LncRNA Inc-RI regulates homologous recombination repair of DNA double-strand breaks by stabilizing RAD51 mRNA as a competitive endogenous RNA

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ABSTRACT

DNA double-strand break (DSB) repair is critical for the maintenance of genome stability. The current models of the mechanism of DSB repair are based on studies of DNA repair proteins. Long non-coding RNAs (lncRNAs) have recently emerged as new regulatory molecules, with diverse functions in biological processes. In the present study, we found that expression of the ionizing radiation-inducible IncRNA, Inc-RI, was correlate negatively with micronucleus frequencies in human peripheral blood lymphocytes. Knockdown of Inc-RI significantly increased spontaneous DSBs levels, which was confirmed to be associated with the decreased efficiency of homologous recombination (HR) repair of DSBs. The expression of RAD51, a key recombinase in the HR pathway, decreased sharply in Inc-RI-depressed cells. In a further investigation, we demonstrated that miR-193a-3p could bind with both Inc-RI and RAD51 mRNA and depressed the expression of Inc-RI and RAD51 mRNA. Lnc-RI acted as a competitive endogenous RNA (ceRNA) to stabilize RAD51 mRNA via competitive binding with miR-193a-3p and release of its inhibition of RAD51 expression. To our knowledge, this is the first study to demonstrate the role of Inc-RI in regulating HR repair of DSBs. The feedback loop established in the current study suggests that Inc-RI is critical for the maintenance of genomic stability.

INTRODUCTION

Genome instability, which is defined as an increased frequency of mutations within the genome of a cellular lineage compared with the normal state, is a double-edged sword: on one hand, genome instability contributes to genetic diversity and biological evolution, while on the other hand, it plays a critical role in the pathology of many genetic-related diseases, including carcinogenesis (1–4). Human genome instability is also a major factor in some neurodegenerative diseases (5,6) and aging (7). Genome instability can be caused by multiple biological processes, including replication dysfunction, failure of DNA repair, and site-specific hotspots of genomic instability, as well as normal cell physiology and metabolism (8). Many genes encoding a series of proteins involved in maintaining genome stability, including those involved in DNA replication, post-replicative repair, DNA damage repair, DNA damage checkpoints, apoptosis, telomere maintenance or mRNA biogenesis, have been well described, and mutations or expression dysregulation of these genes have been confirmed to be associated with carcinogenesis (8–11).

Aberrations in the repair of DNA damage is an important cause of genomic instability (8). DNA double-strand breaks (DSBs) are considered to be the most catastrophic changes in the integrity of the genome during the life-span of a cell and are generally caused by conditions such as replication stress, genotoxic chemicals, ionizing radiation exposure, inflammation, oxidative stress and viral infection (4,12,13). To deal with these continuously produced lesions, eukaryotic cells have developed a complex and efficient DNA damage response (DDR) system, including numerous DNA damage repair pathways, disruption of which has disastrous effects on the genome (4,8,10). Homologous recombination (HR) and non-homologous end-joining (NHEJ) are the major molecular pathways responsible for DSB repair. The NHEJ pathway is an error-prone mechanism, initiated by the binding with the DNA-PK complex to the DNA break ends. In contrast, HR is an error-free repair
pathway that involves the use of homologous DNA sequences as templates in the late S and G2 phases. This process is highly conserved across species ranging from yeast to humans, and occurs via a series of precisely controlled events. In HR repair, the RAD51 protein (phage UvsX, bacterial RecA or archaeal RadA) is the central recombinase responsible for homologous pairing and the DNA strand exchange reaction in an ATP-dependent manner (14,15). The fidelity of RAD51 expression is critical in avoiding illegitimate recombination events during HR that may lead to genetic instability. This recombinase is known to mediate positive and negative regulation at the post-translational level by multiple molecules including BRCA1, BRCA2, PLK1, RAD51 paralogs, or other regulators (16–19). For example, phosphorylation of RAD51 by PLK1 at serine 14(S14) can activate its function in HR repair (19). For example, phosphorylation of RAD51 by PLK1 at serine 14(S14) can activate its function in HR repair via enhanced interaction with the MRE11–RAD50–NBS1 (MRN) complex (18).

Long non-coding RNAs (lncRNAs) are a group of non-coding RNA transcripts comprising more than 200 nucleotides and lacking apparent open reading frames. LncRNAs play important roles in many cellular biological processes, including cell cycle progression, apoptosis, development, pluripotency of stem cells, muscle differentiation and carcinogenesis (20–25). According to the competing endogenous RNA (ceRNA) hypothesis, specific RNAs can act as sinks for pools of miRNAs to control the expression of other transcripts targeted by these miRNAs, and lncRNAs are essential components of the ceRNA cross-talk process (26). Some lncRNAs are now emerging as regulators of DDR. LncRNA LIRR1, gadd7 and lincRNA-p21 were reported to regulate DDR by activating the G1/S checkpoint (27–29). Several lncRNAs have also been revealed to affect DSB repair. Zhang et al. reported that the lncRNA LINP1 contributed to NHEJ by serving as a molecular scaffold between Ku80 and DNA-PKcs (30). PCAT-1, a characterized prostate cancer lncRNA, was demonstrated to have a significant inhibitory effect on HR activity by interacting directly with the 3’UTR of the BRCA2 gene and mediating consequent post-transcriptional repression of BRCA2 (31). LncRNA DDSRI is a DNA damage-inducible lncRNA that positively regulates HR repair by interacting with BRCA1 and hnRNPU1. Defects in DDSRI have been shown to lead to aberrant DSB repair (32). TODRA, another HR pathway-regulating lncRNA, was reported to promote HR efficiency in a RAD51-dependent manner by regulating both RAD51 expression and activity (33). Lnc-RI (long noncoding RNA radiation induced; Gene ID: 401296) with 1423 nucleotides encoded by a gene located in chromosome 7p22.3 (ENST00000382528) is first identified in our laboratory as a radiation-inducible lncRNA. Our previous study indicated that lnc-RI is involved in the control of mitosis by regulating PLK1 expression via competitive targeting of miR-210-3p (34). In that study, an interesting phenomenon was revealed in that knockdown of lnc-RI led to aberrant expression of a set of DDR regulators, indicating that lnc-RI may play a role in DDR and genomic instability.

In the present study, we have revealed the role of lnc-RI in regulating the HR pathway of DSB repair and maintaining genomic stability. It was found that lnc-RI expression correlated negatively with micronucleus frequencies in the peripheral blood lymphocytes of healthy adults. Lnc-RI knockdown led to significantly increased levels of spontaneous DSBs in human cell lines and depression of HR efficiency. Furthermore, HR recombinase RAD51 expression was suppressed by silencing of the lnc-RI gene. Finally, our results indicated that miR-193a-3p targets both RAD51 and lnc-RI, and that lnc-RI regulates the stability of RAD51 mRNA by competitively binding with miR-193a-3p and relieving its inhibition of RAD51 mRNA. Our findings reveal a novel mechanism of HR pathway regulation by lnc-RI via the lnc-RI/miR-193a-3p/RAD51 mRNA 3’UTR axis.

MATERIALS AND METHODS

Isolation of peripheral blood lymphocytes for RNA extraction

Human peripheral blood samples were collected from 21 healthy donors (13 males and 8 females; aged 24–68 years). Written informed consent was obtained from all participants and the protocol was approved by the Ethics Subcommittee on Human Investigation of the Beijing Institute of Radiation Medicine (China). Peripheral blood lymphocytes were isolated from 4 ml EDTA-anticoagulated peripheral blood collected from each person by centrifugation at a speed of 1000 rpm for 20 min using lymphocyte separation medium (Hao Yang Biotechnology company, Tianjin, China). Cell pellet was washed by 10 mL 0.9% sodium chloride solution through repeated centrifugation at the same speed for 15 min and total RNA was extracted using TRIzol reagent (Sigma, USA) according to the manufacturer’s instructions.

CB micronucleus assay

Heparin-anticoagulated whole blood samples (1 ml) were collected from each participant and CB micronucleus assays were performed according to a previously described method (35). One thousand randomly selected binucleate cells per donor were counted to quantify the micronucleus frequency among peripheral blood lymphocytes using an Olympus BX61 microscope (model: BX61TRF; Japan). Micronucleus frequencies were calculated according to the following formula:

\[
\text{Micronucleus frequency (})\%g\\text{) = }\frac{n(\text{number of micronuclei})}{N(\text{number of binucleate cells})} \times 1000; N = 1000.
\]

Cell culture

All cell lines used in this study were maintained in our laboratory and cultured at 37°C in a humidified incubator under 5% CO₂. HeLa cells were cultured in RPMI-1640 medium (HyClone, USA); MCF-7 and LO2 cells were cultured in high glucose DMEM medium (HyClone, USA); U2OS cells were cultured in McCoy’s 5A medium (HyClone, USA); U2OS cells containing the GFP HR assay construct were cultured in high glucose DMEM medium (HyClone, USA). All media were supplemented with 10% prime fetal bovine serum (ExCell Bio, Cat. No. FSP500, China), penicillin (100 units/ml) and streptomycin (100 μg/ml).
Primers, probes, siRNAs and miRNA mimics

Primers, probes, siRNA duplexes and miRNA mimics used in this study were synthesized by Shanghai GenePharma Company (Shanghai, China). Details of the sequences are shown in Supplementary Tables S1–S3.

Transfection

Cells were transfected with 100 nM siRNA or miRNA mimics using Lipofectamine 2000 (Invitrogen, USA). For transfection with plasmids, cells were plated in six-well plates (3.0 × 10⁵ cells/well) ~24 h before transfection with 2.5 μg plasmids per well.

Lentivirus preparation and infection

Lentiviruses expressing shRNA-lnc-RI (LV-KD-lnc-RI) or negative control oligonucleotide sequences shRNA-NC (LV-NC) were prepared by GenePharma (Shanghai, China). The details of shRNA sequences are shown in Supplementary Table S4. HeLa cells were infected with lentivirus at a multiplicity of infection (MOI) of 20 in the presence of polybrene at a final concentration of 5 μg/ml. After 72 h, the cells were cultured in selection medium containing puromycin (1.5 μg/ml) for 7 days. The stably infected cells were then cultured continuously in RPMI-1640 medium supplemented with 0.5 μg/ml puromycin.

Actinomycin D (CHD) or cycloheximide (CHX) exposure

HeLa cells stably infected with 20 MOI lentivirus (LV-KD-lnc-RI or LV-NC) were exposed to 5 μg/ml CHD for 0, 1, 2, 4, 6 or 8 h to block transcription; absolute ethanol was used as the control reagent. Quantitative real-time PCR was performed to analyze RAD51 mRNA expression levels. HeLa cells were exposed to 100 μg/ml CHX for 0, 1, 2, 4, 8 or 12 h to block translation; dimethyl sulfoxide (DMSO) was used as the control reagent. RAD51 protein expression levels were evaluated by western blot analysis.

RNA extraction and real-time PCR

Total RNA from cells were extracted by TRIzol Reagent (Sigma, USA) according to the manufacturer’s instructions. Quality control of the isolated RNA was determined with an ultraviolet spectrophotometer (GE Healthcare GeneQuant 100, USA). 1 μg RNA was employed to synthesize cDNAs using PrimeScriptRT reagent kit with gDNA Eraser (TaKaRa, Japan). TaqMan probe-based real-time PCR analyses were performed on the Bio-Rad MyiQ™2 platform using SuperReal PreMix (Probe) (TIANGEN, China). PCR reactions were performed in a 25 μl volume in triplicate. Amplification steps as follows: step1, initial denaturation at 95°C for 15 min; step 2, denaturation at 95°C for 3 s; step 3, anneal at 60°C for 30 s; 40 cycles form step 2 to step 3. β-Actin was used as a normalizer in the real-time PCR and the relative expression of evaluated genes were calculated by 2−ΔΔCT method.

Western blot analysis

Cells were harvested by 0.25% trypsin digestion and washed twice with ice cold phosphate-buffered saline (PBS). Cell pellets were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.5% sodium deoxycholate; 1% NP-40) containing protease inhibitors and phosphatase inhibitors at the concentrations recommended by the manufacturer for 30 min in ice bath. SDS-PAGE and western blotting were performed to analyze the cell lysates according to standard protocols. The following antibodies were used in western blot analyses: phospho-histone H2AX (Ser139) monoclonal antibody (Millipore, USA; Cat. No. 05-636, 1:1000); β-Actin monoclonal antibody (Proteintech, USA; Cat. No. 60008-1-lg, 1:2000); PLK1 monoclonal antibody (Santa Cruz Biotechnology, USA; Cat. No. sc-17783, 1:500); RAD51 polyclonal antibody (Proteintech; Cat. No. 14961-1-AP, 1:2000); BRCA2 polyclonal antibody (Proteintech; Cat. No. 19791-1-AP, 1:500); NBS1 polyclonal antibody (Proteintech; Cat. No. 55025-1-AP, 1:2000); RAD50 polyclonal antibody (Ruiyingbio, China; Cat. No. RLT3963, 1:500); MRE11 polyclonal antibody (Ruiyingbio; Cat. No. RLT2829, 1:1000); BRCA1 polyclonal antibody (Ruiyingbio; Cat. No. RLT0519, 1:500); BCL2 monoclonal antibody (Ruiyingbio; Cat. No. RLT0519, 1:1000).

Immunofluorescence

Cells were cultured on coverslips in six-well plates (2.5 × 10⁵ cells/well). At 24 h or 48 h post-transfection, HeLa and U2OS cells were fixed in 4% formaldehyde for 15 min at room temperature, permeabilized in 0.3% Triton X-100-PBS buffer and then blocked in 3% bovine serum albumin for 1 h at room temperature. Cells were stained using standard procedures. Specifically, cells were incubated with phospho-histone H2AX (Ser139) monoclonal antibody (Millipore, USA; Cat. No. 05-636, 1:500) at 4°C overnight in a humidifier, and then washed twice in PBS. Subsequently, cells were incubated with a FITC-labeled anti-IgG antibody (KPL, USA; Cat. No. 02-15-06, 1:250) at room temperature for 2 h. DNA was stained with DAPI at a concentration of 2 μg/ml for 10 min in the dark. Images were obtained using a Nikon Ti-A1 capture system (magnification, 60×).

Comet assay

The neutral comet assays were performed using the Reagent Kit for Single Cell Gel Electrophoresis Assay (Trevigen, USA, Cat. No. 4250-050-K) according to the manufacturer’s instructions. DNA was stained using 100 μl propidium iodide (PI, 2 μg/ml) per slide at room temperature for 30 min in the dark. Images were obtained using the Olympus BX61 capture system (magnification, 20×). In each group, 50–100 cells were quantified using CaspLab software.

DR-GFP/I-Sec I HR assay

HR assays of U2OS-DR-GFP were performed as previously described (36). U2OS-DR-GFP cells (provided by Dr T. Ma) were first transfected with 100 nM siRNA-lnc-RI
or siRNA-NC followed by I-Sce I expressing plasmids (or control vectors) in 8 h later using Lipofectamine 2000 according to the manufacturer’s instructions. After 72 h, cells were harvested and the percentage of GFP-positive (GFP+) cells was quantified by flow cytometry (BD FACSCalibur).

NHEJ assay

The mechanism of NHEJ activity assay was well described in Seluanov A and his colleagues’ study (37). HeLa cells stably infected with lentivirus (LV-KD-lnc-RI or LV-NC) were co-transfected with pDsRed2-N1 plasmid which expressing red fluorescent protein (RFP) as transfection control, and NHEJ reporter plasmid which expressing green fluorescent protein (GFP) digested with Hind III, at a ratio of pDsRed2-N1: predigested NHEJ reptoter = 1:3, 2.5 μg total DNA per 3.0 × 10^5 cells. 48 h after transfection, the percentage of GFP+ and RFP+ cells were measured via flow cytometry (BD FACSCalibur). NHEJ activity in HeLa cells were evaluated by the percentage of GFP+ cells in RFP+ cells.

Dual luciferase reporter assay

RAD51 mRNA 3′UTR and lnc-RI sequences were cloned into the pGL3M vector to generate the pGL3M-RAD51 3′UTR and pGL3M-lnc-RI constructs. Subsequently, mutant sequences at the predicted target sites for miR-193a-3p in the RAD51 mRNA 3′UTR or lnc-RI were cloned into the pGL3M vector to generate the pGL3M-RAD51 3′UTR-M and pGL3M-lnc-RI-M constructs. Cells were seeded into 24-well plates (6.0 × 10^4/well) for approximately 24 h before co-transfection with 10 ng of reporter plasmid and 1 ng pRL-CMV internal control plasmid. After 8 h, the medium was replaced and cells were transfected with 100 nM miRNA mimics. After incubation for 48 h, the transfected cells were lysed and luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega, USA, Cat. No. E1910). Firefly luciferase activity was normalized to Renilla luciferase activity and each group was assayed in triplicate.

Statistical analysis

Spearman’s correlation coefficient was calculated to assess the significance of the correlation between expression levels of lnc-RI and micronucleus frequency using Statistical Analysis System (SAS) (P < 0.05 was considered to indicate statistical significance). Data from real-time PCR, comet assay and flow cytometry data were presented as means±SD (all experiments were performed in triplicate or more). Experiments with two experimental groups were statistically analyzed using Two-tailed Student’s t-tests. *P < 0.05, **P < 0.01 or ***P < 0.001 was considered to indicate statistical significance, n.s. meant no significance.

RESULTS

Negative correlation of lnc-RI expression with micronucleus formation

The micronucleus is formed in nucleated cells by chromosome breakage or loss and is generally recognized as a biomarker of genomic instability (38). Our previous findings indicated that lnc-RI is a radiation-inducible lncRNA molecule involved in radiation-induced DDRs. Therefore, we hypothesized that lnc-RI is associated with genome integrity. To test this hypothesis, we first investigated the potential relationship between lnc-RI expression and micronucleus frequency in peripheral blood lymphocytes of 21 healthy donors. The results of real-time PCR analysis of lnc-RI expression levels and analysis of micronucleus frequency using the cytokinesis-block (CB) micronucleus assay are presented in Table 1. The data of Figure 1 indicated that the micronucleus frequency correlated negatively with lnc-RI expression, which implicating that lnc-RI may involve in the maintenance of genome stability.

Knockdown of lnc-RI expression increased accumulation of spontaneous DSBs in multiple cell lines

Our previous study indicated that expression of some DDR-related genes was disrupted in lnc-RI knockdown cells (34). In present study, we found that promoter of lnc-RI contain NF-κB (p65) binding sites (between +71 bp and +81 bp) and then we confirmed that lnc-RI may be induced by radiation in a NF-κB (p65) dependent manner (see in Supplementary Figure S1). Next, we found that lnc-RI knockdown may sensitized HeLa cells to radiation (Supplementary Figure S2). Combined with data of Figure 1, we hypothesized that lnc-RI participates in the maintenance of genomic stability by regulating the efficiency of intrinsic cellular DNA repair processes. To test our hypothesis, lnc-RI expression in HeLa and U2OS cells was silenced using siRNA targeting technology. At 24 h or 48 h after transfection, cells were harvested for analysis of γ-H2AX expression and γ-H2AX foci, which are recognized markers of DSBs. Compared with siRNA-NC, siRNA-lnc-RI #1 and #2 effectively depressed expression of lnc-RI (Figure 2A), with significantly increased γ-H2AX and γ-H2AX foci formation (Figure 2B and C). Similar results were observed in MCF-7 and LO2 cells (Supplementary Figure S3). These results indicated that knockdown of lnc-RI expression significantly increases accumulation of spontaneous DSBs.

In further investigations, neutral comet assays were performed to detect the yield of DSBs in HeLa and U2OS cells at 24 h or 48 h after transfection with specific siRNAs. As shown in Figure 2D, DNA fragments were dramatically increased in the cells transfected with lnc-RI-specific siRNAs. We confirmed that knockdown of lnc-RI increased spontaneous DSBs levels in multiple cell types (Figure 2A–D, Supplementary Figure S3).

lnc-RI regulates homologous repair of DSBs by regulating RAD51 expression

We investigated the effect of lnc-RI on HR activity of DSBs using a well-characterized HR reporter assay (DR-GFP) in U2OS cells. Compared with the control cells, lnc-RI knockdown resulted in a significant decrease in the GFP signal that represents the efficiency of HR repair of DSBs generated in the reporter construct by I-Sce I; RAD51 knockdown cells was performed as a positive control (Figure 3A–C). At the same time, We also investigated the effect of lnc-
Figure 1. The correlation of Inc-RI expression and micronucleus frequency in human lymphocytes. CB micronucleus assays were used to measure the micronucelus frequency in human peripheral blood lymphocytes. 1000 randomly selected binucleated cells per sample were scored to calculate micronucleus frequency ($n = 21$). The correlation between Inc-RI expression and micronucleus frequency was tested by Spearman’s correlation coefficient using SAS (Spearman correlation coefficient: $-0.4409, P < 0.05$).

Table 1. The expression of Inc-RI and micronucleus frequency in human peripheral blood lymphocytes

| Individual | Gender | Age (years) | Relative expression of Inc-RI | Micronucleus frequency(‰) |
|------------|--------|-------------|------------------------------|----------------------------|
| 1          | Female | 25          | 8.93                         | 15                         |
| 2          | Male   | 27          | 3.02                         | 17                         |
| 3          | Male   | 25          | 0.36                         | 17                         |
| 4          | Male   | 27          | 1.00                         | 20                         |
| 5          | Female | 25          | 0.65                         | 20                         |
| 6          | Male   | 24          | 0.28                         | 21                         |
| 7          | Male   | 26          | 1.21                         | 23                         |
| 8          | Female | 30          | 0.46                         | 23                         |
| 9          | Male   | 24          | 0.32                         | 25                         |
| 10         | Female | 28          | 0.49                         | 28                         |
| 11         | Male   | 26          | 0.21                         | 30                         |
| 12         | Male   | 54          | 0.03                         | 32                         |
| 13         | Male   | 62          | 1.42                         | 34                         |
| 14         | Male   | 54          | 1.00                         | 34                         |
| 15         | Male   | 68          | 0.32                         | 37                         |
| 16         | Male   | 64          | 0.33                         | 40                         |
| 17         | Female | 61          | 0.05                         | 43                         |
| 18         | Female | 53          | 0.02                         | 46                         |
| 19         | Female | 63          | 0.11                         | 54                         |
| 20         | Female | 68          | 0.33                         | 60                         |
| 21         | Female | 55          | 0.63                         | 69                         |

Inc-RI on NHEJ activity of DSBs. Our result found that knockdown of Inc-RI may slightly inhibit NHEJ activity (Supplementary Figure S4).

Next, we try to identify the target of Inc-RI by which Inc-RI regulate HR activity, expression of several essential proteins in the HR pathway (MRE11, RAD50, NBS1, RAD51, BRCA1, BRCA2, PLK1 and BCL2) were detected by western blotting. As shown in Figure 3D–E, knockdown of Inc-RI in both HeLa and U2OS cells significantly depressed RAD51 and PLK1 expression. The expression of some HR pathway-associated proteins (RAD50, BRCA1, BRCA2) were also found to be depressed to some extent. Next, we over-expressed RAD51 and PLK1 in Inc-RI knockdown cells, only over expression of RAD51 could attenuate the level of γ-H2AX induced by Inc-RI knockdown (Supplementary Figure S5).

In Figure 3C, we found an interesting phenomenon: knockdown of RAD51 also depressed expression of Inc-RI, which is confirmed in Supplementary Figure S6. Next, HCT116 cells (more sensitive to radiation than HT29 cells) and HT29 cells were selected. And the expression of RAD51 and Inc-RI in HCT116 and HT29 were detected. As shown in Supplementary Figure S7, both levels of Inc-RI and RAD51 in HT29 cells are higher than which in HCT116.

All these evidence above suggested that RAD51 is a critical target through which Inc-RI plays a role in the HR pathway and give us a cue that Inc-RI may regulate RAD51 expression as an ceRNA which is confirm in Figure 4, Figure 5 and Supplementary Figure S6.
Figure 2. Knockdown of lnc-RI led to increased levels of spontaneous DSBs in multiple types of cells. (A) siRNA-mediated depression of lnc-RI expression. HeLa cells were transfected with 100 nM siRNA-lnc-RI or the control siRNA-NC. After 24 h and 48 h, lnc-RI expression was measured by real-time PCR. t-test, mean ± SD, n = 3. (B) Lnc-RI knockdown increased the expression of γ-H2AX. HeLa and U2OS cells were transfected with siRNA-lnc-RI or siRNA-NC. Expression of γ-H2AX was detected by Western blot analysis (n = 6). (C) Lnc-RI knockdown elicited γ-H2AX foci formation. HeLa and U2OS cells were transfected with siRNA-lnc-RI or siRNA-NC. γ-H2AX foci were detected by indirect immunofluorescence staining using an anti-γ-H2AX primary detection antibody and a FITC-conjugated anti-IgG secondary detection antibody; 200 randomly selected cells in each group were scored (t-test, mean ± SD, n = 200). (D) Comet assay analysis of DSBs induced by lnc-RI knockdown. HeLa and U2OS cells were transfected with equivalent concentrations of siRNA-lnc-RI or siRNA-NC. DNA fragments were detected by neutral comet assay performed 24 h and 48 h after transfection. Percentage of tail DNA in each group were measured by CaspLab software. t-test, mean±SD, n = 50–100. Scale bar, 50 μm. The comet assay were repeated three times. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 3. Knockdown of lnc-RI depressed HR efficiency. (A–C) knockdown of lnc-RI suppressed HR efficiency. DR-GFP U2OS cells were co-transfected with I-Sce I expressing plasmids and siRNA-lnc-RI or siRNA-NC. After 72 h, I-Sce I induced homologous recombination was detected via flow cytometry (A) and the results statistically analyzed. t-test, data are presented as mean ± SD, n = 3 (B). The expression of lnc-RI was analyzed by real-time PCR (t-test mean ± SD, n = 3) (C, top); the expression of RAD51 and γ-H2AX was analyzed by Western blot (C, bottom). (D, E) Lnc-RI knockdown decreased the expression of some essential components in HR pathway. HeLa (D) and U2OS (E) cells were transfected with siRNA-lnc-RI and siRNA-NC. After 24 and 48 h, cells were harvested and expression of HR-essential proteins was analyzed by Western blot. Western blot analysis were performed more than three times.*P < 0.05, **P < 0.01, ***P < 0.001.

Lnc-RI is necessary for the stabilization of RAD51 mRNA

The mechanism by which RAD51 depression is induced by lnc-RI knockdown was further investigated. RNAi-mediated lnc-RI knockdown in HeLa and U2OS cells resulted in decreased RAD51 expression at both the protein and mRNA levels (Figure 4A; Supplementary Figure S8). Next, we investigated the ability of lnc-RI to regulate the stability of RAD51 mRNA and protein. HeLa cells were infected with LV-KD-lnc-RI (or control lentivirus LV-NC). Western blot analysis at 1, 2, 4, 8 and 12 h after the addition of cycloheximide (CHX) to block translation indicated that lnc-RI knockdown had no effect on the degradation of RAD51 protein (Figure 4B). In contrast, quantitative real-time PCR analysis after the addition of actinomycin D (CHD) to block transcription revealed rapid degradation of RAD51 mRNA in lnc-RI knockdown cells (Figure 4C). These data indicated that lnc-RI is necessary for stabilization of RAD51 mRNA.
Figure 4. Knockdown of Inc-RI promoted the degradation of RAD51 mRNA and decreased RAD51 protein levels. (A) Inc-RI knockdown reduced RAD51 gene expression at both the mRNA and protein levels. HeLa and U2OS cells were transfected with 100 nM siRNA-Inc-RI or siRNA-NC. After 24 and 48 h, RAD51 protein and mRNA expression was detected by western blot (top) or real-time PCR (bottom), respectively. (B) Inc-RI knockdown had no influence on degradation of RAD51 protein. RAD51 protein expression in HeLa cells infected with lentivirus LV-KD-Inc-RI (or LV-NC) was detected by Western blot at 0, 1, 2, 4, 8, and 12 h after the addition of 100 μg/ml CHX to block translation. (C) Inc-RI knockdown accelerated degradation of RAD51 mRNA. RAD51 mRNA expression in HeLa cells infected with the same lentivirus infected cells was detected by real-time PCR at 0, 1, 2, 4, 8, and 12 h after the addition of 5 μg/ml CHD to block transcription. These experiments were conducted on three independent occasions. t-test, mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, n.s. means no significance.
Figure 5. Lnc-RI stabilized RAD51 mRNA by competitively binding to miR-193a-3p. (A, B) Inhibition of RAD51 and lnc-RI expression induced by enforced expression of miR-193b-3p, miR-193a-3p, miR-34c-5p, miR-328-3p or miR-449a. HeLa cells were transfected with synthetic miRNA mimics (100 nM). After 48 h, expression of lnc-RI (A, left) and RAD51 (A right) was measured by real-time PCR. (B) RAD51 and γ-H2Ax protein expression were detected by Western blot and quantified using Quantity One software normalized to β-Actin. (C–E) MiR-193a-3p directly targets the RAD51 mRNA 3′UTR and lnc-RI. (C) Predicted target sites of miR-193a-3p in lnc-RI and the RAD51 mRNA 3′UTR. (D) Luciferase reporter vectors were constructed by cloning the wild-type lnc-RI and RAD51 mRNA 3′UTR sequences, or mutated lnc-RI (937–959 bp) and RAD51 3′UTR (704–724 bp) sequences into the pGL3M plasmid. (E) HeLa cells were co-transfected with the reporter vectors and miR-193a-3p or NC. Luciferase activity was analyzed at 48 h after transfection. t-test, data are mean ± SD, n = 3, *P < 0.05, **P < 0.01, n.s. means no significance.
**Lnc-RI regulated stability of RAD51 mRNA by competitive binding with miR-193a-3-p**

MiRNAs bind to the 3′UTR region of target mRNA molecules to facilitate their degradation. LncRNAs have been reported to bind miRNAs competitively to regulate mRNA stability (24). Knockdown of lnc-RI facilitated degradation of RAD51 mRNA (Figure 4C). Furthermore, knockdown of RAD51 mRNA also depressed lnc-RI expression, indicating that lnc-RI may regulate RAD51 mRNA stability by competitively binding certain miRNAs (Supplementary Figure S6).

By searching the open databases RegRNA2.0, TargetScan, miRDB, miRTarBase, starBase v2.0, and microRNA.org-Targets and Expression, five candidate miRNAs (miR-328, miR-193a-3p, miR-193b-3p, miR-34c-5p, and miR-449a) were predicted to target both RAD51 mRNA 3′UTR sequences and lnc-RI, all of which have been reported to influence tumor carcinogenesis or DDR (Table 2). The predicted targeting sites of these miRNAs in lnc-RI or RAD51 gene sequences are shown in Supplementary Figure S9.

HeLa cells were transfected with mimics of the five candidate miRNAs respectively (Supplementary Table S3) and the expression of lnc-RI and RAD51 was detected by realtime PCR or Western blot analysis. As shown in Figure 5A, all five miRNA mimics depressed expression of both lnc-RI and RAD51 at the RNA level. Furthermore, miR-193a-3p and miR-34c-5p were found to mediate the most significant inhibition of RAD51 protein expression, with concurrently increased γ-H2AX (Figure 5B). These results implicated miR-193a-3p and miR-34c-5p may be the miRNAs through which lnc-RI regulates RAD51 mRNA stabilization.

To confirm direct interactions of the miRNAs (miR-193a-3p and miR-34c-5p), the sequences of lnc-RI or the 3′UTR region of RAD51 mRNA containing the miR-193a-3p or miR-34c-5p binding sites were cloned separately into downstream of the luciferase gene of the pGL3M plasmid (Figure 5C and D). HeLa cells were then co-transfected with these reporter plasmids and miR-193a-3p or miR-34c-5p. While miR-193a-3p significantly suppressed the luciferase activity of both candidate target sequences (Figure 5E), miR-34c-5p has no effect on either candidate target sequence (data not shown). Thus, we focused on miR-193a-3p in further mechanistic studies.

We mutated the binding sites of miR-193a-3p in the sequences of the lnc-RI and the 3′UTR region of RAD51 mRNA before cloning into pGL3M for similar comparative analyses (Figure 5D). As shown in Figure 5E, analysis of relative luciferase activity at 48 h after co-transfection of HeLa cells with the constructed plasmids and miR-193a-3p mimics (or negative control mimics) revealed that mutation of miR-193a-3p binding site attenuated the suppression of luciferase activity of both candidate target sequences mediated by miR-193a-3p in cells transfected with the lnc-RI-wt and RAD51 3′UTR-wt constructs. However, the RAD51 mRNA 3′UTR 704–724 bp mutant did not completely relieve the repressive effects of miR-193a-3p on RAD51 3′UTR associated luciferase activity, indicating the possible existence of other miR-193a-3p target sites in the RAD51 mRNA 3′UTR sequence. These results indicated that miR-193a-3p regulates the expressions of lnc-RI and RAD51 by direct interaction.

**DISCUSSION**

In the present study, a significant negative correlation between endogenous lncRNA lnc-RI expression and spontaneous micronucleus frequency was identified, and knockdown of lnc-RI was shown to significantly increase DSB accumulation in multiple cell types. We revealed that lnc-RI plays a role in the HR pathway of DSB repair though regulation of RAD51 mRNA stabilization via competition with miR-193a-3p for binding to the 3′UTR region. Lnc-RI functions as a ceRNA to relieve the inhibitory effects of miR-193a-3p on RAD51 expression. These findings provide strong evidence in support of a key role for lnc-RI in the maintenance of genome stability.

HR is generally considered to be a dominant error-free mechanism of DSB repair during the late S and G2 phases. RAD51, which catalyzes DNA strand invasion and leads to the formation of a physical structure known as a ‘D-loop’ between the invading DNA substrate and its homologous DNA sequences, is a critical component of the HR pathway. Dysfunction in RAD51 expression results in deficiency in HR for DSB repair, leading to chromosome aberration, and even embryonic lethality or carcinogenesis (16,39,40). LncRNAs have recently emerged as epigenetic regulatory molecules that play important roles in diverse biological processes and in disease development. However, reports of the involvement of lncRNAs in the HR pathway are relatively rare. In 2015, the lncRNA TODRA, a transcript derived from 69 bp upstream of the RAD51 transcription start site (TSS), was reported to regulate HR repair in a RAD51-dependent manner. To date, TODRA is the only RAD51-associated lncRNA reported to be involved in HR regulation, although no direct interaction between these two genes was identified (33). In the present study, we demonstrated that lnc-RI is a novel lncRNA involved in the regulation of HR repair. Knockdown of lnc-RI expression caused a marked decline in HR efficiency and increased the accumulation of spontaneous DSBs. Expression levels of several critical regulators of HR activity, including RAD51, RAD50, BRCA1 and BRCA2, were found to be depressed by lnc-RI knockdown. As a key protein of HR, RAD51 expression is highest during S/G2 phase of cell cycle (41). So, the decrease of populations of S-phase cells may cause RAD51 reduction. Then we analyzed the cell cycle distribution of HeLa cells after knockdown of lnc-RI. As shown in Supplementary Figure S10, knockdown of lnc-RI induced G2/M arrest but had no effect on populations of cells in S phase, which indicated that RAD51 reduction is not due to decrease of populations of S phase cells. In accordance with this, the expression of lnc-RI showed a negative correlation with micronucleus frequency, which is considered to be a credible biomarker of genomic instability.

LncRNA regulates the expression of target genes through a variety of interactions including RNA-protein, RNA-DNA or RNA-RNA (42,43). It is now widely accepted that lncRNAs act as ceRNA by functioning as decoys of miRNAs, which are a group of small regulatory ncR-
Table 2. Bioinformatic predicted miRNAs target lnc-RI and RAD51

| Target lnc-RI | MiRNAs       | Database       | Target RAD51 3′ UTR | MiRNAs       | Database       |
|---------------|--------------|----------------|--------------------|--------------|----------------|
|               | miR-3192     | RegRNA2.0      | miR-3652           | miRDB        |
|               | miR-328 #    |                | miR-4430           |              |
|               | miR-532-3p   |                | miR-532-3p         |              |
|               | miR-134-5p   | TargetScan     | miR-193b-3p        | miRTarBase   |
|               | miR-148a-3p  |                | miR-3192-5p        |              |
|               | miR-148b-3p  |                | miR-328-3p #       |              |
|               | miR-152-3p   |                | miR-384            |              |
|               | miR-193a-3p #|                | miR-5571-3p        |              |
|               | miR-193b-3p #|                | miR-6819-3p        |              |
|               | miR-3118     |                | miR-6840-3p        |              |
|               | miR-34c-5p # |                | miR-6877-3p        |              |
|               | miR-3652     |                | miR-7975           |              |
|               | miR-384      |                | miR-134-5p         | starBase v2.0|
|               | miR-4430     |                | miR-148a-3p        |              |
|               | miR-449a #   |                | miR-148b-3p        |              |
|               | miR-5571-3p  |                | miR-152-3p         |              |
|               | miR-6819-3p  |                | miR-3118           |              |
|               | miR-6840-3p  |                | miR-34c-5p #       |              |
|               | miR-6877-3p  |                | miR-449a           |              |
|               | miR-7975     | microRNA.org-Targets and Expression | miR-193a-3p # | microRNA.org-Targets and Expression |
|               | miR-384     | microRNA.org-Targets and Expression | miR-193a-3p | |
| Annotations: pounds signed miRNAs were selected for further investigations. |

Figure 6. Schematic diagram of the interaction between miR-193a-3p and lnc-RI or RAD51 in the regulation of HR and genomic stability.

NAs comprising ~22 nucleotides and specifically targeting mRNA 3′ UTR or lncRNA sequences to induce gene silencing (24,26,44). In our study, RNAi-mediated lnc-RI depression accelerated the degradation of RAD51 mRNA. Furthermore, knockdown of lnc-RI or RAD51 led mutual suppression. Based on the ‘ceRNA’ hypothesis, we performed bioinformatics analyses that revealed a number of miRNAs with the potential to target both lnc-RI and the RAD51 mRNA 3′ UTR. MiR-193a-3p is involved in the pathogenesis of various tumors, and it negatively regulates DDR by targeting the ING5 gene (45–47). Our results showed that enforced expression of miR-193a-3p mimics inhibited the expression of both lnc-RI and RAD51, and consequently enhanced DNA damage as indicated by increased γ-H2AX expression. Furthermore, dual luciferase reporter assays confirmed a direct interaction between miR-193a-3p and its predicted target sites in the lnc-RI 937–959 bp region and in the RAD51 mRNA 3′ UTR 704–724 bp region. Thus, these findings suggest that lnc-RI regulates RAD51 expression through competitive binding with miR-193a-3p as a ceRNA.

In the present and previous studies, knockdown of lnc-RI was also shown to depress the expression of PLK1, which has been reported to promote RAD51 activity via phosphorylation at S14 in response to DNA damage (18,34). In the current study however, we did not explore the ability of lnc-RI to regulate RAD51 recombinase activity in a PLK1-dependent manner.

Our studies show that under normal conditions, an appropriate level of lnc-RI is necessary to maintain effective...
HR repair activity via regulation of RAD51 expression. Lnc-RI regulates RAD51 mRNA stability by competitively and specifically binding to miR-193a-3p as a ceRNA and relieves its inhibitory effects on the RAD51 mRNA target. Based on our findings, we propose a model in which down-regulation of Lnc-RI expression leads to an enhanced interaction between miR-193a-3p and the RAD51 mRNA 3’UTR, which accelerates the degradation of RAD51 mRNA to suppress RAD51 expression, eventually inducing DNA damage through HR repair deficiency (Figure 6).

In summary, our study demonstrates a novel role for the lncRNA Lnc-RI in the HR pathway of DSB repair. Knockdown of Lnc-RI suppressed HR repair efficiency and induced the accumulation of spontaneous DSBs. These data indicate that Lnc-RI functions as a ceRNA to regulate the expression of RAD51 via the Lnc-RI/miR-193a-3p/RAD51 axis. This evidence implicates Lnc-RI as a novel regulator of the HR pathway that plays an important role in the maintenance of genome stability.

AVAILABILITY
RegRNA2.0 (http://regrna2.mbc.nctu.edu.tw/), TargetScan (http://www.targetscan.org/vert_71/), miRDB (http://www.mirdb.org/), miRTarBase (http://miirtarbase.mbc.nctu.edu.tw/), starBase v2.0 (http://starbase.sysu.edu.cn/), and microRNA.org-Targets and Expression (http://www.mircomma.org/micorna/home.do) are open sources for microRNA searching.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR online.

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Conflict of interest statement. None declared.

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