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lincRNA-p21 Mediates the Anti-Cancer Effect of Ginkgo Biloba Extract EGb 761 by Stabilizing E-Cadherin Protein in Colon Cancer

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background:

Ginkgo biloba extract EGb 761 is a putative antioxidant and has been used for thousands of years to treat a variety of ailments, including cancer. While it is known that cell behavior can be modulated by long non-coding RNAs (lncRNAs), the contributions of lncRNAs in EGb 761-induced anti-cancer effects are largely unknown.

Material/Methods:

Colon cancer cell lines HT29 and HCT116 were used in this study. RT-qPCR was used to detect the relative expression of lincRNA-p21 in colon cancer cells. Wound-healing assay and Matrigel Transwell assay were performed to investigate the migration and invasion of colon cancer cells. Immunoprecipitation and Western blot experiments were used to verify ubiquitination and the interaction between lincRNA-p21 and E-cadherin, or E-cadherin and b-transducin repeat containing (BTRC) E3 ubiquitin protein ligase.

Results:

Cell function assay verified that treatment with EGb 761 suppressed the migratory and invasive abilities of colon cancer cells in a dose-dependent manner via the suppression of E-cadherin expression level. lincRNA-p21 was upregulated in colon cancer cells after treatment with EGb 761, and knockdown of lincRNA-p21 reversed the EGb 761-induced anti-metastatic effect. Furthermore, lincRNA-p21 was localized in cytoplasm of colon cells and regulated E-cadherin expression at a post-transcriptional level. Specifically, lincRNA-p21 promotes E-cadherin stability by preventing the interaction between BTRC and E-cadherin, which leads to the inhibition of E-cadherin ubiquitination.

Conclusions:

We demonstrated that lincRNA-p21 mediates the anti-cancer effect of Ginkgo biloba extract EGb 761 by stabilizing E-cadherin protein in colon cancer, which may help define the functional role of EGb 761 in cancer treatment.

MeSH Keywords:

Cadherins • Colonic Neoplasms • RNA, Long Noncoding • Ubiquitination

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Background

Colon cancer is the most common gastrointestinal cancer worldwide. Despite great achievements in surgery, chemotherapy, and the development of novel molecular-targeted drugs, the incidence of colon cancer continues to increase [1,2]. Early diagnostic and therapeutic strategies can improve the overall survival of patients with colon cancer. However, for patients diagnosed with advanced-stage disease, colon cancer has often metastasized [2]. The majority of these deaths are caused by the progression of the tumor to metastatic disease. The 5-year survival rate for metastatic colon cancer is 10–15%, compared to 40–90% for non-metastatic colon cancer [3]. Currently, wide use of adjacent chemotherapy following surgery resection has dramatically improved the clinical outcome of colon cancer patients. However, nearly a one-quarter of patients with colon cancer experience metastatic disease and finally become chemoresistant [4]. Early screening and novel therapeutic approaches are essential for improved prognosis of colon cancer.

Herbal medicines such as Ginkgo biloba have long been accepted as useful adjacent therapeutic regimens for various diseases. The use of Ginkgo biloba as an alternative medicine is widespread in the developed world. Leaf extracts have been used in traditional Oriental medicine for several hundred years. Ginkgo biloba extract (EGb 761) is well known for its antioxidant property, which may result from its ability to scavenge free radicals [5] and to neutralize ferryl ion-induced peroxidation [6]. In recent years, many studies have reported anti-cancer effects in different cancer types, including breast cancer and oral cavity cancer [7–10]. Our previous study revealed that EGb 761 inhibited colorectal cancer cell migration via increasing the expression of lincRNA-p21 [11]. However, the underlying regulatory mechanism of EGb 761 is still unclear.

lincRNAs are a class of long non-coding RNAs of greater than 200 nucleotides in length, which play a crucial role in regulating gene expression [12,13]. lincRNA-p21 is a long intergenic non-coding RNA (3100 nt). Because of lincRNA-p21's location on chromosome 17, approximately 15 kb upstream from the CDKN1A (p21) gene, it was named lincRNA-p21 [14]. lincRNA-p21 has been widely reported and well accepted as a tumor suppressor [15–17]. However, the role of lincRNA-p21 in EGb761 functioning in cancer is still unclear.

Based on our previous study, we investigated the underlying regulatory mechanism of lincRNA-p21 during the cancer-suppressive role of EGb 761. We identified that lincRNA-p21 located in cytoplasm enhances the EGb 761-induced anti-metastatic effect by stabilizing E-cadherin in colon cancer cells. More importantly, we revealed that lincRNA-p21 stabilizes E-cadherin protein by inhibition its ubiquitination and decomposition.

Material and Methods

Cell culture and drugs

The human colon cancer cell lines HT29 and HCT116 were purchased from the Chinese Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultured in RPMI 1640 medium (BioWhittaker, Lonza, USA) supplemented with 10 mM Hepes, 1 mM L-glutamine, and 100 U/ml penicillin/streptomycin (BioWhittaker, Lonza) and heat-inactivated 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified incubator with 5% CO₂. Protein synthesis inhibitor cycloheximide (CHX) and proteasome inhibitor MG132 were purchased from Sigma (St. Louis, MO, USA), and was used at the concentration of 50 µg/ml and 20 µM, respectively. Ginkgo biloba extract EGb 761 was purchased from Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany) and used at the concentration of 250 mg/mL or 500 mg/mL.

Reverse transcription-quantitative PCR (RT-qPCR)

RNA extraction from cell fraction was performed using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was reverse transcribed using SuperScript III® (Invitrogen; Thermo Fisher Scientific, Inc.) and amplified by RT-qPCR based on the TaqMan method using a TaqMan Human RNA Assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on a Bio-Rad CFX96 Sequence Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The gene expression levels were normalized to GAPDH expression, and analyzed and expressed relative to quantification cycle values [18]. The primer sequences were: lincRNA-p21 (Forward) 5'-CCCGGGCTTGTCTTTTGT-3', (Reverse) 5'-GAGTGGGTGGCTCACTCTTCTG-3'; E-cadherin (Forward) 5'-TCTTCCAGGAACCTCTGTGATG-3'; (Reverse) 5'-CAATGCCCATCGCTTACACC-3'; GAPDH (Forward) 5'-GCACCGTCAAGGCTGAGAAC-3', (Reverse) 5'-ATGGTGGTGAAGACGCAGT-3'.

Cell transfection

The small-interfering RNA against lincRNA-p21 (si-lincRNA-p21) and BTRC (si-BTRC) and respective overexpression plasmid vectors were synthesized and prepared by GenePharma (Shanghai, China). Negative control siRNA was purchased from Invitrogen (CAT#12935-110, Shanghai, China). All the vectors were labeled with green fluorescence protein (GFP). Cells were plated at 1×10⁵ cells/well in 24-well plates ~24 h prior to transfection or treatment. When the cells had reached 50% confluence, cells were transfected with the vectors described above using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific) for 24 h, following the manufacturer's protocol. Transfection efficiency was evaluated in

every experiment by RT-qPCR 24 h subsequently to ensure that the cells were successfully transfected. The cells were then subjected to RNA/protein extraction and further functional assays. The sequences of small-interfering RNAs were: si-lincRNA-p21#1 5'-UGAAAAGAGCCGUGAGCUAdTdT-3'; si-lincRNA-p21#2 5'-AAATAAAGATGGTGGATGdTdT-3'; si-lincRNA-p21#3 5'-CUGCAAGGCCGAUGAUGAdTdT-3'; si-BTRC 5'-AUCAGCUCGUCGAACCUUdTdT-3'.

Immunoprecipitation (IP)

Colon cancer cells were rinsed with cold PBS and fixed by 1% formaldehyde for 10 min. After centrifugation, cell pellets were collected and re-suspended in the NP-40 lysis buffer. For RNA immunoprecipitation (RIP) assay, the supernatant was incubated overnight with beads conjugated with anti-E-cadherin antibody (Abcam, ab1416, 1: 50) or negative control mouse IgG (Millipore). The beads were then rinsed with cold NT2 buffer, and cultured with proteinase K at 10 mg/ml (Sigma-Aldrich, USA). We also performed IP assay to verify the interaction between BTRC and E-cadherin by using an anti-BTRC antibody (Cell Signaling Technology, D13F10, 1: 50).

Cell migration and invasion assays

Cell migration was evaluated by performing wound-healing assay. For wound-healing assay, cells were seeded at a density of 5×10^5 cell/well onto 6-well plates. Twelve hours after treatment with EGb 761 or (and) transfection with si-lincRNA-p21, the layer of cells was scratched to form wounds by using a sterile 20- μ l pipette tip; the non-adherent cells were washed away with culture medium, and then the cells were further cultured for 48 h, then photographed to identify the gap area. Cell invasive ability was evaluated by using Transwell invasion assay using Boyden chambers (BD Biosciences) with 8- μ m pore size membranes with Matrigel. Cells in serum-free media were placed into the upper chamber of an insert. Media containing 10% FBS was added to the lower chamber. After several hours of incubation, the cells that had invaded through the membrane were stained with methanol and 0.1% crystal violet, imaged, and counted under a microscope at 20 \times magnification in random fields in each well.

Nucleus-cytoplasm fractionation

Nuclear and cytoplasmic RNA from cultured colon cancer cells were isolated by SurePrep Nuclear or Cytoplasmic RNA Purification Kit (product #BP2805-25, Fisher Scientific, Inc.) following the manufacturer's instruction. U1- or GAPDH RNA-processed mRNA was detected in isolated RNAs as control for nuclear RNA or cytoplasm RNA, respectively. Biological triplicates were carried out, followed by RT-qPCR, to detect abundance of lincRNA-p21

Western blot and antibodies

Cell lysates were prepared with RIPA buffer containing protease inhibitors (Sigma, USA). Cell lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, USA). Then, the membrane was blocked with 5% (5 g/100 mL) nonfat dry milk in tri-buffered saline plus Tween (TBS-T) buffer for 2 h at room temperature and incubated with specific primary antibodies (1: 1000 solution) at 4°C overnight, followed by horseradish peroxidase-conjugated (HRP) secondary antibodies at room temperature for 1 h. The primary antibodies used were: anti-E-cadherin (Abcam, ab1416), anti-Ubiquitin (Abcam, ab7780) and anti-BTRC (CST, D13F10), FLAG, and HA (Sigma, F1804 and H3663).

Statistical analysis

Kolmogorov-Smirnov testing was applied for data analysis with the distribution of each group of samples. Data are presented as medians (interquartile range). Comparisons between 2 groups were performed by nonparametric Mann-Whitney U tests. The Kruskal-Wallis test (post hoc Mann-Whitney U test with Bonferroni correction) was used for evaluating the difference among multiple treatment groups. A 2-sided $P < 0.05$ was considered as statistically significant. All statistical data were analyzed using SPSS 18.0 software (SPSS, Chicago, IL) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

Results

EGb 761 suppresses migration and invasion of colon cancer cells

We first investigated whether EGb 761 suppressed cell migration and invasion. As expected, EGb761 significantly inhibited the migratory ability of HT29 and HCT116 cells after treatment for 48 h. Moreover, this effect induced by EGb761 was in a dose-dependent manner as evidenced by the decreased migration in the 500 mg/L EGb 761 group compared to the 250 mg/L EGb 761 group (Figure 1A). In addition, Matrigel Transwell assay showed that EGb 761 suppressed the invasive ability of colon cancer cells in a dose-dependent manner (Figure 1B). Then, we used Western blot analysis to explore the influence of EGb 761 (500 mg, 48 h) on E-cadherin, an epithelial marker downregulated in invasive cells; or vimentin and N-cadherin, the mesenchymal markers upregulated in invasive cells. As shown in Figure 1C, EGb 761 increased the expression of E-cadherin but had little effect on N-cadherin and vimentin expression, indicating that EGb 761 influenced migration and invasion by regulating the E-cadherin expression level.

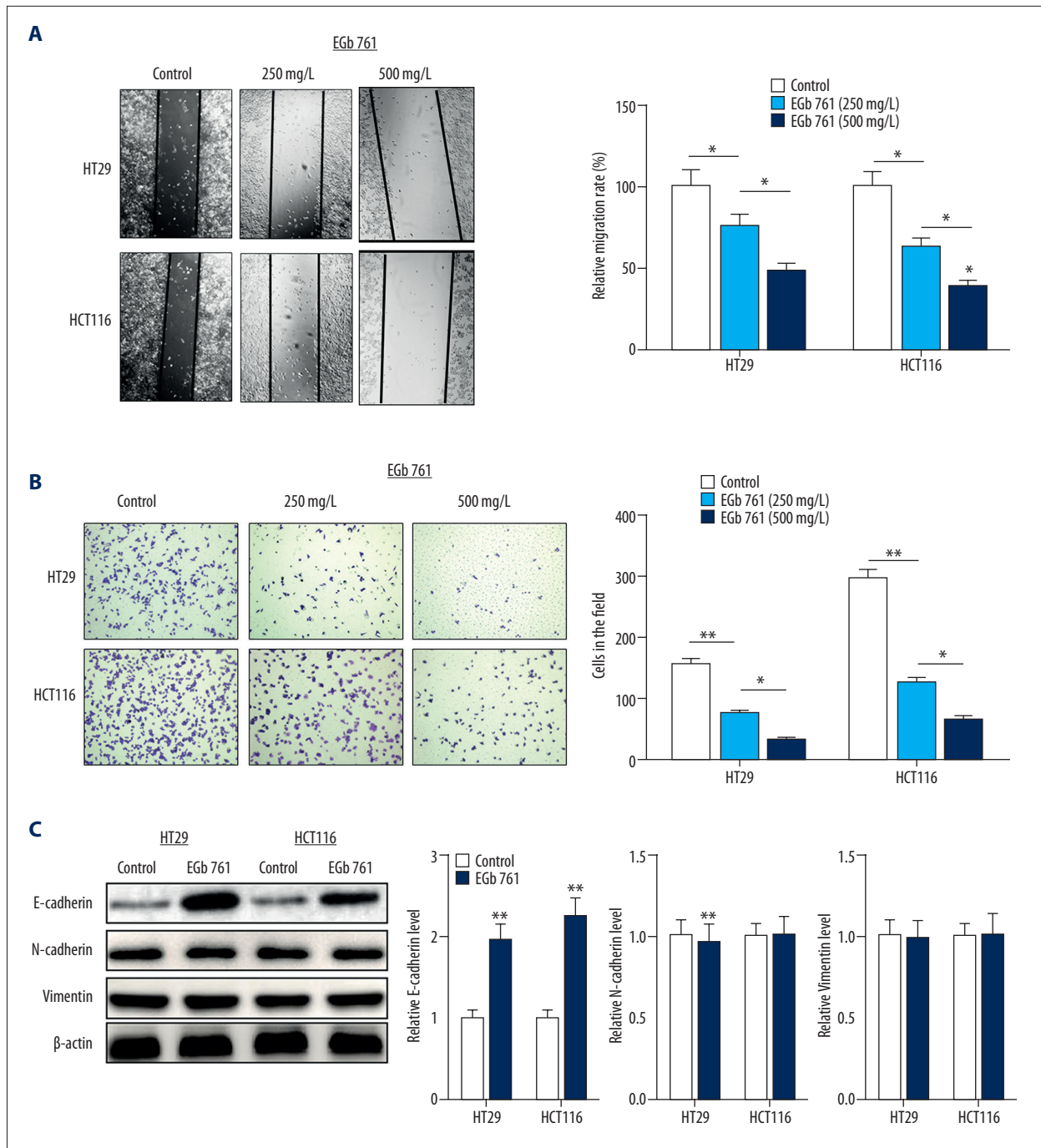


Figure 1. EGb 761 treatment suppressed cell migration and invasion. **(A)** Wound-healing analysis showed the functional effect of EGb 761 treatments (48 h) on the migratory and invasive capacity of colon cancer cells, * $P < 0.05$. **(B)** Transwell invasion assay showed that EGb 761 treatment (48 h) suppressed cell invasion in a dose-dependent manner, * $P < 0.05$, ** $P < 0.01$. **(C)** Western blot assay was performed to identify the influence of EGb 761 (500 mg/mL, 48 h) on the expression of indicated proteins, ** $P < 0.01$ compared to controls.

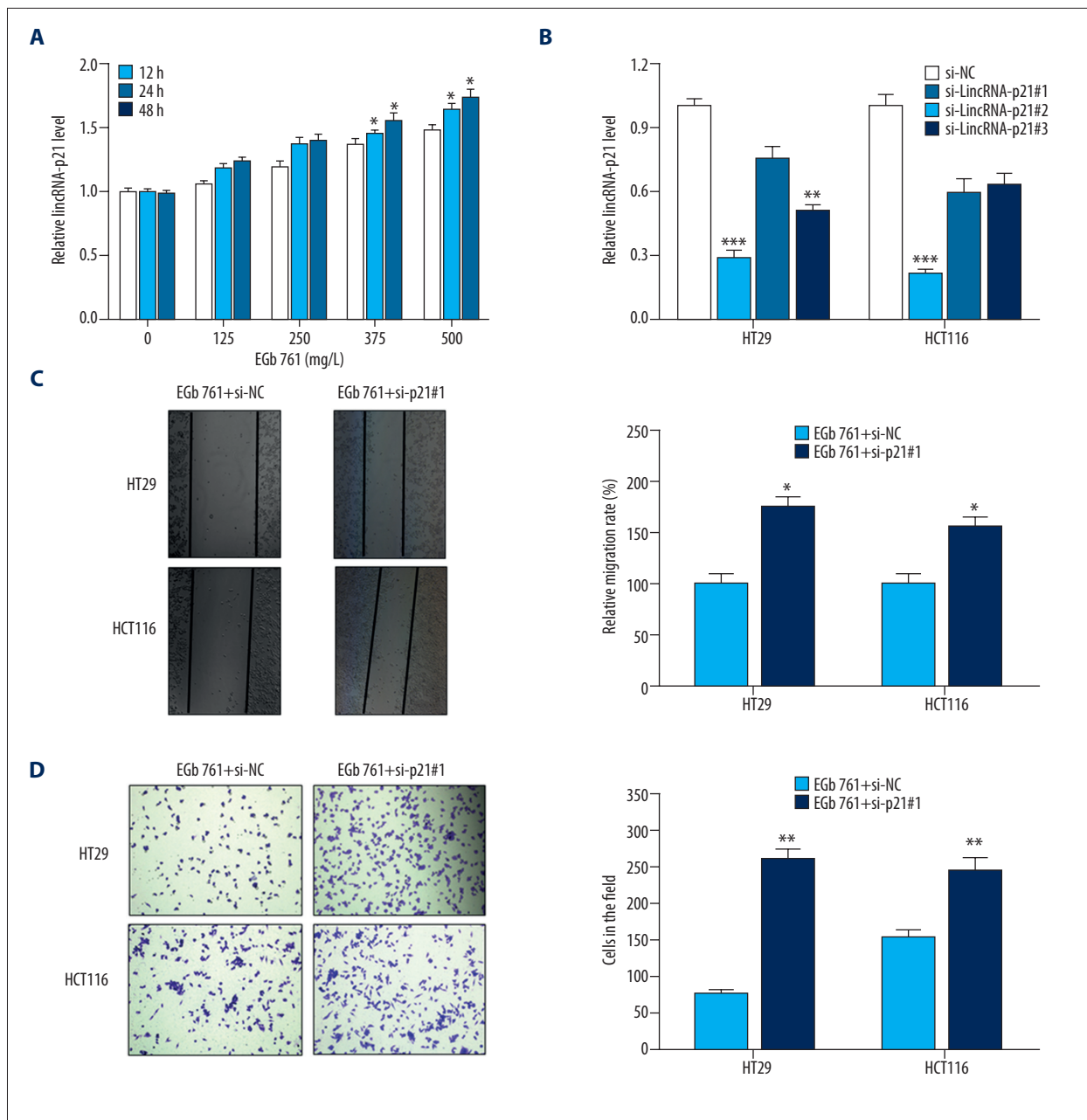


Figure 2. lincRNA-p21 is essential for the functional effect of EGb 761 in colon cancer. **(A)** RT-qPCR analysis of lincRNA-p21 expression in HCT116 cells after treatment of EGb 761, * $P < 0.05$ compared to cells free from EGb 761 treatment (0 mg/mL group). **(B)** RT-qPCR analysis of lincRNA-p21 expression after transient transfection of silencing vectors, ** $P < 0.01$; *** $P < 0.001$ compared to si-NC. **(C, D)** Wound-healing assay indicated that knockdown of lincRNA-p21 reversed the effect of EGb 761 on cell migration **(C)** and invasion **(D)**, * $P < 0.05$; ** $P < 0.01$ compared to (EGb 761 + si-NC)-treated group.

LincRNA-p21 is essential for the anti-metastatic effect of EGb 761

We then investigated the role of lincRNA-p21 in the EGb 761-induced anti-metastatic effect. RT-qPCR showed that lincRNA-p21 was upregulated in colon cancer cells treated with EGb 761 when compared to controls (Figure 2A). Then, we silenced

lincRNA-p21 expression in colon cells by transfection of specific silencing oligonucleotides (Figure 2B), and si-lincRNA-p21#1 was selected for further experiments. As shown in Figure 2C and 2D, downregulated lincRNA-p21 abrogated the functional effect of EGb 761 on colon cancer cell migration and invasion, suggesting that EGb 761 regulates metastasis of colon cancer by targeting and upregulation of the lincRNA-p21 expression.

Table 1. Score of lincRNA-p21 at different subcellular locations by lncLocator (<http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/>).

Subcellular locations	Score
Cytoplasm	0.308986935516
Nucleus	0.27269052156
Ribosome	0.149984719047
Cytosol	0.174993349377
Exosome	0.0933444745007

lincRNA-p21 promotes E-cadherin expression in a post-transcriptional-dependent manner

As we have verified that EGb 761 regulates the expression level of E-cadherin protein, and that lincRNA-p21 is essential for the function of EGb 761, we then investigated whether lincRNA-p21 was associated with E-cadherin. It is well known that lncRNAs in cytoplasm can exert their functional roles at post-transcriptional level via sponging microRNAs or protein modifications, and mediate transcriptional regulation via the chromatin modification when they are located in the nucleus [19]. Therefore, we localized the subcellular location of lincRNA-p21 in CRC cells. By using the online lncRNA location prediction software lncLocator (<http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/>), we identified that intracellular lincRNA-p21 was predicted to be located in both nuclei and cytoplasm (Table 1). In addition, nucleus-cytoplasm fractionation followed by RT-qPCR assay indicated that lincRNA-p21 was enriched in nuclear and cytoplasm sections (Figure 3A). We detected the transcript and protein levels of E-cadherin after knockdown of lincRNA-p21. Interestingly, we found that silencing of lincRNA-p21 decreased the endogenous E-cadherin level but did not affect the E-cadherin mRNA level (Figure 3B, 3C). Herein, we hypothesized that lincRNA-p21 in cytoplasm affects the E-cadherin protein level via molecular interactions in a post-transcriptional-dependent manner. To verify this hypothesis, we performed RNA immunoprecipitation (RIP) assay. As shown in Figure 3D, a sufficient interaction was found between lincRNA-p21 and E-cadherin as evidenced by the enrichment of lincRNA-p21 by E-cadherin antibody, but no enrichment of IgG antibody. To validate this interaction, we performed RNA pull-down assay, and found that lincRNA-p21 specially pulled down endogenous E-cadherin protein but not GAPDH protein, which served as a negative control (Figure 3E). These data suggest that lincRNA-p21 increases E-cadherin expression at the post-transcriptional level.

lincRNA-p21 stabilizes E-cadherin by impeding E-cadherin ubiquitination

To elucidate the underlying mechanism of the interaction between lincRNA-p21 and E-cadherin, we examined the E-cadherin stability after overexpression of lincRNA-p21. By treating cells with cycloheximide (CHX), a protein synthesis inhibitor, we found that enhanced lincRNA-p21 increased the E-cadherin stability by impeding degradation (Figure 4A), suggesting that overexpression of lincRNA-p21 extended E-cadherin half-life. To further verify whether lincRNA-p21 affects E-cadherin stability by regulating ubiquitination, we used proteasome inhibitor MG132 followed by immunoprecipitation and Western blot analysis to identify the ubiquitination level caused by dysregulation of lincRNA-p21. As expected, E-cadherin ubiquitination was dramatically repressed by enhanced expression of lincRNA-p21 in HCT116 cells (Figure 4B), whereas silencing of lincRNA-p21 increased the expression level of endogenous E-cadherin ubiquitination (Figure 4C). Collectively, we revealed that lincRNA-p21 stabilized E-cadherin protein by blocking its ubiquitination process.

lincRNA-p21 blocks BTRC-mediated E-cadherin ubiquitination

To further investigate how lincRNA-p21 suppresses E-cadherin ubiquitination, we focused on the b-transducin repeat containing (BTRC) E3 ubiquitin protein ligase, which is critical for ubiquitination. As shown in Figure 5A, overexpression of BTRC in HCT116 cells decreased E-cadherin protein levels. Moreover, overexpression of lincRNA-p21 reversed the degradation of E-cadherin induced by BTRC (Figure 5B). Immunoprecipitation assay results suggest that enhanced expression of lincRNA-p21 blocks the association between E-cadherin and BTRC (Figure 5C). These data suggest that lincRNA-p21 inhibits BTRC-induced E-cadherin ubiquitination by preventing the interaction between BTRC and E-cadherin.

Discussion

Advanced colon cancer patients who develop resistance to chemotherapy have limited therapeutic clinical options at present. Many patients therefore turn to alternative treatment [9]. In recent years, the interest in herbal remedies has grown rapidly in the industrialized world, since these drugs are increasingly considered as effective and safe alternatives to synthetic drugs. In this study, we focused the herbal remedy EGb 761, one of the few well-established plant products supported by clinical trials, which is the standardized special extract from the leaves of the plant *Ginkgo biloba*. Our study suggests that treatment with EGb 761 inhibited colon cancer migration and invasion via the upregulation of lincRNA-p21. Moreover,

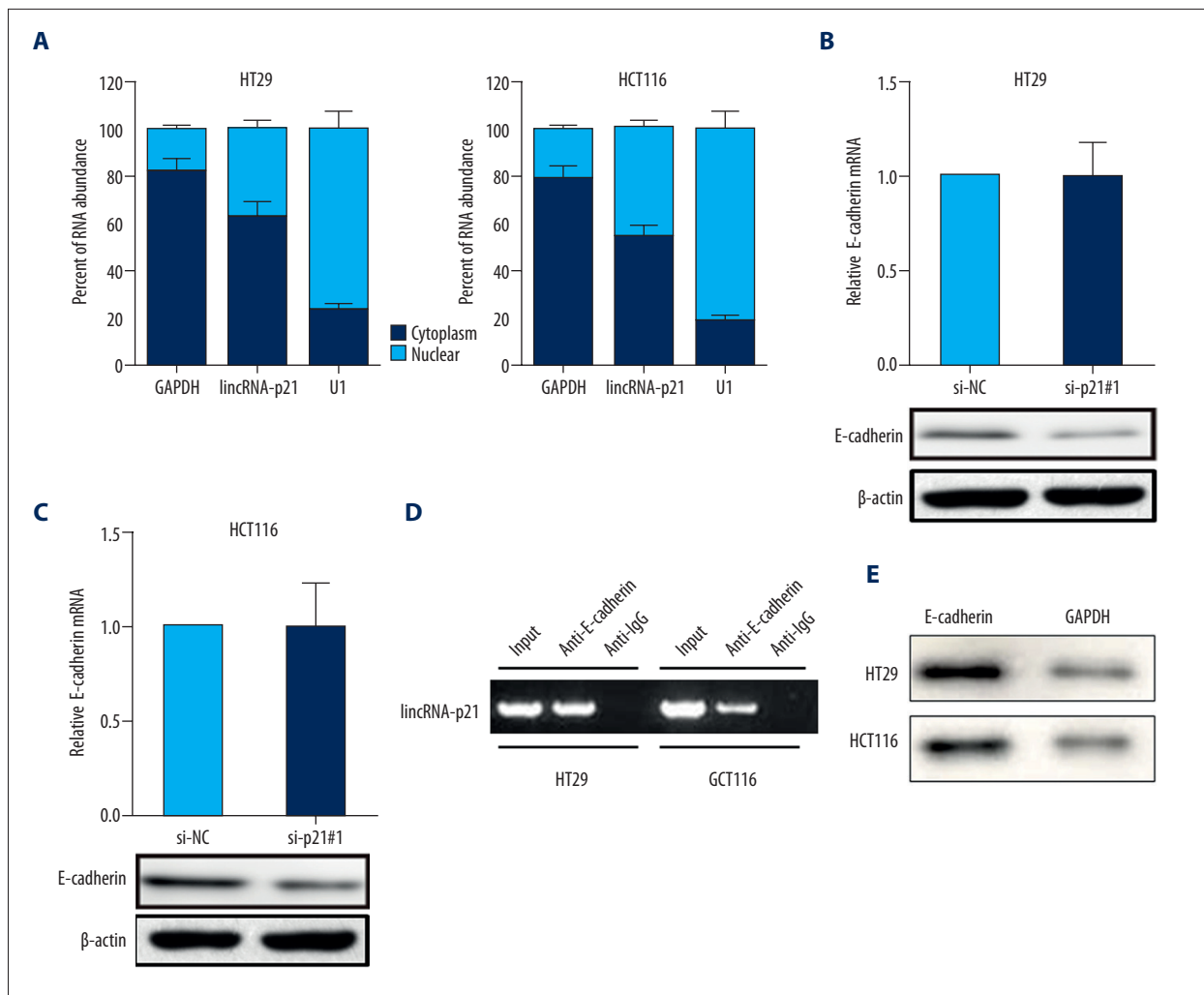


Figure 3. lincRNA-p21 promotes E-cadherin expression in a post-transcriptional-dependent manner. (A) The expression levels of lincRNA-p21 in nuclei and cytoplasm of colon cancer cells. U1 (nuclear retained) and GAPDH (exported to cytoplasm) were used as controls. (B, C) E-cadherin protein levels, but not those of E-cadherin mRNA, are upregulated by lincRNA-p21 in HT29 (B) and HCT116 cells (C). (D) RNA immunoprecipitation (RIP) was performed to identify the enrichment of lincRNA-p21 by anti-E-cadherin antibody with anti-IgG antibody as a negative control. (E) RNA pull-down assay was done to verify the interaction between lincRNA-p21 and E-cadherin, and GAPDH served as control.

a series of *in vitro* experiments showed that lincRNA-p21 inhibited E-cadherin ubiquitination process by preventing the interaction between BTRC and E-cadherin, thus inducing the stabilizing E-cadherin protein.

The roles of lncRNAs in cancer progression have long been researched, and lincRNA-p21 is widely accepted as a tumor suppressor gene in cancers, including colon cancer. Previously, Wang et al. demonstrated that lincRNA-p21 suppresses β -catenin signaling and tumorigenicity of colorectal cancer stem cells [20]. Another study showed that lincRNA-p21 affects prognosis in resected non-small cell lung cancer patients through angiogenesis regulation [21]. A clinical study revealed that lincRNA-p21 level is downregulated by and associated with

CRC stage, tumor tissue invasion, and vascular invasion [22]. In our previous study, we clearly showed that EGb 761 treatment of colorectal cells suppressed the invasive ability by targeting lincRNA-p21 [11]. Moreover, experimental investigations revealed that nuclear lincRNA-p21 affects the expression of fibronectin via binding to the promoter of the fibronectin gene. On this basis, we extended our study and revealed that cytoplasmic lincRNA-p21 stabilized E-cadherin protein by inhibition of ubiquitination process during EGb 761 treatment.

The realization that histones can be modified by the protein ubiquitin through Lys-linked isopeptide bonds marked a new era in post-translational signaling [23]. Ubiquitination is a post-translational protein modification that consists of

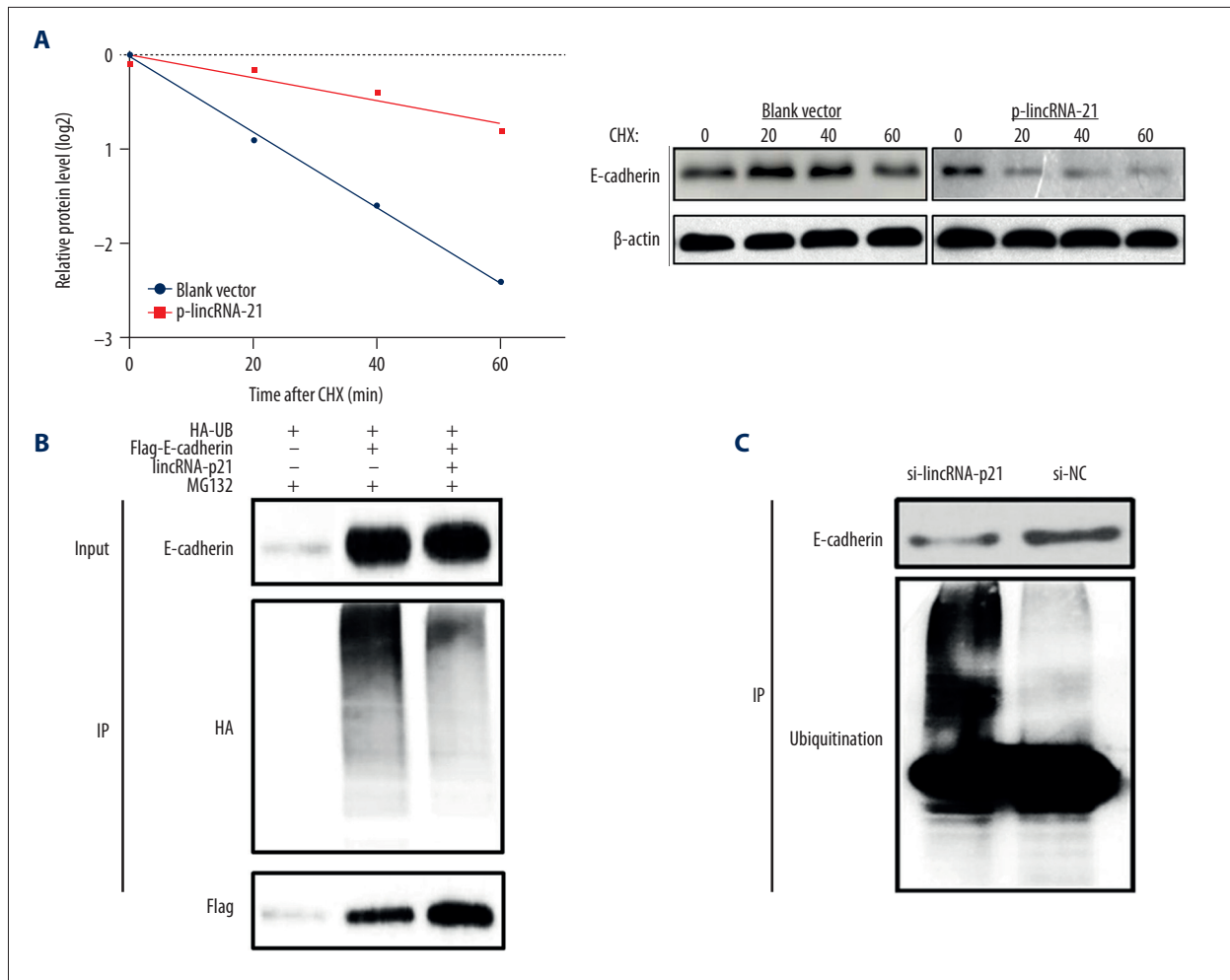


Figure 4. LincRNA-p21 stabilizes E-cadherin by impeding E-cadherin ubiquitination. **(A)** Blank vector- or lincRNA-p21-transfected HCT116 cells were cultured with medium containing 50 µg/ml cycloheximide (CHX) for 0–60 min, then subjected to Western blotting analysis. **(B)** HCT116 cells were transfected with HA-UB, Flag-E-cadherin, or lincRNA-p21 in combination as indicated, and 24 h after transfection, proteasome inhibitor MG132 was used to treat cells for 6 h, followed by immunoprecipitation assay and Western blot analysis. **(C)** HCT116 cells were transfected with si-lincRNA-p21, and 48 h post-transfection, cells were subjected to Western blot analysis.

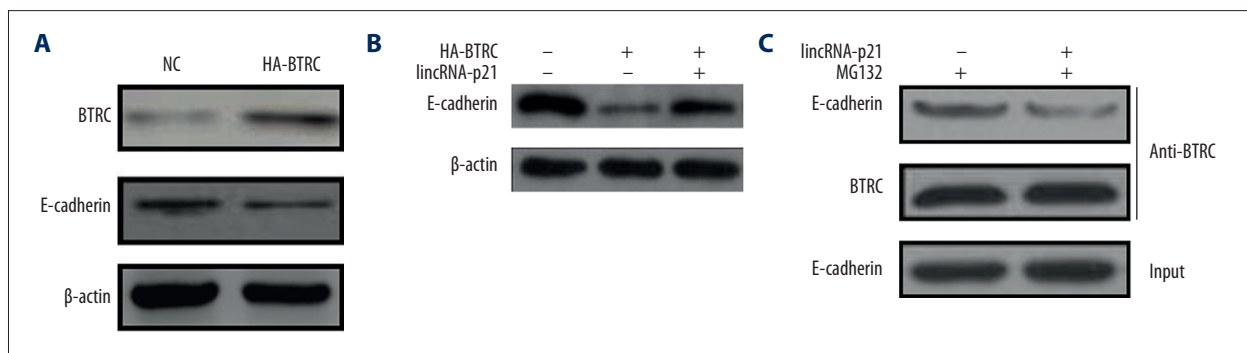


Figure 5. LincRNA-p21 blocks BTRC-mediated E-cadherin ubiquitination. **(A)** Western blot analysis of BTRC and E-cadherin level in HCT116 cells transfected with BTRC overexpression vector. **(B)** Western blot analysis of E-cadherin level in HCT116 cells treated with HA-BTRC or lincRNA-p21 in combination, as indicated. **(C)** HCT116 cells were overexpressed with lincRNA-p21 and treated with MG132 for 6 h, then anti-BTRC antibody was used for immunoprecipitation assay.

the attachment of 1 or more ubiquitins to a target protein. Ubiquitination starts by the attachment of a single ubiquitin molecule to a substrate Lys residue. These monoubiquitinating events are abundant [24] and have many roles in cells [25]. Ubiquitination involves the sequential transfer of ubiquitin molecules through an enzyme cascade consisting of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3), until an isopeptide bond is formed between the C-terminus of ubiquitin and the ϵ -amino group of a lysine residue on a substrate protein [26]. Previous reports showed that 2 paralogues of the F-box protein β -TrCPs E3, β -TrCP1/ β -transducin repeat containing E3 ubiquitin protein ligase (BTRC) and β -TrCP2/F-box and WD repeat domain containing 11 (FBXW11), widely participated in protein ubiquitination [27]. In this study, we identified that lincRNA-p21 regulated E-cadherin stabilization via the blockage

of BTRC-mediated ubiquitination process, which explained how lincRNA-p21 regulates EGb 761-mediated inhibition of migration and invasion of colon cancer cells.

Conclusions

Our study demonstrates that lincRNA-p21 is essential for the EGb 761-induced suppressive effect of colon cancer progression. Moreover, functional analysis revealed that lincRNA-p21 upregulated E-cadherin expression via the blockage of BTRC-mediated ubiquitination. Therefore, lincRNA-p21 may serve as a promising therapeutic target in colon cancer.

Conflict of interest

None.

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