or co-downregulated), but few RNAs showed the opposite regulatory direction (i.e., upregulated in IR-only group but downregulated in radioprotectant-only group or vice

Table 3. Selected GO Enrichment Analysis of Histone Cluster Genes in Downregulated DEGs of IR-Only or Radioprotectant + IR Groups.

	GO term	Description	IR downregulated genes			
BP	GO:0006334	nucleosome assembly	HIST1H1D, SHPRH, RSF1, HIST1H1A, HIST1H2BG, HIST1H3A, MCM2, HIST3H2BA, SMARCA4			

			Radioprotectant + IR downregulated genes			
	GO Term	Description	Common Genes	Amifostine	CBLB502	Nilestriol
BP	GO:0006334	nucleosome assembly	HIST1H4 M, HIST4H4, HIST1H4 K, HIST1H2BM, HIST1H4C, HIST1H4D, HIST1H4 J, HIST3H2BA, HIST1H2BA, HIST1H2BB, HIST1H1E, HIST1H1B, HIST1H1A, HIST1H2BG, HIST2H4, HIST2H2BB, HIST1H3A, HIST1H3C, HIST1H3F, HIST1H3G, SMARCA4, HIST1H3I	H1FX, H2AFB1, HIST1H4I, HIST2H3B, HIST3H2BB-PS, MCM2, HIST1H1 T, HIST1H3D, CHAF1B	H1FX, HIST1H4I, HIST2H3B, HIST3H2BB-PS, HIST1H1 T, HIST1H3D, CHAF1B	MCM2
BP	GO:0032776	DNA methylation on cytosine	HIST1H4 M, HIST2H3B, HIST1H4 K, HIST2H4, HIST1H3A, HIST1H3C, HIST1H4C, HIST1H4D, HIST1H3F, HIST1H3G, HIST1H4 J, HIST1H3I	UHRF1, HIST1H3D, HIST1H4I	HIST4H4, HIST1H3D, HIST1H4I	UHRF1, HIST4H4
BP	GO:0006335	DNA replication-dependent nucleosome assembly	HIST2H3B, HIST1H4 M, HIST4H4, HIST1H4 K, HIST1H3A, HIST1H3C, HIST1H4C, HIST1H4D, HIST1H3F, HIST1H3G, HIST2H4, HIST1H4 J, HIST1H3I	HIST1H3D, HIST1H4I, CHAF1B	RBBP4, HIST1H3D, HIST1H4I, CHAF1B	RBBP4
BP	GO:0006336	DNA replication-independent nucleosome assembly	HIST1H4 M, HIST4H4, HIST1H4 K, HIST1H4C, HIST1H4D, HIST1H4 J, HIST2H4	HIST1H4I	RBBP4, HIST1H4I	RBBP4
BP	GO:0006352	DNA-templated transcription, initiation	HIST1H4 M, HIST4H4, HIST1H4 K, HIST1H4C, HIST1H4D, HIST1H4I, HIST1H4 J, HIST2H4	/	/	/
BP	GO:0045815	positive regulation of gene expression, epigenetic	HIST2H3B, HIST1H4 M, HIST4H4, HIST1H4 K, HIST2H4, HIST1H3A, HIST1H3C, HIST1H4C, HIST1H4D, HIST1H3F, HIST1H3G, HIST1H4 J, HIST1H3I	HIST1H3D, HIST1H4I	HIST1H3D, HIST1H4I	/
CC	GO:0000788	nuclear nucleosome	HIST1H2BA, HIST2H3B, HIST1H2BB, HIST1H2BG, HIST1H2BM, HIST2H2BB, HIST1H3A, HIST1H3C, IRF4, HIST1H3F, HIST1H3G, TCF3, HIST3H2BA, HIST1H3I	HIST3H2BB-PS, H2AFB1, HIST1H3D	HIST3H2BB-PS, HIST1H3D	/
CC	GO:0000228	nuclear chromosome	HIST2H3B, HIST1H4 M, HIST4H4, HIST1H4 K, HIST2H4, HIST1H3A, HIST1H3C, HIST1H4C, HIST1H4D, HIST1H3F, HIST1H3G, HIST1H4 J, HIST1H3I	HIST1H3D, HIST1H4I	HIST1H3D, HIST1H4I	SPO11
MF	GO:0042393	histone binding	HIST1H4 M, HIST4H4, HIST1H4 K, TONSL, RAG1, LEF1, HIST2H3B, HIST2H4, HIST1H4C, HIST1H4D, HIST1H4 J, HIST1H3A, USP49, HIST1H3C, HIST1H3F, HIST1H3G, SMARCA4, HIST1H3I	CD1D1, HIST1H4I, MCM2, UHRF1, BRDT, HIST1H3D, CHAF1B, APBB1	CD1D1, BRDT, HIST1H3D, HIST1H4I, APBB1, CHAF1B	MCM2, UHRF1
MF	GO:0031492	nucleosomal DNA binding	HIST2H3B, HIST1H3A, HIST1H3C, HIST1H3F, HIST1H3G, HIST1H3I, SMARCA4	HIST1H3D	RBBP4, HIST1H3D	RBBP4

IR: ionizing radiation.
Fold-changes of DEGs were set to 3.0.

Table 4. Selected GO Enrichment Analysis of Radioprotectant + IR Groups.

GO term	Description	Amifostine		CBLB502		Nilestriol	
		Count	P value	Count	P value	Count	P value
Upregulated genes							
GO:0045087	innate immune response	25	1.98E-6	32	2.63E-9	/	/
GO:0006955	immune response	16	4.19E-4	20	1.48E-5	19	4.14E-4
GO:0035458	cellular response to interferon-beta	7	1.56E-4	7	2.97E-4	/	/
GO:0071347	cellular response to interleukin-1	9	2.01E-4	7	0.0095	8	0.0056
GO:0006954	inflammatory response	19	3.21E-4	22	4.02E-5	15	0.0801
GO:0042771	intrinsic apoptosis response to DNA damage by p53	6	3.51E-4	5	0.0050	5	0.0091
GO:0070374	positive regulation of ERK1 and ERK2 cascade	13	4.29E-4	12	0.0038	10	0.0672
GO:0032874	positive regulation of stress-activated MAPK cascade	4	0.0071	/	/	/	/
GO:0000165	MAPK cascade	/	/	6	0.0199	6	0.0376
GO:0035457	cellular response to interferon-alpha	3	0.0232	4	0.0022	/	/
GO:0071346	cellular response to interferon-gamma	6	0.0118	7	0.0043	/	/
GO:0071356	cellular response to tumor necrosis factor	/	/	9	0.0035	6	0.0096
GO:2000778	positive regulation of interleukin-6 secretion	/	/	/	/	3	0.0828
GO:0030818	negative regulation of cAMP biosynthetic process	/	/	4	0.0085	4	0.0135
GO:0010862	positive regulation of SMAD phosphorylation	/	/	4	0.0944	5	0.0394
Downregulated genes							
GO:0000122	negative regulation of transcription of RNA polymer. II	46	0.0055	/	/	30	0.0560
GO:0030890	positive regulation of B cell proliferation	/	/	/	/	7	0.0015
GO:0032725	positive regulation of GM-CSF factor production	3	0.0409	/	/	/	/
GO:0042102	positive regulation of T cell proliferation	9	0.0049	8	0.0184	9	5.21E-4
GO:0042130	negative regulation of T cell proliferation	/	/	/	/	5	0.0029

Fold-changes of DEGs were set to 3.0.

versa). Specifically, there were 21/6, 21/7, and 23/9 miRNAs; 830/24, 868/18, and 146/79 lncRNAs; and 165/15, 206/10, and 55/28 mRNAs showing the same/opposite direction of regulation upon induction by amifostine, CBLB502 and nilestriol alone, respectively (Supplementary Table 2). However, many more differentially expressed RNAs showed opposite regulatory direction against IR-only treatment in the radioprotectant + IR/IR groups as compared to those in radioprotectant/PBS groups. Specifically, 1/35, 2/37, and 5/7 miRNAs; 12/2187, 28/2279, and 60/257 lncRNAs; and 26/356, 42/367, and 39/97 mRNAs showed the same/opposite regulatory direction by amifostine, CBLB502 and nilestriol, respectively, in irradiated mice (Supplementary Table 2). These results suggested that miRNAs, lncRNAs, and mRNAs might protect against IR-induced damage by inverting the expression profile of IR-induced RNAs during radioprotection but not before irradiation.

Radioprotectants Commonly Downregulate the Expression of Histone Genes

To investigate the global mechanism during radioprotection, we next focused on the common RNAs regulated by the 3 radioprotectants. The intersecting set of differentially expressed miRNAs in radioprotectant + IR groups was functionally annotated and shown in Supplementary Table 3 and Table 4. The radioprotectants induced miRNAs related to similar KEGG pathways and GO functions with variable gene counts and p-values. Meanwhile, the GO and KEGG pathway analysis of downregulated mRNAs (differentially expressed genes, DEGs) in the radioprotectant + IR groups, demonstrated the involvement of similar biological processes, including nucleosome assembly, DNA methylation of cytosine, DNA replication-dependent and DNA replication-independent nucleosome assembly, and DNA-templated initiation of transcription (Figure 4; see the full list in the Supplementary Files).

PPI network analysis revealed a prominent subnetwork mainly consisting of histone and related genes in the intersecting set of DEGs downregulated by the radioprotectants, but not in radioprotectant-upregulated DEGs (Supplementary Figure 1 and Figure 2). To confirm this, we selected some GO terms enriched from the intersecting set of downregulated DEGs by radioprotectants to examine their relationship with histones related genes. However, for IR-only-induced DEGs, only 9 histones and related genes were identified among the downregulated DEGs related to of nucleosome assembly, which was fewer than that observed for the radioprotectant + IR-induced downregulated DEGs (Table 3, Supplementary Tables 5 and 6), and there were no histone genes among the upregulated IR-only or radioprotectant + IR-induced DEGs (Supplementary Table 5 and 7). Interestingly, no DEGs in the radioprotectant-only groups were related to the regulation of histone genes (Supplementary Figures 3-5, Supplementary Table 8). We further verified the downregulation of the expression of selected histone cluster genes for histones H1–H4 in irradiated mice by RT-PCR, whereas some were generally
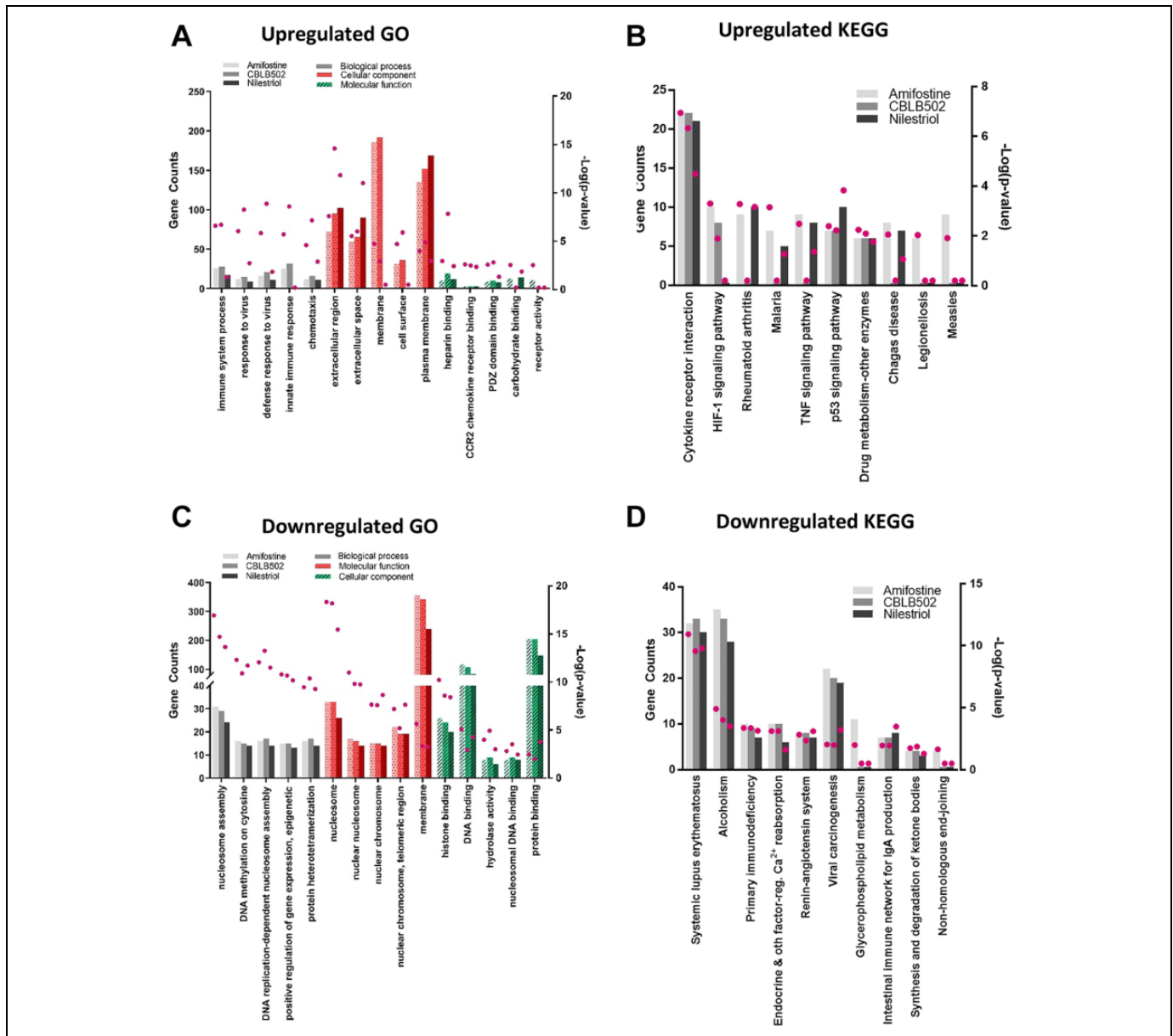
Figure 4. GO enrichment and KEGG pathway analysis of DEGs in radioprotectant treatment. Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of upregulated DEGs (A, B) and downregulated DEGs (C, D) associated with pretreatment with radioprotectants in irradiated mice. The gene counts (histogram) and –log (p-value) (pink dot) were set to 0 when the GO terms or KEGG pathways were not in the list of GO or KEGG enrichment results. Light color: amifostine, medium color: CBLB502, dark color: nilestriol.

reversed by amifostine and nilestriol but not CBLB502. However, they were downregulated in non-irradiated mice treated with amifostine and CBLB502 but not nilestriol (Figure 5).

Moreover, all radioprotectant + IR groups showed the activation of commonly-modulated, specific regulatory pathways, such as immune response, cellular response to IL-1, p53-mediated intrinsic apoptotic signaling pathway, and positive regulation of the ERK1 and ERK2 cascade. Other processes and pathways involved in either 1 or 2 of the radioprotectant treatments include IFN signaling, TNF, cAMP biosynthesis process, and SMAD phosphorylation (Table 4).

Nilestriol Demonstrates Complementation of Amifostine and CBLB502 Effects

Either amifostine or CBLB502 and nilestriol showed lower overlap for differentially expressed RNAs having the same regulatory direction and an increase in the overlap for differentially expressed RNAs having opposite regulatory directions, indicating that these radioprotectants induced different radioprotective mechanisms involving some unique pathways (Figure 6A, Supplementary Table 9). Therefore, we examined the potential complementary effects of combining radioprotectants. Although
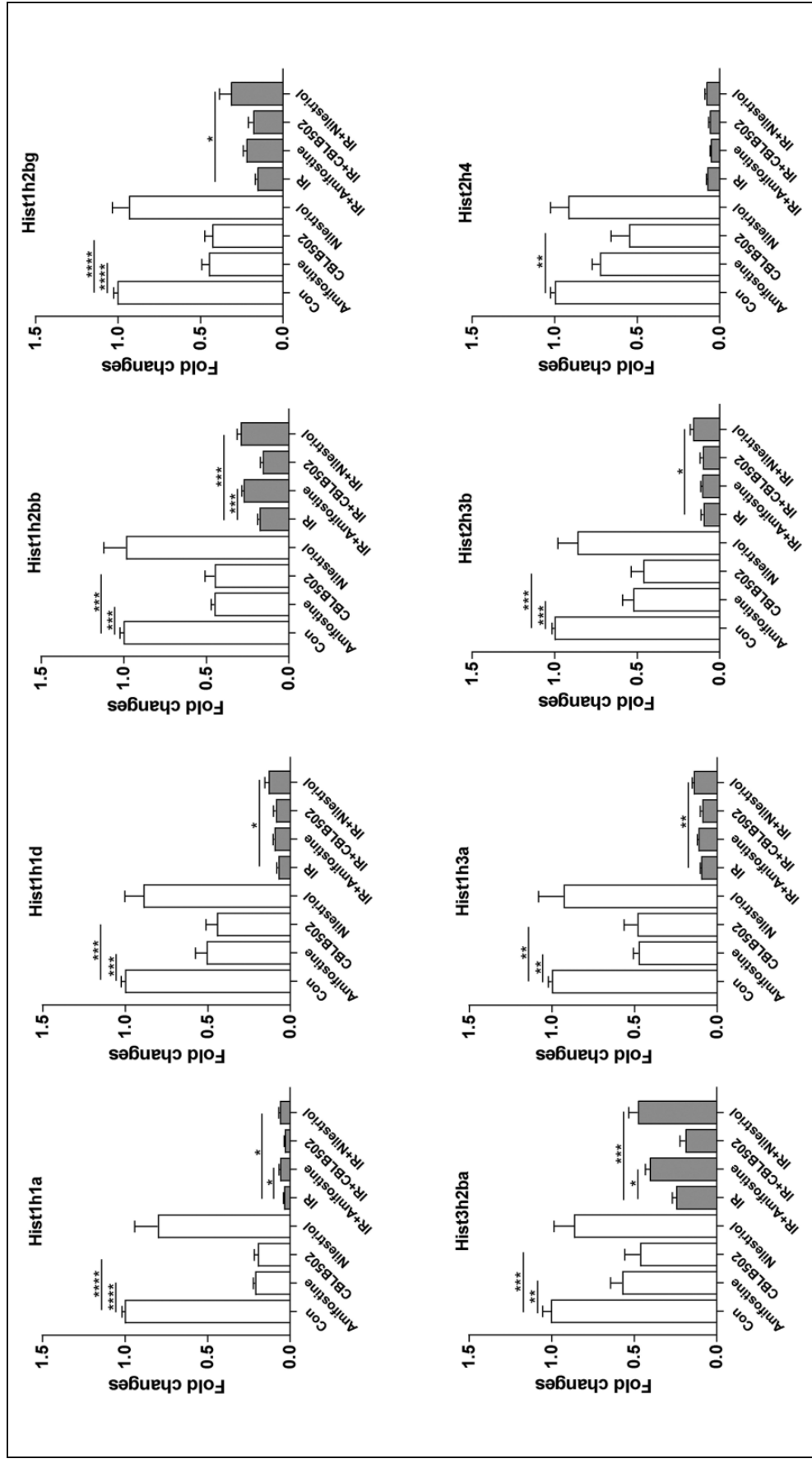
Figure 5. Validation of expression of selected histone cluster genes. Total RNA was extracted from the bone marrow of irradiated or non-irradiated mice with or without radio-protectant pretreatment, followed by real-time PCR analysis of *Hist1h1a, Hist1h1d, Hist1h2bb, Hist1h2bg, Hist3h2ba, Hist1h3a, Hist3h3b, and Hist2h4* genes. *Gapdh* was used as an internal control. Con: Control, IR: irradiation. The experiments were performed using 3 mice, and data are presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.
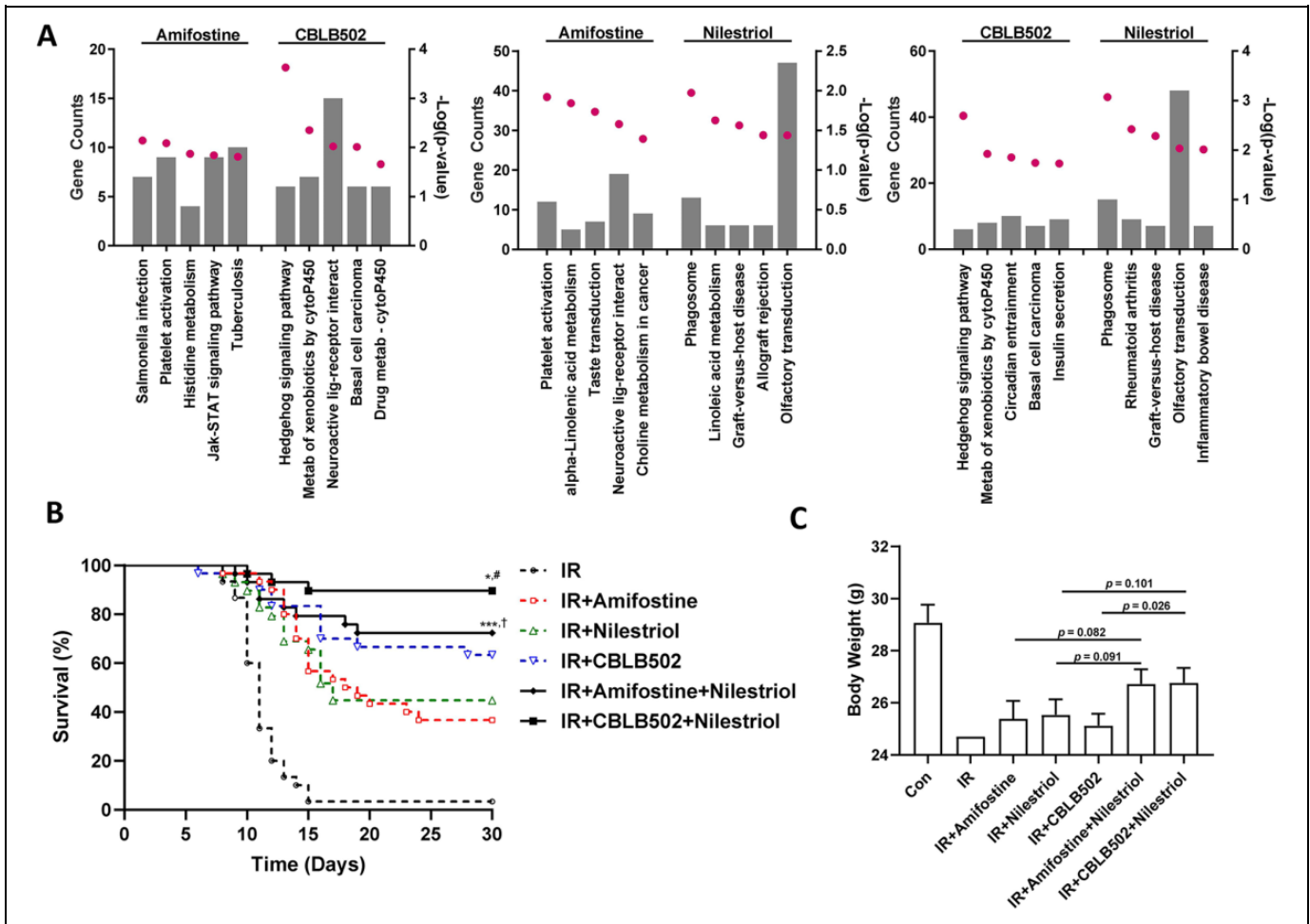
Figure 6. Complementary effects of combined radioprotectants during radioprotection. (A) KEGG analysis of DEGs in irradiated mice exclusively induced by radioprotectant-only in the overlap of amifostine + CBLB502 (left), amifostine + nilestriol (middle), and CBLB502 + nilestriol (right). Top 5 KEGG items are shown. (B) Kaplan–Meier survival curve of C57BL/6 irradiated mice pretreated with 75 mg/kg amifostine, 0.02 mg/kg CBLB502, 5 mg/kg nilestriol, combination of amifostine + nilestriol, or combination of CBLB502 + nilestriol. Statistical analysis of survival was performed by the log-rank test (n = 30 per group, [#] $p < 0.05$ for amifostine *vs.* amifostine + nilestriol; [*] $p < 0.05$ for nilestriol *vs.* amifostine + nilestriol; [***] $p < 0.001$ for nilestriol *vs.* CBLB502 + nilestriol, [†] $p < 0.05$ for CBLB502 *vs.* CBLB502 + nilestriol). (C) Body weights of surviving mice on day 30. Statistical analysis was performed by 1-tailed Student's *t*-test; *p* values are indicated.

pre-treatment of irradiated mice with a low-dose combination of radioprotectants offered limited survival benefits, the combination of low-dose amifostine and nilestriol, or CBLB502 and nilestriol showed higher radioprotective effects than a single administration of corresponding radioprotectants (all $p < 0.05$) (Figure 6B). Moreover, on day 30, the body weights of irradiated mice pretreated with combinations of radioprotectants showed an increasing trend. Most differences were not significant, except the comparison of groups of CBLB502 *vs.* CBLB502 + nilestriol ($p = 0.026$) (Figure 6C). Thus, the lower overlap of differentially expressed RNA profiles suggested a complementary effect between radioprotectants.

Discussion

The expression profiles of non-coding RNAs and coding RNAs have been described in irradiating human cells at low and high

doses of IR[20,29]; however, the changes in the RNA levels response to radioprotectant treatment upon IR were unclear. In this study, we showed that pre-treatment with different radioprotectants induced the differential expression of a more extensive set of miRNAs, lncRNAs, and mRNAs in the mouse bone marrow after exposure to high-dose γ-rays. The amifostine and CBLB502 pre-treatments resulted in similar patterns of differentially expressed miRNAs, lncRNAs, and mRNAs, which differed from those induced with nilestriol, in both IR and non-IR exposed mice. Further analysis of the intersecting set of differentially expressed mRNAs suggested that radioprotectants downregulate histone gene expression and the nucleosome assembly process under IR stimulation. Importantly, RNAs differentially expressed in irradiated mice pretreated with radioprotectants showed opposite regulatory direction to those of RNAs differentially expressed in mice in the IR-only group. Pre-treatment of irradiated mice with a

low-dose of either amifostine and nilestroil or CBLB502 and nilestriol produced a better radioprotective effects.

Although the mechanisms of amifostine and CBLB502-mediated radioprotection are poorly understood,[39] they are both likely to have a cytoprotective effect and rapid response against IR-induced stress within several hours,[5,40] with achieving complex radiation-cytoprotective effects by interfering with genes involved in scavenging free radicals, cell cycle regulation, apoptosis, and inflammation.[6,7,41-46] However, nilestriol requires a longer time, usually hours or days, to exert its protective effects, and its post-irradiation mechanism does not involve primary radiation-induced cellular processes.[3,9] These differences in reaction time and mechanism may explain the similarities between amifostine and CBLB502 in terms of the influence on IR-induced RNAs, which was distinct from that of nilestriol. Nevertheless, the high similarity in the RNA profile of amifostine and CBLB502 groups provide insights into possible shared regulatory mechanisms of radioprotection.

Interestingly, our results also showed that radioprotectants mostly exert their radioprotective effects after irradiation but not at the pre-irradiation stage. The radioprotectants, particularly amifostine and CBLB502, induced opposite expression of RNAs to that induced by IR mostly in irradiated mice, but rarely in non-irradiated mice. Several reports have shown conflicting results regarding the effects of radioprotectants at the pre-irradiation stage. For instance, CBLB502 pre-treatment in non-irradiated mice increased IL-6 and G-CSF levels, which are pivotal to radioprotection[5,6]; however, pre-administration of amifostine significantly reduced radiation-induced increases in the excretion rates of prostaglandins and thromboxane in irradiated rats, but not in non-irradiated rats.[47] Thus, our results provide evidence at the genome-wide RNA expression level that some radioprotectants might not show prominent effects if administered before irradiation.

Notably, we found that IR reduces the expression levels of several histone cluster genes involved in nucleosome assembly, which is consistent with previous reports showing that IR downregulated histone expression in parallel with inhibition of DNA synthesis.[48,49] IR exposure induces highly lethal DNA damage resulting in local and global decondensation of chromatin,[50,51] which resulted in chromatin remodeling, including nucleosome disruption and histone eviction at double-strand break sites, which may facilitate DDR.[52,53] However, based on our results, pre-treatment with radioprotectants before IR may further downregulate histone cluster genes, including most histone proteins involved in nucleosome assembly, DNA methylation, and a variety of similar biological processes. This finding indicates that a rapid response of radioprotectants upon IR facilitates subsequent nucleosome remodeling inducing DDR and DNA repair. Our findings provide novel evidence that the downregulation of histone cluster genes is the primary biological response to IR-induced damage and that radioprotectants regulate this response following exposure regardless of their specific radioprotective mechanisms.

Finally, our data showed that differentially expressed RNA profiles of amifostine and CBLB502 had a lower overlap with those of nilestriol, indicating that they have different radioprotective mechanisms, which potentially exert complementary effects. Therefore, the unique RNAs and pathways induced by amifostine or CBLB502 might be complementary to those induced by nilestriol and vice versa. Nonetheless, extensive investigations are required to clarify the differences in their mechanisms.

Conclusion

Our comparison of the common miRNAs, lncRNAs, and mRNAs with altered expression in radioprotectant-pretreated mice with or without IR revealed different patterns between amifostine, CBLB502, and nilestriol. Pathway enrichment and biological process analyses of the genes commonly altered by all radioprotectants confirmed the involvement of these genes in nucleosome assembly and their antagonistic regulation against IR-induced RNAs while demonstrating a complementary radioprotection effect of radioprotectants as a combination. These findings will facilitate future research on the functional genes and regulatory mechanisms that mediate radioprotection.

Authors' Note

CG, XZ and YD conceived and designed the experiments. CG, FS, HF, YW, BT, BL, and JZ performed the experiments and data analysis. CG wrote the manuscript with comments provided by XZ and YD.

ORCID iD

Changhui Ge https://orcid.org/0000-0002-4215-3828

Supplemental Material

Supplemental material for this article is available online.

References

1. Singh VK, Newman VL, Romaine PL, Wise SY, Seed TM. Radiation countermeasure agents: an update (2011-2014). *Expert Opin Ther Pat*. 2014;24(11):1229-1255.

2. Singh VK, Garcia M, Seed TM. A review of radiation counter-measures focusing on injury-specific medicinals and regulatory approval status: part II. Countermeasures for limited indications, internalized radionuclides, emesis, late effects, and agents demonstrating efficacy in large animals with or without FDA IND status. *Int J Radiat Biol*. 2017;93(9):870-884.

3. Vasin MV. Comments on the mechanisms of action of radiation protective agents: basis components and their polyvalence. *Springerplus*. 2014;3:414.

4. Andreassen CN, Grau C, Lindegaard JC. Chemical radioprotection: a critical review of amifostine as a cytoprotector in radiotherapy. *Semin Radiat Oncol*. 2003;13(1):62-72.

5. Burdelya LG, Krivokrysenko VI, Tallant TC, et al. An agonist of Toll-like receptor 5 has radioprotective activity in mouse and primate models. *Science*. 2008;320(5873):226-230.

6. Krivokrysenko VI, Shakhov AN, Singh VK, et al. Identification of granulocyte colony-stimulating factor and interleukin-6 as candidate biomarkers of CBLB502 efficacy as a medical radiation countermeasure. *J Pharmacol Exp Ther*. 2012;343(2):497-508.

7. Li W, Ge C, Yang L, Russe HP. CBLB502, an agonist of Toll-like receptor 5, has antioxidant and scavenging free radicals activities in vitro. *Int J Biol Macromol*. 2016;82:97-103.

8. Thompson JS, Reilly RW, Crawford M, et al. The effect of estradiol and estriol on the survival of sublethally and lethally irradiated mice. *Radiat Res*. 1965;26(4):567-583.

9. Thompson JS, Simmons EL, Crawford MK, Severson CD. Studies on the mechanisms of estradiol-induced radioprotection. *Radiat Res*. 1969; 40(1):70-84.

10. Liao EY, Luo XH, Deng XG, et al. The effect of low dose nylestriol-levonorgestrel replacement therapy on bone mineral density in women with postmenopausal osteoporosis. *Endocr Res*. 2003;29(2):217-226.

11. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009;136(2):215-233.

12. Amiel J, de Pontual L, Henrion-Caude A. miRNA, development and disease. *Adv Genet*. 2012;80:1-36.

13. Fernandez-Hernando C, Ramirez CM, Goedeke L, Suárez Y. MicroRNAs in metabolic disease. *Arterioscler Thromb Vasc Biol*. 2013;33(2):178-185.

14. Deiuliis JA. MicroRNAs as regulators of metabolic disease: pathophysiologic significance and emerging role as biomarkers and therapeutics. *Int J Obes (Lond)*. 2016; 40(1):88-101.

15. Shenoy A, Blelloch RH. Regulation of microRNA function in somatic stem cell proliferation and differentiation. *Nat Rev Mol Cell Biol*. 2014;15(9):565-576.

16. Simone NL, Soule BP, Ly D, et al. Ionizing radiation-induced oxidative stress alters miRNA expression. *PLoS One*. 2009;4(7): e6377.

17. Dickey JS, Zemp FJ, Martin OA, Kovalchuk O. The role of miRNA in the direct and indirect effects of ionizing radiation. *Radiat Environ Biophys*. 2011;50(4):491-499.

18. Girardi C, De Pitta C, Casara S, et al. Analysis of miRNA and mRNA expression profiles highlights alterations in ionizing radiation response of human lymphocytes under modeled microgravity. *PLoS One*. 2012;7(2):e31293.

19. Lu J, Chen C, Hao L, Zheng Z, Zhang N, Wang Z. MiRNA expression profile of ionizing radiation-induced liver injury in mouse using deep sequencing. *Cell Biol Int*. 2016; 40(8):873-886.

20. Bugden M, Billing S, Mak KC, Norton F, Klokov D, Wang Y. Ionizing radiation affects miRNA composition in both young and old mice. *Int J Radiat Biol*. 2019;95(10):1404-1413.

21. Fu H, Su F, Zhu J, Zheng X, Ge C. Effect of simulated microgravity and ionizing radiation on expression profiles of miRNA, lncRNA, and mRNA in human lymphoblastoid cells. *Life Sci Space Res*. 2020;24:1-8.

22. Czochor JR, Glazer PM. microRNAs in cancer cell response to ionizing radiation. *Antioxid Redox Signal*. 2014;21(2):293-312.

23. Bertoli G, Cava C, Castiglioni I. MicroRNAs: new biomarkers for diagnosis, prognosis, therapy prediction and therapeutic tools for breast cancer. *Theranostics*. 2015;5(10):1122-1143.

24. Zhao L, Bode AM, Cao Y, Dong Z. Regulatory mechanisms and clinical perspectives of miRNA in tumor radiosensitivity. *Carcinogenesis*. 2012;33(11):2220-2227.

25. Goeman F, Strano S, Blandino G. MicroRNAs as key effectors in the p53 network. *Int Rev Cell Mol Biol*. 2017;333:51-90.

26. Terradas M, Martin M, Repulles J, Huarte M, Genescà A. Distinct sets of lncRNAs are differentially modulated after exposure to high and low doses of X rays. *Radiat Res*. 2016;186(6):549-558.

27. Beer L, Nemec L, Wagner T, et al. Ionizing radiation regulates long non-coding RNAs in human peripheral blood mononuclear cells. *J Radiat Res*. 2017;58(2):201-209.

28. Ouyang J, Hu J, Chen JL. lncRNAs regulate the innate immune response to viral infection. *Wiley Interdiscip Rev RNA*. 2016;7(1): 129-143.

29. Aryankalayil MJ, Chopra S, Levin J, et al. Radiation-induced long noncoding RNAs in a mouse model after whole-body irradiation. *Radiat Res*. 2018;189(3):251-263.

30. Zhou Y, He L, Liu XD, et al. Integrated analysis of lncRNA and mRNA transcriptomes reveals new regulators of ubiquitination and the immune response in silica-induced pulmonary fibrosis. *Biomed Res Int*. 2019;2019:6305065.

31. Su M, Wang H, Wang W, et al. LncRNAs in DNA damage response and repair in cancer cells. *Acta Biochim Biophys Sin (Shanghai)*. 2018;50(5):433-439.

32. Tian B, Fu H, Liu B, Zhu J, Zheng X, Ge C. Effects of amifostine pre-treatment on miRNA, lncRNA, and mRNA profiles in the hypothalamus of mice exposed to ^{60}Co gamma radiation. *Health Physics*. 2020: In Press.

33. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res*. 2000;28(1):27-30.

34. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44-57.

35. Vlachos IS, Zagganas K, Paraskevopoulou MD, et al. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. *Nucleic Acids Res*. 2015;43(W1): W460-466.

36. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res*. 2019;47(D1):D607-D613.

37. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003;13(11):2498-2504.

38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408.

39. Koukourakis MI, Giatromanolaki A, Zois CE, et al. Normal tissue radioprotection by amifostine via Warburg-type effects. *Sci Rep*. 2016;6:30986.

40. Choi NC. Radioprotective effect of amifostine in radiation pneumonitis. *Semin Oncol*. 2003;30(6 suppl 18):10-17.

41. Maurici D, Monti P, Campomenosi P, et al. Amifostine (WR2721) restores transcriptional activity of specific p53 mutant proteins in a yeast functional assay. *Oncogene*. 2001;20(27): 3533-3540.

42. Ormsby RJ, Lawrence MD, Blyth BJ, et al. Protection from radiation-induced apoptosis by the radioprotector amifostine (WR-2721) is radiation dose dependent. *Cell Biol Toxicol*. 2014;30(1):55-66.

43. Huang B, He T, Yao Q, et al. Amifostine suppresses the side effects of radiation on BMSCs by promoting cell proliferation and reducing ROS production. *Stem Cells Int*. 2019;2019:8749090.

44. Xu Y, Dong H, Ge C, et al. CBLB502 administration protects gut mucosal tissue in ulcerative colitis by inhibiting inflammation. *Ann Transl Med*. 2016;4(16):301.

45. Wang L, Zhang W, Ge CH, et al. Toll-like receptor 5 signaling restrains T-cell/natural killer T-cell activation and protects against concanavalin A-induced hepatic injury. *Hepatology*. 2017;65(6): 2059-2073.

46. Bai H, Sun F, Yang G, et al. CBLB502, a Toll-like receptor 5 agonist, offers protection against radiation-induced male reproductive system damage in mice. *Biol Reprod*. 2019;100(1): 281-291.

47. Donlon M, Steel L, Helgeson EA, Wolfe WW, Catravas GN. WR-2721 inhibition of radiation-induced prostaglandin excretion in rats. *Int J Radiat Biol Relat Stud Phys Chem Med*. 1985;47(2): 205-212.

48. Su C, Gao G, Schneider S, et al. DNA damage induces down-regulation of histone gene expression through the G1 checkpoint pathway. *EMBO J*. 2004;23(5):1133-1143.

49. Datta R, Weichselbaum R, Kufe DW. Ionizing radiation down-regulates histone H1 gene expression by transcriptional and post-transcriptional mechanisms. *Radiat Res*. 1993;133(2): 176-181.

50. Kruhlak MJ, Celeste A, Dellaire G, et al. Changes in chromatin structure and mobility in living cells at sites of DNA double-strand breaks. *J Cell Biol*. 2006;172(6):823-834.

51. Dellaire G, Kepkay R, Bazett-Jones DP. High resolution imaging of changes in the structure and spatial organization of chromatin, gamma-H2A.X and the MRN complex within etoposide-induced DNA repair foci. *Cell Cycle*. 2009;8(22):3750-3769.

52. Goldstein M, Derheimer FA, Tait-Mulder J, et al. Nucleolin mediates nucleosome disruption critical for DNA double-strand break repair. *Proc Natl Acad Sci U S A*. 2013;110(42): 16874-16879.

53. Hauer MH, Seeber A, Singh V, et al. Histone degradation in response to DNA damage enhances chromatin dynamics and recombination rates. *Nat Struct Mol Biol*. 2017;24(2):99-107.