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

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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, IncRNA, and mRNA expression patterns were similar with amifostine and CBLB502, but not nilestriol. The radioprotectants exhibited mostly opposite effects against IR-induced miRNAs, IncRNAs, 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, IncRNA, and mRNA, to explain the mechanism underlying radioprotection.

Keywords
RNA profile, bone marrow, ionizing radiation, radioprotectant, histone gene cluster

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
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-kB signaling, which modulates the expression of numerous genes, including apoptosis inhibitors, scavengers of reactive oxygen species, and a spectrum of cytokines. 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. The estrogenic activity of nilestriol is 230-fold and 510-fold greater than that of estriol when used intravenously or orally, respectively.

Nevertheless, the complex mechanisms underlying the altered biological processes after irradiation and regulation by radioprotectants are not completely understood. In addition to the miRNAs, 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.

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, and nilestriol), and radioprotectant + IR (3 mice per group). Animals were irradiated using a 60Co 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 Metro 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. 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).

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).
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™ 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
Survival and Body Weight Analysis Following Radioprotectant Combination

Mice (30 per group) were administered 75 mg/kg amifostine and 0.02 mg/kg CBLB502 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 ± 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.
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 other 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 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) or 3.0 (lncRNAs and mRNAs). Bars represent the sum of the gene count values shown in Supplementary Table 2.

### 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.

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

|         | Groups                        | Total number | Both upregulated | Both downregulated | Opposite regulated |
|---------|-------------------------------|--------------|------------------|--------------------|-------------------|
| miRNA   | Amifostine/CBLB502/Nilestriol | 51           | 24               | 22                 | 5                 |
|         | Amifostine/Nilestriol         | 72           | 35               | 36                 | 1                 |
|         | CBLB502/Nilestriol            | 47           | 20               | 20                 | 7                 |
| LncRNA  | Amifostine/CBLB502/Nilestriol | 1325         | 477              | 630                | 218               |
|         | Amifostine/CBLB502/Nilestriol | 3619         | 1906             | 1700               | 13                |
|         | CBLB502/Nilestriol            | 1319         | 363              | 726                | 230               |
| mRNA    | Amifostine/CBLB502/Nilestriol | 620          | 217              | 374                | 29                |
|         | Amifostine/CBLB502/Nilestriol | 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).

(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.
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, RSFI, HIST1H1A, HIST1H2BG, HIST1H3A, MCM2, HIST3H2BA, SMARCA4       |

| GO Term               | Description                                             | Common Genes       | Amifostine     | CBLB502      | Nilestriol   |
|-----------------------|---------------------------------------------------------|--------------------|----------------|--------------|--------------|
| BP GO:0006334         | nucleosome assembly                                     | HIST1H4 M, HIST4H4, HIST1H4 K, HIST1H4 J, HIST1H4 C, HIST1H2BM, HIST1H4C, HIST1H4 D, HIST1H4 F, HIST1H4 G, HIST1H4 H, HIST1H3A, HIST1H3C, HIST1H3D, HIST1H3F, HIST1H3G, HIST1H3I | MCM2             | UHRF1, HIST1H4I, HIST1H4J | RBBP4, HIST1H4D, HIST1H4I |
| BP GO:0032776         | DNA methylation on cytosine                            | HIST1H4 M, HIST2H3B, HIST1H4 K, HIST1H4 J, HIST1H4 H, HIST1H4I | MCM2             | UHRF1, HIST1H4I, HIST1H4J | RBBP4, HIST1H4D, HIST1H4I |
| BP GO:0006335         | DNA replication-dependent nucleosome assembly           | HIST1H4 J, HIST1H4 M, HIST1H4 K, HIST1H4 C, HIST1H4 D, HIST1H4 F, HIST1H4 G, HIST1H4 H, HIST1H3A, HIST1H3C, HIST1H3D, HIST1H3F, HIST1H3G, HIST1H3I | RBBP4, HIST1H4D, HIST1H4I | RBBP4, HIST1H4D, HIST1H4I |
| BP GO:0006336         | DNA replication-independent nucleosome assembly         | HIST1H4 J, HIST1H4 M, HIST1H4 K, HIST1H4 C, HIST1H4 D, HIST1H4 F, HIST1H4 G, HIST1H4 H, HIST1H3A, HIST1H3C, HIST1H3D, HIST1H3F, HIST1H3G, HIST1H3I | RBBP4, HIST1H4D, HIST1H4I | RBBP4, HIST1H4D, HIST1H4I |
| BP GO:0006352         | DNA-templated transcription, initiation positive regulation of gene expression, epigenetic | HIST1H4 M, HIST2H3B, HIST1H4 K, HIST1H4 J, HIST1H4 H, HIST1H4I | /               | /            | /            |
| BP GO:0045815         | DNA-templated transcription, initiation positive regulation of gene expression, epigenetic | HIST1H4 M, HIST2H3B, HIST1H4 K, HIST1H4 J, HIST1H4 H, HIST1H4I | /               | /            | /            |
| CC GO:0000788         | nuclear nucleosome                                      | HIST1H2BA, HIST2H3B, HIST1H2BB, HIST1H2BM, HIST2H2B, HIST1H3A, HIST1H3C, HIST1H3D, HIST1H3F, HIST1H3G, HIST1H3I, IRF4, HIST1H3F, HIST1H3G, TCF3, HIST3H2BA, HIST1H3I | /               | /            | /            |
| CC GO:0000228         | nuclear chromosome                                      | HIST1H2BA, HIST2H3B, HIST1H2BB, HIST1H2BM, HIST2H2B, HIST1H3A, HIST1H3C, HIST1H3D, HIST1H3F, HIST1H3G, HIST1H3I, IRF4, HIST1H3F, HIST1H3G, TCF3, HIST3H2BA, HIST1H3I | /               | /            | /            |
| MF GO:0042393         | histone binding                                         | HIST1H4 M, HIST4H4, HIST1H4 K, TNSL, RAG1, LEF1, HIST2H3B, HIST2H4, HIST1H4C, HIST1H4D, HIST1H4 J, HIST1H3A, HIST1H3C, HIST1H3D, HIST1H3F, HIST1H3G, HIST1H3I, IRF4, HIST1H3F, HIST1H3G, TCF3, HIST3H2BA, HIST1H3I, SMARCA4, HIST1H3I | CD1DI, CD1HI4I, MCM2, UHRF1, BRDT, HIST1H3D, CHAF1B, APBB1, CHAF1B, MCM2, UHRF1 | MCM2, UHRF1, HIST1H4I, APBB1, CHAF1B |
| MF GO:0031492         | nucleosomal DNA binding                                 | HIST1H3D           | RBBP4, HIST1H3D | RBBP4, HIST1H3D |

IR: ionizing radiation.
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 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 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

### 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:000122 | 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.
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 radioprotective + 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 radioprotective 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

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**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.
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 radioprotectant pretreatment, followed by real-time PCR analysis of Hist1h1a, Hist1h1d, Hist1h2bb, Hist1h2bg, Hist3h2ba, Hist1h3a, Hist2h3b, 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.
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, IncRNAs, and mRNAs in the mouse bone marrow after exposure to high-dose $\gamma$-rays. The amifostine and CBLB502 pre-treatments resulted in similar patterns of differentially expressed miRNAs, IncRNAs, and mRNAs in the mouse bone marrow after exposure to high-dose $\gamma$-rays. The amifostine and CBLB502 pre-treatments resulted in similar patterns of differentially expressed miRNAs, IncRNAs, 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 pre-treated 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 nilestriol or CBLB502 and nilestriol produced a better radioprotective effects.

Although the mechanisms of amifostine and CBLB502-mediated radioprotection are poorly understood, they are both likely to have a cytoprotective effect and rapid response against IR-induced stress within several hours, with achieving complex radiation-cytoprotective effects by interfering with genes involved in scavenging free radicals, cell cycle regulation, apoptosis, and inflammation. 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. 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 is consistent with previous reports showing that IR downregulated histone expression in parallel with inhibition of DNA synthesis. IR exposure induces highly lethal DNA damage resulting in local and global decondensation of chromatin, which resulted in histone eviction at double-strand break sites, which may facilitate DDR. 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.

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