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Original Article

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lncRNA LINC00960 promotes apoptosis by sponging ubiquitin ligase Nrdp1-targeting miR-183-5p

Hao Yang[1,2], Tianxia Jiang[2], Libin Fan[1,*], and Xiaobo Qiu[2,*]

[1]College of Life Sciences, Anhui Medical University, Hefei 230032, China, and [2]Key Laboratory of Cell Proliferation and Regulation Biology, Ministry of Education, and College of Life Sciences, Beijing Normal University, Beijing 100875, China
*Correspondence address. Tel: +86-10-58808117; E-mail: xqiu@bnu.edu.cn (X.Q.) / Tel: +86-551-65167282; E-mail: lfan@ahmu.edu.cn (L.F.)

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Abstract
The ubiquitin ligase Nrdp1/RNF41 promotes the ubiquitin-dependent degradation of multiple important substrates, including BRUCE/BIRC6, a giant ubiquitin-conjugating enzyme inhibiting both apoptosis and autophagy. miR-183-5p is associated with various malignancies potentially by targeting dozens of genes. Here, we show that the lncRNA LINC00960 binds to the Nrdp1-targeting miR-183-5p and promotes apoptosis. Compared to other known miR-183-5p targets, Nrdp1 mRNA is among the few with top scores to complement miR-183-5p. miR-183-5p binds to the 3′UTR of Nrdp1 mRNA and downregulates Nrdp1 at both the mRNA and protein levels. The miR-183-5p mimics inhibit DNA damage-induced apoptosis probably by upregulating BRUCE level, whereas the miR-183-5p inhibitor suppresses the effects of miR-183-5p. LINC00960 is the noncoding RNA with the highest score to complement miR-183-5p. LINC00960 overexpression reduces, but its knockdown increases, the level of miR-183-5p, whereas LINC00960 overexpression increases, but its knockdown decreases, the level of Nrdp1 and apoptosis. Importantly, the expression of LINC00960, which is associated with multiple types of tumors, positively correlates with that of Nrdp1 in several tumors but inversely correlates with that of miR-183-5p in multiple human tumor cell lines, as analysed by quantitative PCR. Thus, miR-183-5p downregulates Nrdp1 expression and inhibits apoptosis, whereas LINC00960 upregulates Nrdp1 and promotes apoptosis by inhibiting miR-183-5p. These results may provide new ideas for the prevention, diagnosis and treatment of apoptosis-related diseases, such as tumors and neurodegenerative diseases.

Key words Nrdp1, BRUCE, apoptosis, lncRNA, miRNA

Introduction

Apoptosis is critical to the maintenance of cellular homeostasis by removing excess, damaged or dangerous cells in the body without causing damage to surrounding cells or tissues [1]. Dysregulation of apoptosis may lead to various diseases [2]. Caspase-dependent apoptosis is driven by a cascade of caspase activation. Autophagy is an evolutionarily conserved important process for the turnover of intracellular substances in eukaryotes [3]. During macroautophagy (hereafter referred to as autophagy), cytoplasmic LC3 (*i.e.*, LC3-I) is cleaved into a smaller size and then modified by a phospholipid to form LC3-II on the autophagosome membrane [4]. The balance between apoptosis and autophagy is vital to the development of an individual and the pathogenesis of various severe diseases [5].

BRUCE/BIRC6, a large membrane-associated protein (about 530 kDa), serves as an inhibitor of apoptosis protein (IAP) by catalyzing the ubiquitination and degradation of proapoptotic proteins such as caspase 3 and Smac in certain cells [6–8]. On the other hand, BRUCE promotes proteasomal degradation of LC3-I, suppresses the formation of autophagosomes and thus inhibits autophagy under certain circumstances [9]. Degradation of BRUCE can be promoted by Nrdp1, a RING-finger ubiquitin ligase (E3), which catalyzes ubiquitination of BRUCE under certain stress conditions [10]. Nrdp1 also promotes ubiquitination and proteasomal degradation of ErbB3 (a member of the epidermal growth factor receptor family) and Parkin (a ubiquitin ligase involved in mitochondrial autophagy and the pathogenesis of a subtype of Parkinson's disease) [11,12]. Nrdp1 not only increases neuronal apoptosis by downregulating BRUCE during intracerebral haemorrhage [13], but also inhibits glioma cell migration by downregulating ErbB3 and reducing the cytoplasmic localization of p27(Kip1) [14]. Thus, Nrdp1 is an

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important ubiquitin ligase potentially implicated in the pathology of various diseases, including intracerebral haemorrhage, cancer and Parkinson's disease, by regulating both apoptosis and autophagy.

Noncoding RNAs refer to RNAs that are not translated into polypeptides. Small noncoding RNAs with lengths shorter than 200 nt include snRNA, snoRNA, miRNA, piRNA, and circRNA. Those longer than 200 nt are classified as long noncoding RNAs (lncRNAs). lncRNAs are poorly conserved among species and show tissue-specific expression patterns. lncRNAs are involved in multiple cellular regulatory processes, such as X chromosome inactivation, chromatin modification, telomere elongation, transcriptional activation and nuclear trafficking [15,16]. The miRNA miR-183-5p is frequently associated with various types of tumors, probably by targeting dozens of genes.

In this study, we demonstrate that miR-183-5p downregulates the level of Nrdp1 mRNA and inhibits apoptosis, and the lncRNA LINC00960 binds to miR-183-5p probably by serving as a competing endogenous RNA (ceRNA) to restore the level of Nrdp1 mRNA.

Materials and Methods

Cell culture

HEK293T human embryonic kidney cells, HeLa human cervical cancer cells, MCF-7 human breast adenocarcinoma cells, HCT116 human colon carcinoma cells, HepG2 human liver cancer cells, and U2OS human osteosarcoma cells were purchased from American Type Culture Collection (ATCC, Manassas, USA). Cells were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, USA) containing 10% fetal bovine serum (Sijiqing, Beijing, China), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, St Louis, USA). We used PCR to detect mycoplasma contamination and confirmed that there was no contamination for any cell lines used in this study.

Cell transfection

LINC00960 siRNA (si-LINC00960) and siRNA control oligos were obtained from Gene Pharma (Suzhou, China), and miR-183-5p mimics, mimics NC, inhibitor, and inhibitor NC were from RIBOBIO (Guangzhou, China) (Table 1). The plasmids of WT and MUT LINC00960 were constructed in pcDNA-6B (Invitrogen). Cells were plated in growth medium one day before transfection so that they were 50%–60% confluent at the time of transfection. siRNA oligos or plasmids, which were diluted in Opti-MEM (Invitrogen), were transfected into cells using LipoMax (Sudgen Biotechnology, Nanjing, China) according to the manufacturer's protocol.

Table 1. The oligo sequences used in this study

Oligo name	Sequence
si-LINC00960#1	Sense: 5′-GGCGUGAGAGUAAAGC-3′ Antisense: 5′-GUGCUUAGGCUUAGAG-3′
si-LINC00960#2	Sense: 5′-UUAAGAGCUGGUCACG-3′ Antisense: 5′-UAAGGCACUGGAGCAA-3′
siRNA control	Sense: 5′-UCAUACUAUAUGACAG-3′ Antisense: 5′-GACGGUAAGUAGGCGA-3′
hsa-miR-183-5p mimics	Sense: 5′-UAUGGCACUGGUAGAAUUCACUG-3′ Antisense: 5′-UGAAUUCUACCAGUGCCAUAUU-3′
mimics NC	Sense: 5′-UUCUCCGAACGUGUCACGUTT-3′ Antisense: 5′-ACGUGACACGUUCGGAGAATT-3′
hsa-miR-183-5p inhibitor	Sense: 5′-CAGUGAAUUCUACCAGUGCCAUA-3′
Inhibitor NC	Sense: 5′-CAGUACUUUUGUGUAGUACAA-3′

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells by using TRIzol reagent (15596026; Thermo, Waltham, USA) and reverse transcribed into cDNA using a Reverse Transcription Kit (Roche, Basel, Switzerland). The reaction system consists of 10 μL SYBR Green mix (4367659; Thermo), 1 μL forward primer , 1 μL reverse primer, 1 μL cDNA and 7 μL deionized water. Amplification was performed using a reaction cycle at 95°C for 3 min, 95°C for 10 s, 60°C for 30 s and 72°C for 30 s (40 cycles), 72°C for 5 min, 4°C hold. *GAPDH* was selected as the internal control for Nrdp1 while *U6* was used for miR-183-5p and LINC00960. Primers are listed in Table 2.

Western blot analysis

Cells were washed with phosphate-buffered saline (PBS) three times, and total protein was immediately extracted with Nrdp1 lysis buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.2% Nonidet P-40, 10% glycerol, 1 mM $ZnCl_2$, 10 mM β-glycerophosphate, 5 mM tetrasodium pyrophosphate, 1 mM NaF, 1 mM Na_3VO_4, and a protease inhibitor mixture. The sample was sonicated at 200 W for 4 s twice and centrifuged at 16,000 *g* for 10 min at 4°C. Next, 40 μg of protein was separated by 1% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane (Millipore). Following incubation with antibodies, the protein bands were subsequently visualized with an enhanced chemiluminescence detection kit (5200; Tanon, Shanghai, China). The primary antibodies included anti-Nrdp1 (SC365622, 1:1000; Santa Cruz Biotechnology, Santa Cruz, USA), anti-β-actin (A5441, 1:10,000; Sigma-Aldrich), anti-active caspase 3 (9665S, 1:1000; Cell Signaling Technology, Boston, USA), anti-BRUCE (622291, 1:500; BD Bioscience, New Jersey, USA), and anti-LC3B (l7543, 1:1000; Sigma-Aldrich). Peroxidase-conjugated anti-mouse IgG (ZB-2305, 1:3000; ZSGB-BIO, Beijing, China) or anti-rabbit IgG (ZB-2301, 1:3000; ZSGB-BIO) was used as the secondary antibody.

Luciferase reporter assay

The wild-type (WT) or mutant (MUT) LINC00960 sequence and the 3′UTR of the Nrdp1 sequence containing miR-183-5p binding sites, which were amplified by PCR, were cloned into the luciferase reporter vector pGL3-promoter (Fenghbio, Changsha, China). miR-183-5p mimics and miR-NC were transfected into HEK 293T cells together with LINC00960 or the 3′UTR of the Nrdp1 reporter for

Table 2. The PCR primers used in this study

Gene	Primer sequence
Nrdp1	Forward: 5′-TGCATTAAGCACCTGCGC-3′ Reverse: 5′-AGCAGGACAGCCACTCTCC-3′
LINC00960	Forward: 5′-CCTCTAAGCCTAAGCACCGCC-3′ Reverse: 5′-GGAAGCCTGGGCAAGGAATGG-3′
hsa-miR-183-5p	Forward: 5′-TCACTTAAGATGGTCACGGTAU-3′ Reverse: 5′-ATAGACCAACAGGTGTACTGA-3′
GAPDH	Forward: 5′-CTCAGACACCATGGGGAAGGT-3′ Reverse: 5′-ATGATCTTGAGGCTGTTGTCATA-3′
U6	Forward: 5′-CTCGCTTCGGCAGCACA-3′ Reverse: 5′-AACGCTTCACGAATTTGCGT-3′

24 h, and firefly luciferase activity was detected uisng a dual-luciferase reporter assay kit (E1910; Promega, Madison, USA), while Renilla luciferase served as a reference.

Immunofluorescence microscopy

Cells on glass coverslips in 6-well plates were fixed in 4% polyformaldehyde for 10 min at room temperature, followed by membrane permeation using 100 μg/mL digitonin in PBS for 5 min. Cells were blocked in 3% BSA (bovine serum albumin) before primary antibodies were applied. Cells were incubated with primary antibodies in a moist container at room temperature for 1 h. Then, secondary antibodies conjugated with Alex-594 (ZSGB-BIO, Beijing, China) were applied to the cells in a moist container at room temperature for 1 h. The DNA in the nuclei was stained with 4′,6-diamidino-2-phenylindole (DAPI) at the final preparation step. The slides were viewed on an LSM700 fluorescence microscope (Zeiss, Oberkochen, Germany) using a 63 × oil objective and laser at 488 or 594 nm for excitation.

TUNEL assay

Apoptosis was analysed using a TUNEL assay kit (G3250; Promega, Madison, USA) according to the manufacturer's instructions. Briefly, after growing cells on slides and treatment, slides were immersed in 4% paraformaldehyde (PFA) diluted in PBS for 25 min at 4°C, washed twice with PBS for 5 min, immersed in 0.2% Triton X-100 diluted in PBS for 5 min, and washed twice with PBS for 5 min. Then, 100 μL of equilibration buffer was added to cover the slides for 10 min. The rTdT incubation buffer, which contained 45 μL of equilibration buffer, 5 μL of nucleotide mix and 1 μL of rTdT enzyme, was prepared. The slides were covered with 50 μL of rTdT incubation buffer in a dark box at 37°C for 1 h. The slides were washed three times with PBS for 5 min. DAPI was diluted with mounting solution (DAPI:mounting solution = 1:20,000). Finally, the cells were observed under the LSM700 fluorescence microscope.

Statistical analysis

Unless stated elsewhere, significance levels for comparisons between two groups were determined by one-way ANOVA tests. Data are presented as the mean ± SEM. Sample size was based on empirical data from pilot experiments. No additional randomization or blinding was used to allocate experimental groups. All images were chosen at random and quantitated using ImageJ. $P < 0.05$ indicated a significant difference.

Results

miR-183-5p inhibits apoptosis but promotes autophagy by downregulating Nrdp1 expression

To explore whether any miRNAs could regulate Nrdp1's function, we searched for miRNAs that could potentially bind with the 3′UTR of Nrdp1 mRNA by using the miRTarbase database (http://mirtarbase.mbc.nctu.edu.tw/index.html) (Figure 1A). Several miRNAs were predicted to potentially bind with the 3′UTR of

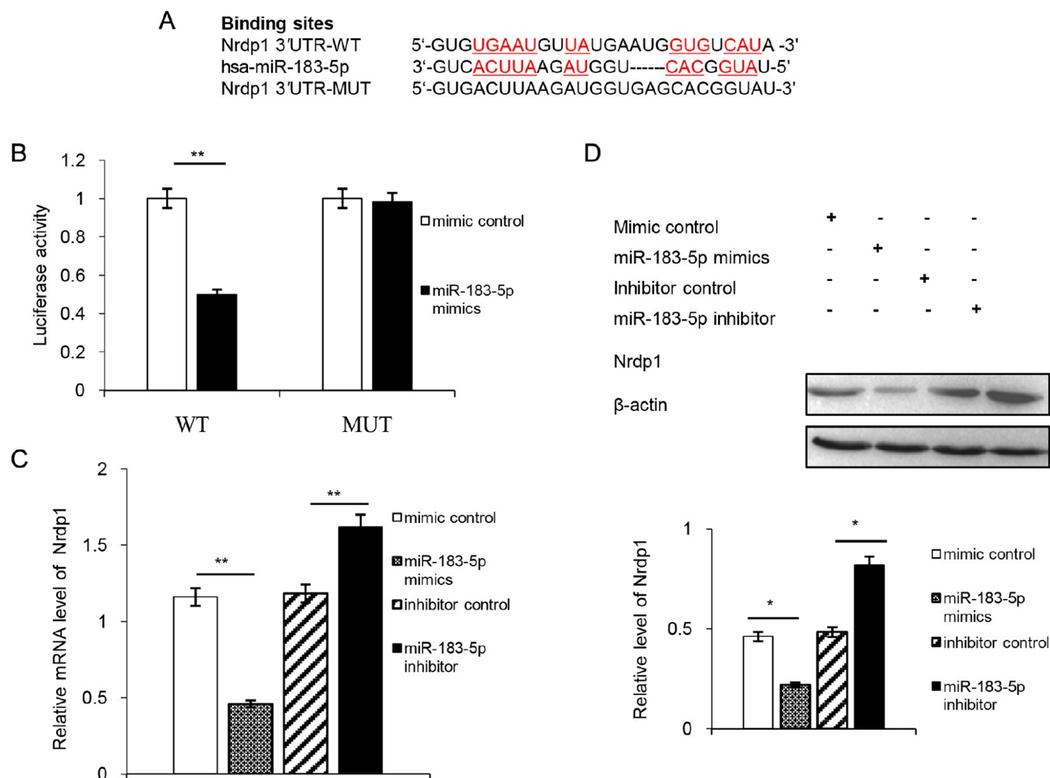

Figure 1. miR-183-5p downregulates the expression of Nrdp1 (A) Binding sites between miR-183-5p and the 3′UTR of Nrdp1 are underlined. (B) miR-183-5p mimic control or miR-183-5p mimics were cotransfected with the luciferase reporter containing the wild-type 3′UTR of Nrdp1 (Nrdp1 3′UTR-WT) or its mutant (Nrdp1 3′UTR-MUT) in HeLa cells. Then, the luciferase activities in each group were determined. (C,D) Analyses of the mRNA by RT-PCR (C) and protein levels of Nrdp1 by western blot analysis (D) after transfecting HeLa cells as in B. Nrdp1 levels were quantified by densitometry (normalized to β-actin). Data are representative of one experiment with two independent biological replicates. $*P < 0.05$, $**P < 0.01$ (two-tailed unpaired, mean ± SEM).

Nrdp1 mRNA, but miR-183-5p had the highest score. Among all known miR-183-5p target genes, Nrdp1 mRNA ranked second next to THEM4 to complement miR-183-5p (Table 3). Since the top-ranked THEM4 has been shown to be targeted by miR-183-5p in mediating the PI3K/AKT and NF-κB pathways [17], we tried to study the regulation of Nrdp1 by miR-183-5p. Dual-luciferase reporter assay indicated that miR-183-5p repressed the luciferase activity of the wild-type 3′UTR of the Nrdp1 reporter vector but did not markedly change that of the reporter vector containing mutated binding sites in HeLa cells (Figure 1B). We successfully transfected the mimics or inhibitor of miR-183-5p into HeLa cells and found that Nrdp1 expression declined at both the mRNA and protein levels in the cells transfected with miR-183-5p mimics but was elevated in the cells transfected with the miR-183-5p inhibitor (Figure 1C,D).

We previously reported that transfection with Nrdp1 sensitizes cells to caspase 3 activation and apoptosis [10]. To explore whether miR-183-5p regulates apoptosis, we transfected HeLa cells with the miR-183-5p mimics or inhibitor for 48 h. Following treatment with the DNA topoisomerase II inhibitor etoposide at 0, 5, 10, or 20 μM for 24 h, the levels of active caspase 3 were reduced by the miR-183-5p mimics but were elevated by the miR-183-5p inhibitor (Figure 2A). Furthermore, the level of BRUCE was reciprocally regulated by miR-183-5p in comparison to those of Nrdp1 (Figure 2A) because Nrdp1 promotes ubiquitination and proteasomal degradation of BRUCE upon initiation of apoptosis, as we reported previously [10]. Notably, overexpression of Nrdp1 abolished the enhancing effect of miR183-5p mimics on the level of BRUCE (Figure 2B). Next, we employed the TUNEL (TdT-mediated dUTP nick-end labelling) assay to detect apoptosis and found that the miR-183-5p mimic reduced, but its inhibitor enhanced, etoposide-induced apoptosis (Figure 2C,D). Furthermore, we attempted to use the autophagy marker LC3-II to monitor the effect of miR-183-5p on autophagy. After transfecting HeLa cells with the miR-183-5p mimics or inhibitor for 48 h, etoposide (20 μM) was added and incubated for 24 h. Immunostaining analyses showed that the number of LC3-II puncta in autophagosomes was increased by the miR-183-5p mimics but decreased by the miR-183-5p inhibitor (Figure 2E). These results suggest that miR-183-5p inhibits apoptosis but promotes autophagy by downregulating Nrdp1 expression.

Table 3. Comparison of pairing sequences of miR-183-5p targets

Name	Score (%)	Sequence
hsa-miR-183-5p	100	3′-GUCACUUAAGAUGGUCACGGUAU-5′
THEM4	60.87	5′-GAGAUUAAACCACU–GUGCCAU–U-3′
Nrdp1	56.52	5′-G-----UGAAUGUUAUG--GUG--CAUA-3′
Erbin	34.78	5′-UGGACCAGGAAAAAGUGCCAUA-3′
ERG	30.43	5′-AGUCUUAAGGACAAAGUGCCA––A-3′
EZR	30.43	5′-UUCCCCAGUUGUAAAGUGCCA––A-3′
FOXP1	30.43	5′-A–––UGUGUUAACUCU–GUGCCAUC-3′
GDNF	30.43	5′-GUUUCUUUUCAAAG––GUGCCA––A-3′
GREM1	30.43	5′-AACUUUCAUUGAAAA––UGCCAUA-3′
MDM4	30.43	5′-CACGUCUGAUAUCAC–––UGCCAUA-3′
MTA1	30.43	5′-GUGUUCUGUUGAAG––GUGCCAUU-3′
PDCD4	30.43	5′-AGCUACCUUUUGUAAGUGCCAUG-3′
PIK3CA	30.43	5′-CUGUGUUUUACCCGAGUGCCA––A-3′
PLK1	30.43	5′-UGGUUGGCUCCCGCGGUGCCAUG-3′
RGS2	30.43	5′-CAGUGUCCGUUA––––AGUGCCA––A-3′
SMAD4	30.43	5′-ACUUCAAAAAUAAU–GUGCCAU–G-3′
ZEB2	30.43	5′-CUGUGUUUCUGCAA–GUGCCAU–C-3′
FOXO1	26.09	5′-CUGUAGAUAAGGACUGUGCCA––U-3′
FOXO4	26.09	5′-AGGGUUUUUCUCACUGUGCCA––A-3′
TMED5	26.09	5′-AUACAACAAAGUUUC–UGCCAU–C-3′
TP53	26.09	5′-CAUCUCUCCCUCCCC––UGCCAU–U-3′
TUSC2	26.09	5′-UUCUUCCGACCUACC––UGCCAU–U-3′

Target sequences are underlined. The pairing score of each target in percentile was obtained by normalizing the number of bases paired with miR-183-5p to that of total bases.

LINC00960 upregulates Nrdp1 by sponging miR-183-5p

To define any lncRNAs that might regulate miR-183-5p, we predicted potential miR-183-5p-binding lncRNAs using the DIANA database (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php) and found that LINC00960 contains the sequence to potentially bind with miR-183-5p (Figure 3A). LINC00960 bears closer potential binding sites with miR-183-5p than Nrdp1 (Figure 3A). A few miRNAs have been reported to be targeted by LINC00960 [18–21], but LINC00960 bears the highest score to complement miR-183-5p (Supplementary Table S1). Several lncRNAs or circRNAs have also been shown to target miR-183-5p [22–28], but LINC00960 ranks at the top among all these noncoding RNAs (Supplementary Table S2). We next transfected LINC00960 (pcDNA6B-LINC00960) or its siRNA oligos into HeLa cells and found that LINC00960 upregulated, but its siRNA oligos down-regulated, the protein level of Nrdp1 (Figure 3B). miR-183-5p markedly repressed the luciferase activity of the wild-type LINC00960 reporter but did not change that of the reporter containing mutated binding sites (Figure 3C). RT-PCR analyses demonstrated that transfection with LINC00960 reduced, but its siRNA oligos increased, the level of miR-183-5p in HeLa cells (Figure 3D). These results suggest that LINC00960 upregulates Nrdp1 by sponging miR-183-5p.

Next, we analysed apoptosis following transfection of HeLa cells with LINC00960 or its siRNA oligos. As predicted, etoposide increased the level of active caspase 3 in a dose-dependent manner. The decrease in the level of Nrdp1 induced by si-LINC00960 was only visible after treatment with high dose of etoposide (20 μM), raising the possibility that etoposide by itself might upregulate the expression of Nrdp1 (Figure 4A). Overexpression of LINC00960 enhanced, but siRNA oligos for LINC00960 suppressed, the etoposide-induced activation of caspase 3 in HeLa cells (Figure 4A). The TUNEL assay showed that overexpression of LINC00960 elevated, but siRNA oligos for LINC00960 reduced, the level of apoptosis in the presence of etoposide (Figure 4B,C). Conversely, overexpression of LINC00960 decreased, but siRNA oligos for LINC00960 increased, the numbers of LC3-II puncta in autophagosomes of the HeLa cells treated with etoposide (Figure 4D). These results suggest that LINC00960 upregulates the expression of Nrdp1 and promotes apoptosis probably by competitively inhibiting miR-183-5p, while miR-183-5p downregulates Nrdp1 expression.

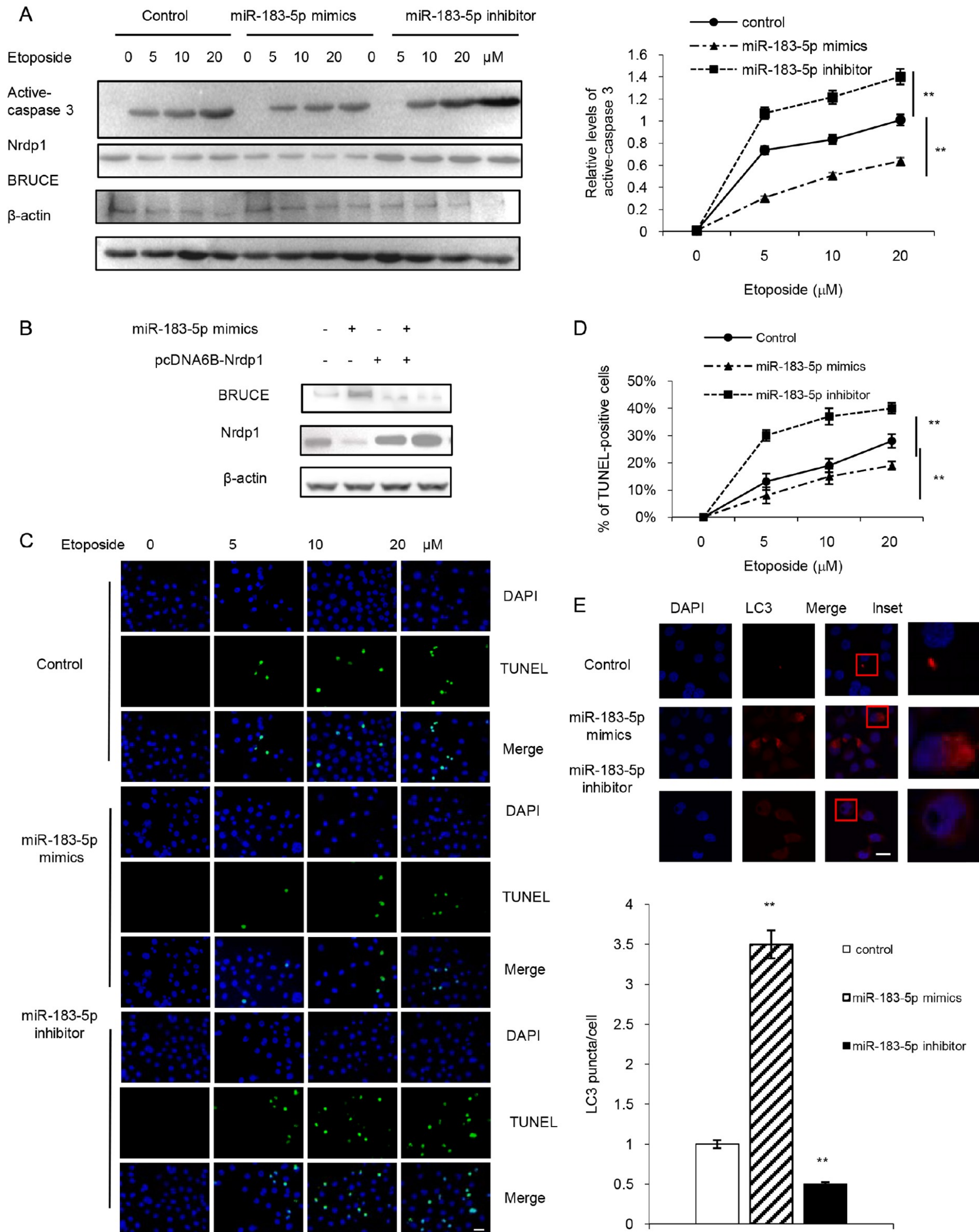

Figure 2. miR-183-5p inhibits apoptosis (A) The protein levels of active caspase 3, Nrdp1, and BRUCE were detected by western blot analysis after transfection of HeLa cells with the miR-183-5p mimics or inhibitor for 48 h and treatment with etoposide at 0, 5, 10 or 20 μM for 24 h. Active caspase 3 levels were quantified by densitometry (normalized to β-actin). (B) Following transfection of HeLa cells with the Nrdp1 and/or miR-183-5p mimics for 48 h, protein levels were analysed by western blot analysis. (C and D) TUNEL assay was used to detect the levels of apoptosis in HeLa cells transfected and treated as in (A). Scale bar = 10 μm (n = 20 images). (E) After transfection of HeLa cells with the miR-183-5p mimics or inhibitor for 48 h and treatment with etoposide (20 μM) for 24 h, LC3-II-containing autophagosomes were visualized by immunostaining. Scale bar = 10 μm (n = 20 images). Data are representative of one experiment with two independent biological replicates. *P < 0.05, **P < 0.01 (two-tailed unpaired, mean ± SEM).

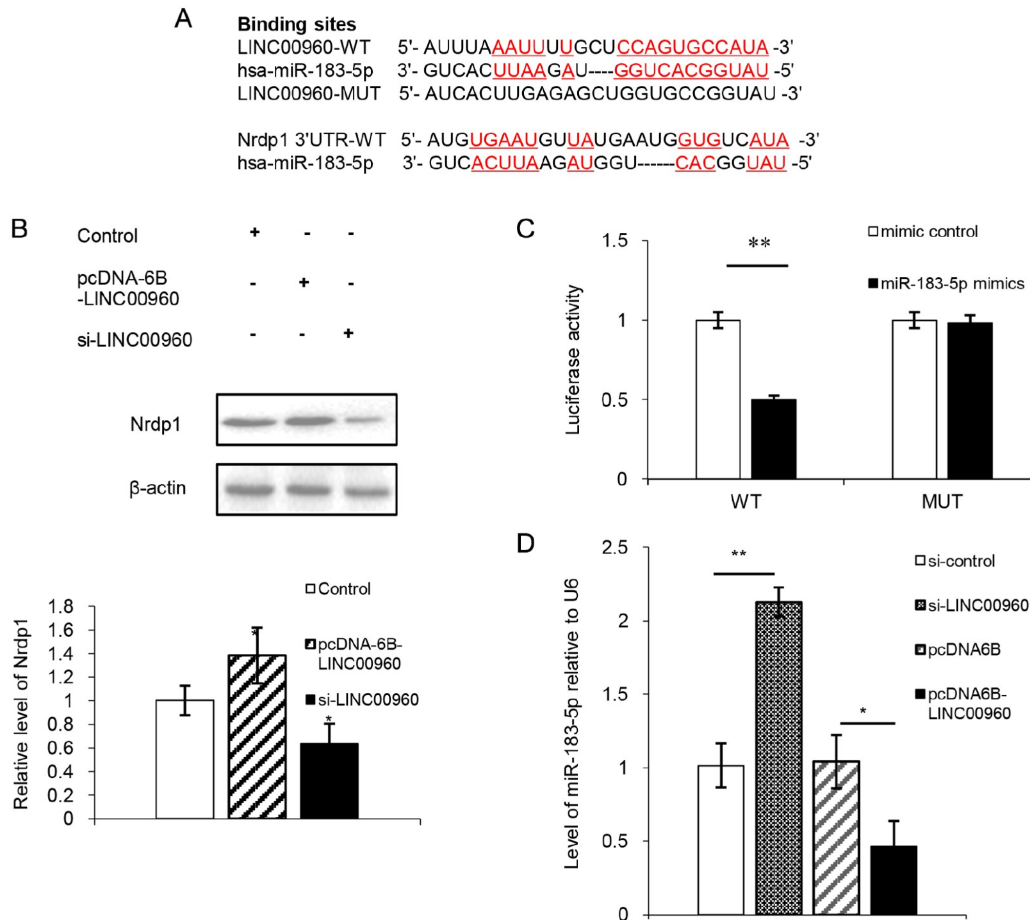

Figure 3. LINC00960 upregulates Nrdp1 by sponging miR-183-5p (A) Binding sites between miR-183-5p and LINC00960 are underlined. (B) The protein levels of Nrdp1 were detected after transfection of HeLa cells with LINC00960 or its siRNA oligos. Nrdp1 levels were quantified by densitometry (normalized to β-actin). (C) The miR-183-5p mimic control or miR-183-5p mimic was cotransfected with a luciferase reporter containing the wild-type miR-183-5p-binding sequence of LINC00960 (LINC00960-WT) or its mutant (LINC00960-MUT). Then, the luciferase activity of the LINC00960 reporter in the cells in each group was determined. (D) Detection of the levels of miR-183-5p by RT–PCR after transfection of HeLa cells with LINC00960 or its siRNA oligos. Data are representative of one experiment with two independent biological replicates. *$P < 0.05$, **$P < 0.01$ (two-tailed unpaired, mean ± SEM).

Expression levels of LINC00960 and miR-183-5p are inversely correlated in multiple cancer cell lines

To explore the relationships of LINC00960 and miR-183-5p with cancers, we analysed the expression of LINC00960 in 33 types of cancer by using the GEPIA database (http://gepia.cancer-pku.cn/index.html). Compared with that in the normal tissues, the expression of LINC00960 was low in CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma), SKCM (skin cutaneous melanoma), and TGCT (testicular germ cell tumors) but high in THYM (thymoma) (Figure 5A). Furthermore, a positive correlation between the expression of LINC00960 and Nrdp1 was found in these four cancers by using the GEPIA database (Figure 5B). As reported previously [29–34], the expression of miR-183-5p is lower in CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma) and LUAD (lung adenocarcinoma) but higher in ACC (adrenocortical carcinoma), BRCA (breast invasive carcinoma), COAD (colon adenocarcinoma), and NSCLC (non-small cell lung cancer) than in normal tissues (Table 4). Finally, we analysed the expression of LINC00960 and miR-183-5p by RT-PCR, and an inverse correlation was observed in multiple cancer cell lines, including HeLa human cervical cancer cells, MCF-7 human breast adenocarcinoma cells, HCT116 human colon carcinoma cells, HepG2 human liver cancer cells, and U2OS human osteosarcoma cells (Figure 5C).

We next analysed the relationship of LINC00960 and miR-183-5p with patient survival probability in various cancers by using the Kaplan-Meier Plotter database (http://kmplot.com/analysis/index.php?p = background) and found that the expression of LINC00960 was positively associated with patient survival in BRCA (breast invasive carcinoma) and KIRP (kidney renal papillary cell carcinoma) but negatively associated with patient survival in CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma) (Figure 6A). Meanwhile, the expression of miR-183-5p was negatively associated with patient survival in KIRC (kidney renal clear cell carcinoma), LIHC (liver hepatocellular carcinoma), READ (rectum adenocarcinoma), and SARC (sarcoma) but positively associated with patient survival in LUSC (lung squamous cell carcinoma) and STAD (stomach adenocarcinoma), as documented in the Kaplan–Meier Plotter database (Figure 6B). Thus, the expression levels of LINC00960 and miR-183-5p are inversely correlated in multiple types of cancer cell lines.

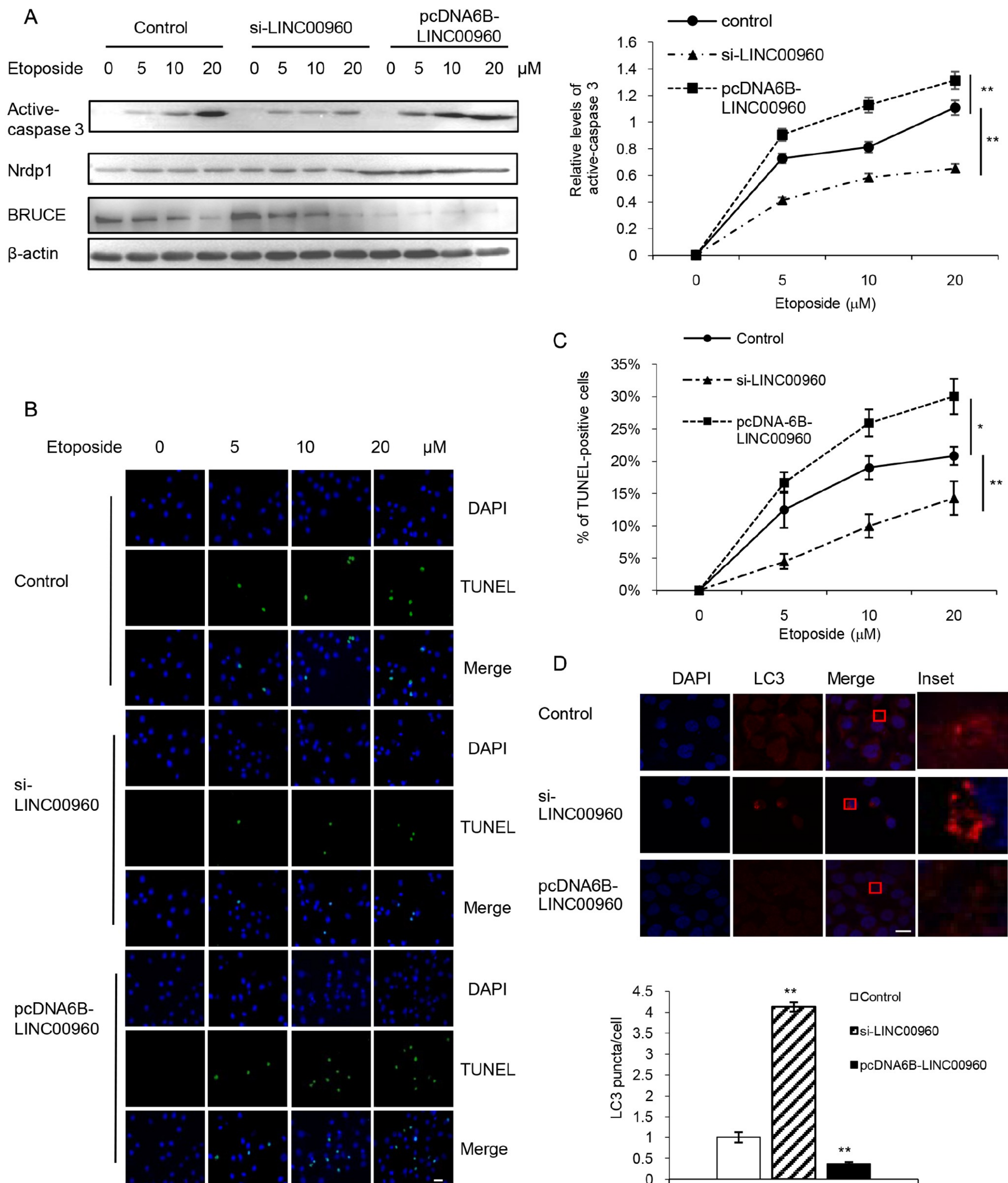

Figure 4. LINC00960 promotes apoptosis but inhibits autophagy (A) The protein levels of active caspase 3, Nrdp1, BRUCE and β-actin were detected by western blot analysis after transfection of HeLa cells with LINC00960 or its siRNA oligos for 48 h and treatment with etoposide at 0, 5, 10 or 20 μM for 24 h. Active caspase 3 level was quantified by densitometry (normalized to β-actin). (B and C) TUNEL assay was used to detect the levels of apoptosis after treatment of HeLa cells as in (A). Scale bar = 10 μm (n = 20 images). (D) After transfection of HeLa cells with LINC00960 or its siRNA oligos for 48 h and treatment with etoposide (20 μM) for 24 h, LC3-II-containing autophagosomes were visualized by immunostaining. Scale bar = 10 μm (n = 20 images). Data are representative of one experiment with two independent biological replicates. *P < 0.05, **P < 0.01 (two-tailed unpaired, mean ± SEM).

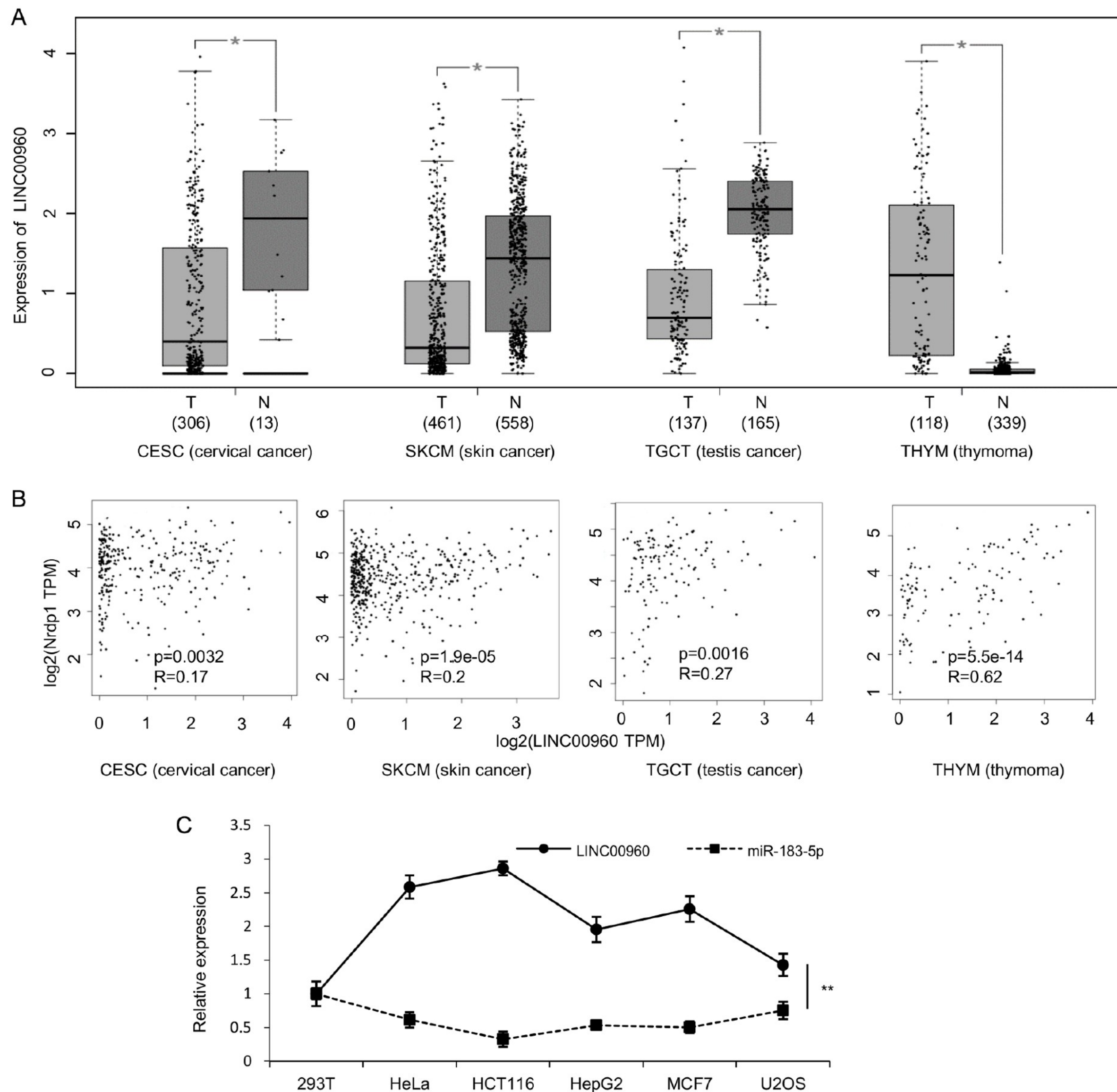

Figure 5. The expression levels of LINC00960 and miR-183-5p are inversely correlated in multiple cancer cell lines (A) Expression of LINC00960 in CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma), SKCM (skin cutaneous melanoma), TGCT (testicular germ cell tumors) and THYM (thymoma) (T) compared to normal tissues (N). Sample numbers are shown in parentheses. (B) Positive correlation of LINC0960 and Nrdp1 expression in CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma), SKCM (skin cutaneous melanoma), TGCT (testicular germ cell tumors) and THYM (thymoma). (C) The expression levels of LINC00960 and miR-183-5p were analysed by RT-PCR in multiple cancer cell lines. The 293T cell line served as a control. Data are representative of one experiment with two independent biological replicates. *$P<0.05$, **$P<0.01$ (two-tailed unpaired, mean ± SEM).

Table 4. Summary of reported expression of miR-183-5p in six tumors compared to normal tissues

Tumor	miR-183-5p level	Ref.
ACC	High ($n=41$)	[30]
BRCA	High ($n=18$)	[29]
CESC	Low ($n=43$)	[33]
COAD	High ($n=11$)	[28]
LUAD	Low ($n=15$)	[31]
NSCLC	High ($n=64$)	[32]

ACC, adrenal cortical carcinoma; BRCA, breast cancer; CESC, cervical cancer; COAD, colorectal cancer; LUAD, lung cancer; NSCLC, non-small cell lung cancer.

Discussion

Nrdp1 is a RING finger-type ubiquitin ligase that potentially ubiquitinates a number of proteins, including BRUCE [10], ErbB3 [11], USP8 [35], Parkin [12], HPV 16 E2 [36], HBV-encoded X protein (HBx) [37], SIP/CACYBP [38] and LCP1 [39], and regulates cell proliferation, apoptosis and oxidative stress. Nrdp1 expression levels appear to be significantly decreased in hepatocellular carcinoma tissues [40], glioblastoma [41], renal cell carcinoma [42], and mammary tumors [43], implying that Nrdp1 may function as a tumor-suppressive protein. miR-183-5p, which is located on human chromosome 7q32.2, forms a cluster with miR-96 and miR-182 in certain cases [44,45]. miR-183-5p regulates the proliferation, viability, migration and invasion of cancer cells as well as many

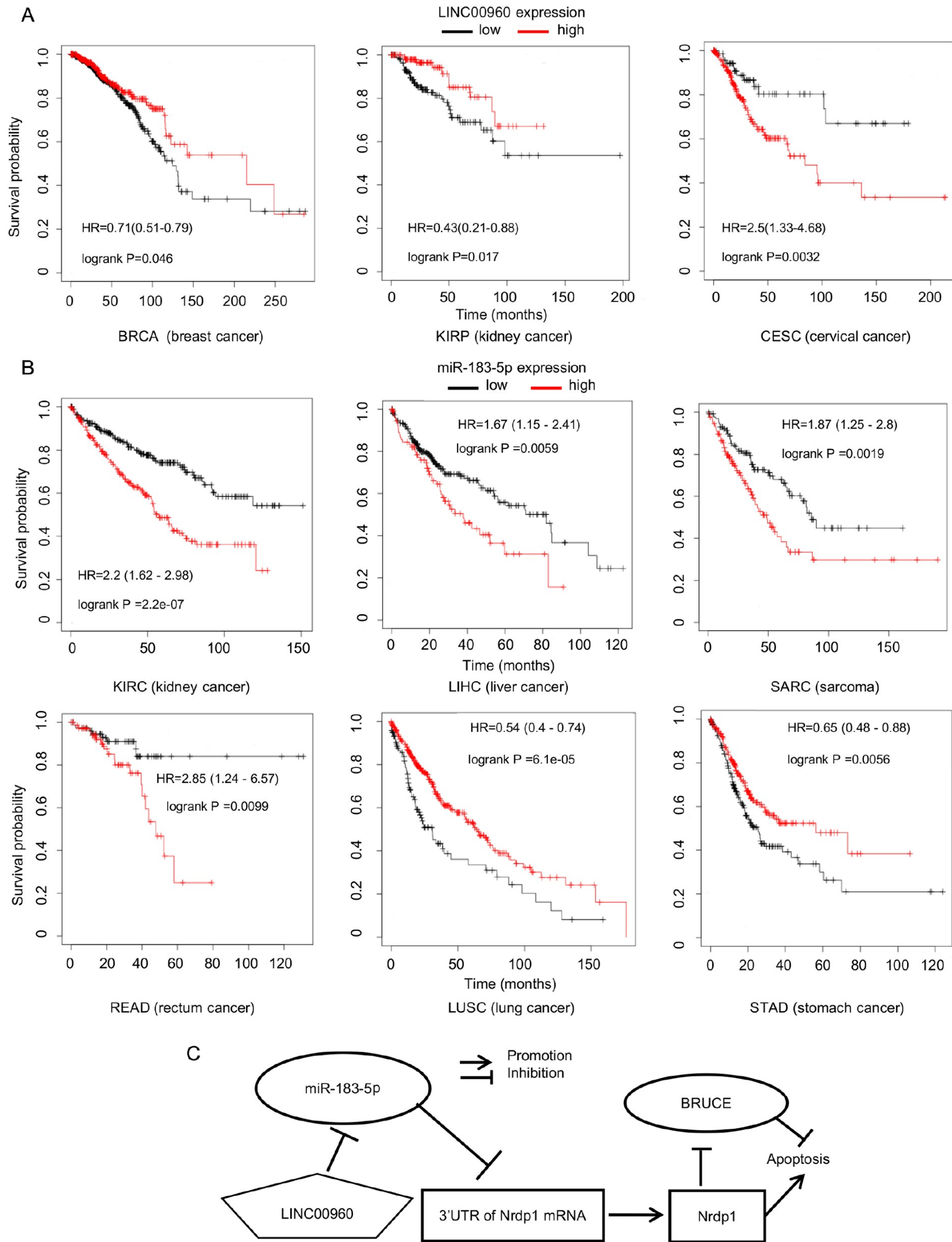

Figure 6. LINC00960 and miR-183-5p are potentially associated with patient survival in certain types of cancer (A) Expression of LINC00960 is associated with the survival of patients with BRCA (breast invasive carcinoma), KIRP (kidney renal papillary cell carcinoma), and CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma). (B) Expression of miR-183-5p is associated with survival of patients in KIRC (kidney renal clear cell carcinoma), LIHC (liver hepatocellular carcinoma), READ (rectum adenocarcinoma), SARC (sarcoma), LUSC (lung squamous cell carcinoma) and STAD (stomach adenocarcinoma). HR means hazards ratio, which was calculated based on the Cox PH Model. The group cut-off standard of high or low is 50%. (C) Model mechanisms by which LINC00960 upregulates Nrdp1 and promotes apoptosis by sponging miR-183-5p.

other processes, such as viral infection and obesity- or diabetes-related cellular activities, because it might directly target a broad spectrum of diversified genes, including transcription factor ERG [24], four and a half LIM domains protein 1 (FHL1) [46], FOXO1 [23, 47], FOXO4 [48], FOXP1 [49], GREM1 [22], insulin receptor substrate 1 (IRS1) [50], MTA1 [51], programmed cell death 4 (PDCD4) [52], PLK1 [53], EGR1 [54], regulator of G protein signaling 2 (RGS2) [55], SMAD4 [56], thioesterase superfamily member 4 (THEM4) [17], TMED5 [25], tumor suppressor candidate 2 (TUSC2) [27], and ZEB2 [57]. miR-183-5p is one of the most frequent cancer-associated miRNAs with oncogenic properties in various malignancies, such as adrenal cortical carcinoma, colorectal cancer, breast cancer, lung cancer, cervical cancer and non-small cell lung cancer [29–34]. miR-183-5p was detected with different expression tendencies in different types of tumors, probably because there are different pathways regulating miR-183-5p expression in different types of tumors. On the other hand, miR-183-5p might play a tumor-suppressive role in several types of cancer. For example, miR-183-5p was shown to inhibit the growth of lung cancer by suppressing PIK3CA [32]. How these miR-183-5p-mediated activities against different targets are coordinated remains elusive. Following cleavage of precursor RNA transcripts by Ribonuclease III enzymes, miRNAs are usually loaded to the effector protein Argonaute to form the miRNA-induced silencing complex (miRISC). The specific miRISC binds with the complementary sequence in its mRNA target and thus silences the target by destabilizing mRNA and/or repressing translation [58]. Most likely, miR-183-5p targets and suppresses the expression of Nrdp1 in this manner because the 3′UTR of Nrdp1 possesses a binding sequence for miR-183-5p. Our results suggest that miR-183-5p may play an oncogenic role in HeLa cells, at least partially by targeting Nrdp1, which is among the few with top scores to complement miR-183-5p.

The expression of miR-183-5p has been reported to be regulated by several circRNAs and lncRNAs. miR-183-5p promotes the development of glioma by suppressing the expression of TUSC2, whereas circ-EGFR can regulate the TUSC2 level by sponging miR-183-5p [27]. circ_DHRS3 enhances GREM1 expression by competitively targeting miR-183-5p to regulate chondrocyte proliferation and apoptosis [22]. lncRNA PVT1 in exosomes promotes osteosarcoma growth and metastasis by sponging miR-183-5p [24]. LINC00960 was found to play an active role in promoting the malignant behaviors of bladder cancer cells [59], pancreatic ductal adenocarcinoma [19], lung adenocarcinoma [18], and osteosarcoma [60]. Interestingly, LINC00960 was also shown to promote lung adenocarcinoma by sponging miR-124a, the latter of which suppresses the expression of SphK1 [18] and regulates cell viability, migration and invasion in pancreatic ductal adenocarcinoma by modulating miR-146a-5p, which targets interleukin 1 receptor-associated kinase 1 [19]. However, LINC00960 is the noncoding RNA with the highest score to complement miR-183-5p. This study demonstrates that the expression of LINC00960 is positively correlated with that of Nrdp1 in several tumors but inversely correlated with that of miR-183-5p in multiple cancer cell lines.

In conclusion, this study suggests that miR-183-5p can suppress apoptosis by reducing Nrdp1 expression at both the mRNA and protein levels, whereas LINC00960 upregulates Nrdp1 and promotes apoptosis probably by sponging miR-183-5p (Figure 6C). Our results suggest that the LINC00960-miR-183-5p-Nrdp1 axis might regulate tumorigenesis or tumor progression, providing important clues for the prevention, diagnosis, and treatment of various types of tumors.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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