Research article

LINC02257 regulates colorectal cancer liver metastases through JNK pathway

Xiangan Wu [a,e,1], Xiaokun Chen [d,e,1], Xiao Liu [a], Bao Jin [a], Yuke Zhang [a,e], Yuxin Wang [a,e], Haifeng Xu [a], Xueshuai Wan [a], Yongchang Zheng [a], Lai Xu [c], Yi Xiao [c], Zhengju Chen [f], Haiwen Wang [f], Yilei Mao [a], Xin Lu [a], Xinting Sang [a], Lin Zhao [b,**], Shunda Du [a,*]

[a] *Department of Liver Surgery, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China*
[b] *Department of Medical Oncology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China*
[c] *Department of General Surgery, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China*
[d] *Department of Surgery, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China*
[e] *Graduate School, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China*
[f] *Pooling Medical Research Institutes, Beijing, China*

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ABSTRACT

Background: Long noncoding RNAs (lncRNAs) have emerged as critical regulators of colorectal cancer (CRC) progression, but their roles and underlying mechanisms in colorectal cancer liver metastases (CRLMs) remain poorly understood.
Methods: To explore the expression patterns and functions of lncRNAs in CRLMs, we analyzed the expression profiles of lncRNAs in CRC tissues using the TCGA database and examined the expression patterns of lncRNAs in matched normal, CRC, and CRLM tissues using clinical samples. We further investigated the biological roles of LINC02257 in CRLM using in vitro and in vivo assays, and verified its therapeutic potential in a mouse model of CRLM.
Results: Our findings showed that LINC02257 was highly expressed in metastatic CRC tissues and its expression was negatively associated with overall survival. Functionally, LINC02257 promoted CRC cell growth, migration, metastasis, and inhibited cell apoptosis in vitro, and enhanced liver metastasis in vivo. Mechanistically, LINC02257 up-regulated phosphorylated c-Jun N-terminal kinase (JNK) to promote CRLM.
Conclusions: Our study revealed that LINC02257 played a key role in the proliferation and metastasis of CRC cells through the LINC02257/JNK axis. Targeting this axis may represent a promising therapeutic strategy for the treatment of liver metastases in patients with CRC.

1. Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed gastrointestinal malignancies and the third leading cause of cancer death globally [1]. There are approximately 2,000,000 new cases of CRC worldwide [2]. Notably, the liver emerges as the primary target organ for CRC metastasis [3,4], with approximately 25 % of initially diagnosed CRC patients displaying concurrent liver metastases [5]. Moreover, an additional 25 % of CRC patients eventually experience liver metastases following curative intestinal surgery during postoperative follow-up [6]. Colorectal cancer liver metastases (CRLMs) emerge as the predominant contributors to mortality in CRC patients. Consequently, delving into the underlying mechanisms of CRLMs and identifying therapeutic markers constitutes a pivotal focus within CRC research. Previous studies have shown that liver metastasis in CRC is governed by a complex interplay of multiple factors and biological processes [7]. The promotion of epithelial-mesenchymal transition (EMT) is closely associated with the metastatic process in CRLM [8]. Furthermore, mutations in key oncogenes such as RAS, BRAF, TP53, and PIK3CA have been linked to an increased risk of CRLM [9]. Additionally, the tumor microenvironment and the immune status of the liver are crucial in facilitating the CRLM seeding process [10,11].

Recent studies have revealed that RNA processing is systematically changed in cancers [12–15]. These modifications may be detected not only in protein-coding RNA (mRNAs), but also in noncoding RNAs (ncRNAs) such as long noncoding RNAs (lncRNAs), miRNAs, and circular RNAs (circRNAs). RNAs larger than 200 nucleotides that do not code for proteins are considered as lncRNAs. LncRNAs are one of the most important regulators in the human genome. They regulate gene expression at the epigenetic, transcriptional, and translational levels, which is closely related to the occurrence and development of many malignant tumors [16–18]. LncRNAs serve a variety of functions, including nuclear roles such as regulation of gene expression in cis or trans, modulation of splicing, and nucleation of subnuclear regions [19].

Various lncRNAs have been shown to impact the development or progression of cancer, including CRC. Yari et al. discovered that the lncRNA REG1CP is commonly up regulated in CRC cells. They observed that lncRNA REG1CP promotes carcinogenesis by recruiting FANCJ helicase for REG3A transcription via an enhancer complex [20,21]. Currently, numerous studies have reported that various lncRNAs influence the development of CRC [21–24]. Wang X et al. [24] analyzed the data of 51 normal intestinal tissues and 646 colorectal cancer tissues based on The Cancer Genome Atlas (TCGA) database and found that 15 kinds of lncRNAs, including LINC02257, were related to the prognosis of CRC and speculated that these lncRNAs might play a role through the mechanism of competitive endogenous RNA (ceRNA). Huang X et al. [25] also found that 1176 lncRNAs were differentially expressed in the data analysis of 41 normal intestinal tissues and 480 colorectal cancer tissues based on the TCGA database. Further LASSO regression analysis found that 6 kinds of lncRNAs, including LINC02257, could be used to establish a prediction model for the overall survival of CRC patients, which may be valuable for individualized treatment. Recently, some researchers reported that LINC02257 is the most significant enhancer RNA related to the prognosis of CRC based on TCGA database analysis, and its role may be related to PI3K-Akt, MAPK and many other signal pathways [26]. A recent study has reported that LINC02257 has potential in CRC diagnosis and prognosis prediction, and the silence of LINC02257 inhibits the proliferation of CRC cells [27]. Therefore, we speculated that LINC02257 may also play a role in CRC distant metastasis. Given that liver metastases are the primary cause of death in CRC patients, it is crucial to determine the underlying mechanism of CRLM. However, as far as we know, the role of LINC02257 in liver metastases of colorectal cancer has not been reported.

In this study, we functionally characterized LINC02257, a CRC-associated lncRNA with significantly increased expression levels from CRC to CRLM tissues, and selected it for further analysis concerning CRC development. Additionally, we validated the therapeutic potential of LINC02257 in a mouse model of CRLM.

2. Methods

2.1. Patient samples

The tissues of 30 CRC patients who underwent surgical treatment at Peking Union Medical College Hospital from October 2019 to October 2020 were collected, including 15 CRC patients with liver metastasis and 15 patients without liver metastasis. Only the normal adjacent colon or rectum was used in patients without liver metastasis as a negative control. This study was approved by the Ethics Committee of Peking Union Medical College Hospital (ID: K23C1279), and all patients involved signed a written informed consent form.

2.2. Database (DB) search

This project utilized the RNAseq count and colorectal cancer clinical data from the TCGA database, including 448 COAD and 91 READ cases. In the screening of the TCGA database, the Primary sites were restricted to the colon and rectum, and only the data from these major sites were analyzed. This project's entire data analysis and processing is based on the R programming language. We performed differential analysis and survival analysis using edgeR, survminer, survival, and other R packages. Based on edgeR, differential expression analysis of screened lncRNAs was performed. We establish logFoldchange (logFC) ≥ 5, ≤ -3.5, and FDR ≤ 0.05 (BH multiple correction) as the differential gene threshold. Genes with logFC ≥ 5 and FDR ≤ 0.05 were considered up-regulated. Down-regulated genes were identified by logFC ≤ -3.5 and FDR ≤ 0.05. For lncRNAs with differential expression, the Cox proportional hazards regression model was used to examine the association between gene expression and prognosis, after adjusting for covariables

(age, gender, distant metastasis). The results were evaluated using P ≤ 0.05 as the threshold. Using the TGGA database, we evaluated the prognostic significance of LINC02257 in CRC patients using the Kaplan-Meier plot. The median of transcript values was used to determine high and low expression.

2.3. RNA extraction and qRT-PCR

Total RNA was isolated from the tissues or cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total complementary DNA (cDNAs) was reverse transcribed using PrimeScript™ RT reagent Kit (TAKARA, Japan). Real-time quantitative RT-PCR (qRT-PCR) was performed using the SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (TAKARA, Japan). The mRNA levels for target genes were analyzed by a LightCycler96 system (Roche, Switzerland). The specific primers for target genes in this study are listed in Table 1. Levels of the housekeeping gene β-actin was used as an internal control for the normalization of RNA quantity and quality differences among the samples. We calculated fold changes in gene expression normalized to β-actin by the $2{-}\Delta Ct$ method. The results were shown as fold changes compared to the control group.

2.4. Cell culture and cell lines

All cell lines were purchased from Procell Life Science&Technology Co.,Ltd. All cells, except LoVo cell lines, were cultured in RPIM-1640 (GIBCO, USA) medium and enriched with 10 % fetal bovine serum (GIBCO, USA) and 1 % penicillin-streptomycin. LoVo cell lines were maintained in DMEM (GIBCO, USA) with 10 % fetal bovine serum (GIBCO, USA), 1 % penicillin and streptomycin. All cell lines were cultured under 5 % CO_2 at 37 °C.

2.5. Cellular growth

To test the influence of LINC02257 on cellular growth, we measured the short-term effects (up to 72 h at 24 h interval) by applying cell counting kit 8 CCK, MCE (HY–K0301). Cells were seeded at a density of 5000 cells per well in a 96-well plate. We used transient transfection protocols with siRNAs according to the manufacturer protocols (Lipofiter™, Hanbio Biotechnology). Overexpression cells were also seeded in 96-well plates. Cells were incubated from 24 to 72 h and every 24 h the CCK8 proliferation reagent was added in the wells according to the manufacturer's recommendations. Colorimetric changes were measured using a Flexstation® 3 (Molecular Devices, USA) at a wavelength of 450 nm. Three independent biological replicates were performed each.

2.6. Transfection and siRNA experiments

We designed siRNAs against LINC02257. Each of three highest-ranking siRNA sequences for LINC02257 were tested in our experiments. The performance was assessed at 48 h intervals post-transfection by qRT-PCR. The cells were transfected with the respective siRNA by using Lipofiter™ (Hanbio Biotechnology) according to the manufacturer's protocol. For the final experiments, we selected the siRNA with the highest knock-down efficacy (LINC02257 siRNA#2 sense sequence: F: GCUGGGAUUGCAGAGCAAATT, R: UUUGCUCUGCAAUCCCAGCTT). The non-specific siRNA (sense sequence : F:UUCUCCGAACGUGUCACGUTT, R:ACGUGA-CACGUUCGGAGAATT) was used as the negative control.

To identify the mechanisms of action of LINC02257, we used second-generation sequencing technology to analyze the differential

Table 1
Primers sequence for qRT-PCR.

Target	Primer sequence
LNC02257-F	TACTAGAGGATCTATTTCCGGTGAATTCAGATGCACATGAAATTTGGTG
LNC02257-R	CAGATCCTTACTAGTATCGATGGATCCTTTTTTTTTTAAGACGACCACTG
LINC00261-F	AAGACCAGCTCAACCATCGC
LINC00261-R	TGCCATTTCCTGTGAATTGATGA
LINC01550-F	GGTGCAGTCTCCTCAGAACTAC
LINC01550-R	GGGAGAGGGAGAACGACTGT
β-actin –F	GGCTGTGCTATCCCTGTACG
β-actin –R	AGGTAGTCAGTCAGGTCCCG
ERK-F	GGCTGTTCCCAAATGCTGAC
ERK-R	AACTTGAATGGTGCTTCGGC
JNK-F	TGTGTGGAATCAAGCACCTTC
JNK-R	AGGCGTCATCATAAAACTCGTTC
VHL-F	CATCCACAGCTACCGAGGTC
VHL-R	GGCAAAAATAGGCTGTCCGTC
HIF-1α-F	TGCTGAAGACACAGAAGCAAAG
HIF-1α-R	CTTTCAGGGCTTGCGGAACT
IL-17-F	TCTGTCCCCATCCAGCAAGA
IL-17-R	CCCACGGACACCAGTATCTTC
CXCL2-F	CACAGTGTGTGGTCAACATTTCT
CXCL2-R	TGCTCTAACACAGAGGGAAACA

gene expression of CRC cells with either control or upregulation (overexpression) of LINC02257. The reconstituted and synthesized siRNA blank plasmid, or si-LINC02257, was transfected into CRC LoVo cells, which were used for transcriptome sequencing.

2.7 Lentiviral overexpressing vector establishment

For construction of lentiviral overexpressing vector, the LINC02257 was PCR-amplified by phanta Max-Super-Fidlity DNA polymerase (Stratagene, P505-D1) and subcloned into the EcoRI and *Bam*HI sites of pHBLV-CMV-MCS-EF1-ZsGreen-T2A-puro lentiviral vector. Stably LINC02257-overexpressed HCT116 and HT29 cells were established using lentivirus mentioned above according to the recommended infection methods.

2.8. Apoptosis assay by conventional flow cytometry (CFC)

LINC 02257 siRNA transfected LoVo and SW480 cells and LINC 02257 overexpressed HCT116 and HCT 29 cells were resuspended in PBS and were stained by Annexin V-FITC and propyl iodide (PI). The above indicators were detected by flow cytometry (BD Biosciences FACSCalibur) according to the manufacturer's instructions.

2.9. Cell migration and invasion assays

The cells (2×10^5 cell/mL, 200 μL) in 800 μL of MEM medium containing 10 % FBS were seeded into the upper Transwell chambers (Corning) without (migration assay) or pre-coated with (invasion assay) Matrigel (BD Bioscience, USA) according to the manufacturer's instructions. After incubating for 24 h, the migrated/invaded cells were fixed with 70 % ice ethanol for 1h, stained with 0.5 % crystal violet for 20 min, and counted under an inverted microscope (ECLIPSE Ts2, Nikon).

2.10. Western Blotting

Mice colorectal tissues as well as cultured cells were resuspended in RIPA buffer (150 mM NaCl, 0.5 % sodium deoxycholate, 10 % SDS, 1 % NP40, 1 mM EDTA and 50 mM Tris pH 8.0). Equal concentration proteins were separated by polyacrylamide gel electrophoresis and followed by being transferring to polyvinylidene difluoride (PVDF; Millipore) membranes, which were immediately placed in 5 % non-fat milk in Tris-buffered saline -Tween buffer for blocking (2 h in room temperature). Membranes were then washed 5 times in TBS-Tween buffer for 25 min, followed by incubation with specific primary antibodies (dilution 1:1000) at 4 °C overnight. Membranes were then washed 5 times for 25 min in TBS-Tween buffer, and incubated with an anti-rabbit antibody (1:10,000) at room temperature for 2 h. The catalog numbers for the antibodies are as follows: JNK: Cat No. 17572-1-AP, proteintech; *p*-JNK: Cat No. 80024-1-RR, proteintech; Jun: Cat No. 80024-1-RR, proteintech; *p*-Jun: Cat No. 80086-1-RR, proteintech; β-actin: Cat No. 81115-1-RR, proteintech; second antibody: ab6795, abcam. Proteins were visualized on autoradiographic film using an ECL Plus Western blot detection system (Bio-Rad, USA).

2.11. Histological analysis

Liver metastases tissues were fixed in 10 % neutral formalin and paraffin-embedded sections were stained with hematoxylin and eosin (H&E) to analyze the histology of samples.

2.12. Gene ontology (GO) and KEGG pathway enrichment analysis

The core enrichment genes of LncRNA set revealed by GSEA were subject to TCGA http://cancergenome.nih.gov/for GO biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichments. The differentially expressed genes (DEGs) between LINC02257^{High} and LINC02257^{Low} patients were identified by the edge R package. Genes with $|Log_2 FC| \geq 1$ and adjusted p-value <0.05 were considered statistically significant. The distribution of DEGs was shown in volcano plots. The upregulated DEGs were subjected to the TCGA database for KEGG pathway enrichment analysis. The enrichment pathways were visualized by the "ggplot2" R package.

2.13. In vivo treatment of colorectal cancer metastases

The experiment used SPF grade BALB/C nude mice, 6–8 weeks old, 18–20 g. The LoVo colorectal cancer cell line was used in the experiment. LoVo cells stably expressing the luciferase gene were established. After 6 weeks of feeding, mice were imaged in vivo to observe the size and distribution of tumors. The model [28] of liver metastasis of colorectal cancer in situ was established by injecting cecal serosa into mice. To ensure that five animals per group could be included in subsequent studies, we initially used ten mice for the modeling process. The tumor implantation was maintained for 35 days (5 weeks), after which the animals were sacrificed on day 35, following IVIS imaging. As shown in Fig. 6, we harvested and photographed liver tissue to illustrate the distribution of tumor deposits within the liver tissues. We observed a higher distribution of tumors in the group that received the tumor transplant compared to the negative control (NC) group or those transfected with siR-LINC02257. Regrettably, we did not calculate the exact number of liver metastases due to the complex distribution of the tumors. This study was approved by the Ethics Research Board of Peking Union

Medical College Hospital (PUMCH), ID: K23C1279.

2.14. Live imaging of small animals

An IVIS Spectrum small-animal imaging system (IVIS Spectrum, PerkinElmer Inc, US) was used to measure the distribution of transplanted colorectal cancer cells in vivo. After the LoVo cells stably expressing luciferase gene were constructed and screened, the fluc-LoVo cells transfected with NC plasmid or si-RNA LINC02257 plasmid were injected into the serous membrane of the cecum. After 5 weeks of feeding, imaging was performed to observe tumor size and distribution.

After anesthesia, the nude mice were placed flat in the prone position in the recording dark box of the small-animal imaging system. Cy7 was diluted and 200 μL (0.5 mg/mL) was injected into the tail vein of nude mice. After 10 min, the nude mice were put into the detection system, and the signal was collected within the time range of 1–5 min according to the signal intensity. The imaging pictures of fluorescence emitted by the nude mice in vivo were recorded, and the distribution of fluorescent drugs was analyzed. Control nude mice were not injected with drugs and simultaneous recordings were performed. For Cy7 detection, the excitation wavelength was 700–770 nm, the emission wavelength was 790 nm, and the exposure time was 500 ms.

2.15. Quantification and statistical analysis

Statistical analyses were performed with SPSS statistical software package (SPSS 22.0; IBM Corp., Armonk, NY). Differences between groups were estimated by the $\chi 2$ test and Student's t-test, where appropriate. For time-to-event analyses, survival estimates were calculated using the Kaplan-Meier analysis, and groups were compared with the log-rank test. Throughout all figures: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and Significance was concluded at $p < 0.05$.

3. Results

1 LINC02257 expression in colorectal cancer correlates with the occurrence of CRLM and overall survival.
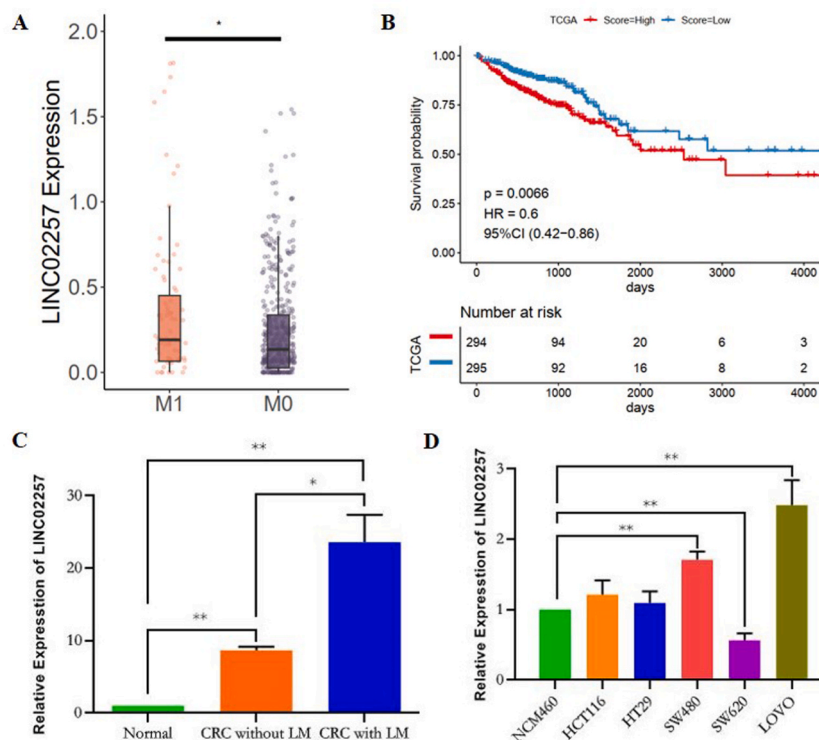

Fig. 1. LINC02257 is upregulated in CRC patients with CRLM and correlates with patients' overall survival. **(A)** LINC02257 expression in primary colorectal cancer without metastasis and metastasis. LINC02257 is expressed at a higher level in patients with metastatic colorectal cancer. (B) Survival of colorectal cancer patients with different LINC02257 expression levels. High expression levels of LINC02257 were associated with poor survival. (C) LINC02257 expression in normal tissues adjacent to cancer (n = 15), colorectal cancer tissues without liver metastasis (n = 15), and primary colorectal cancer tissues with liver metastasis (n = 15). (D) LINC02257 expression in a colon epithelial cell line (NCM460) and a colorectal cancer cell line (HCT116, HT29, SW480, SW620, LoVo). Data are presented as the means ± s.d.s (**$p < 0.01$; ***$p < 0.001$).
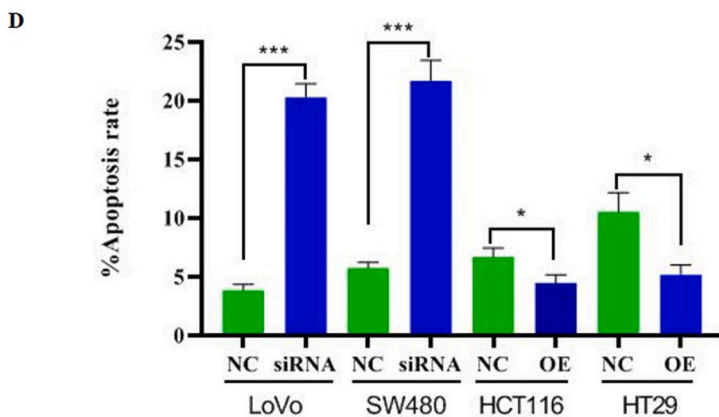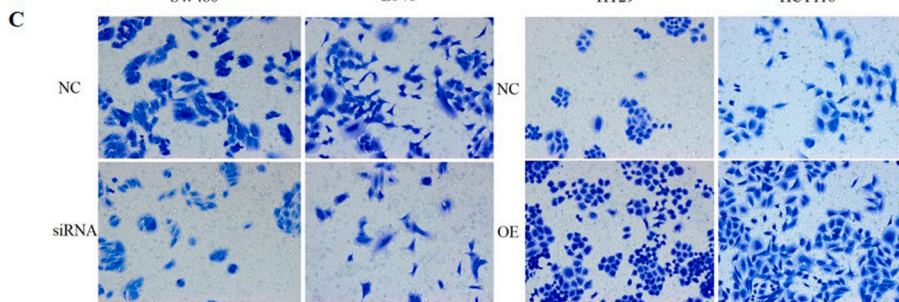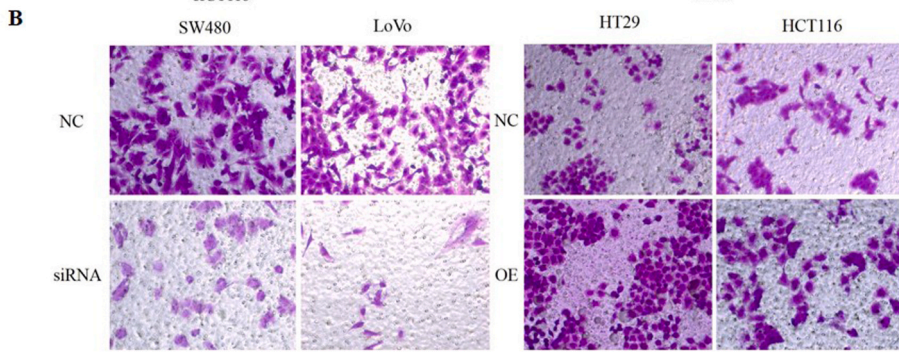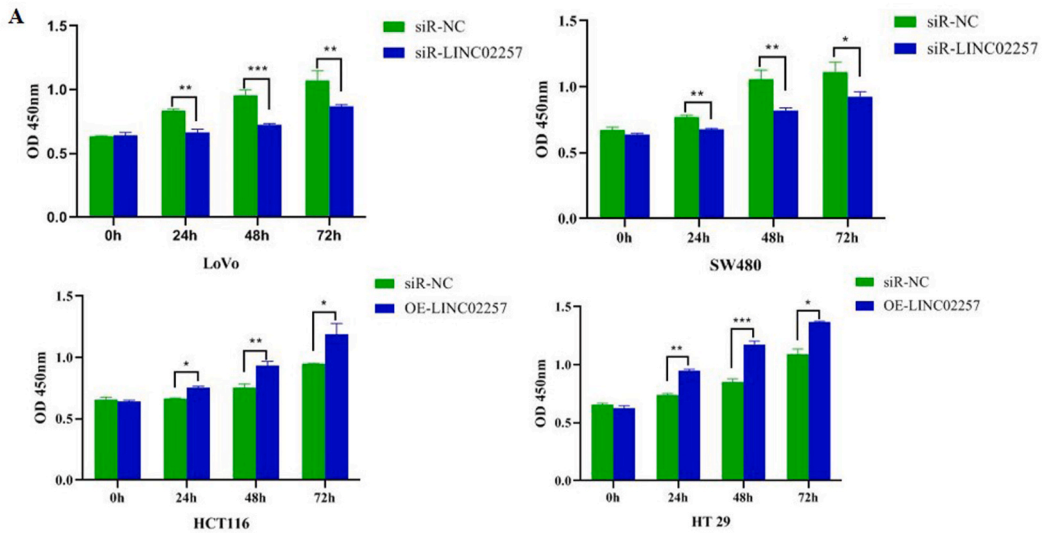
(caption on next page)

Fig. 2. LINC0227 promotes cell proliferation, migration, invasion, and anti-apoptosis in CRC cells. (**A-C**) Knockdown of LINC02257 in LoVo and SW480 cell lines inhibited cell proliferation, migration, invasion and increased cell apoptosis. On the contrary, overexpression of LINC02257 in HCT116 and HT29 cell lines promoted cell proliferation, migration, invasion and inhibits cell apoptosis. siR-NC: siRNA Negative control. OE: Overexpression. (*p < 0.05; **p < 0.01; ***p < 0.001).

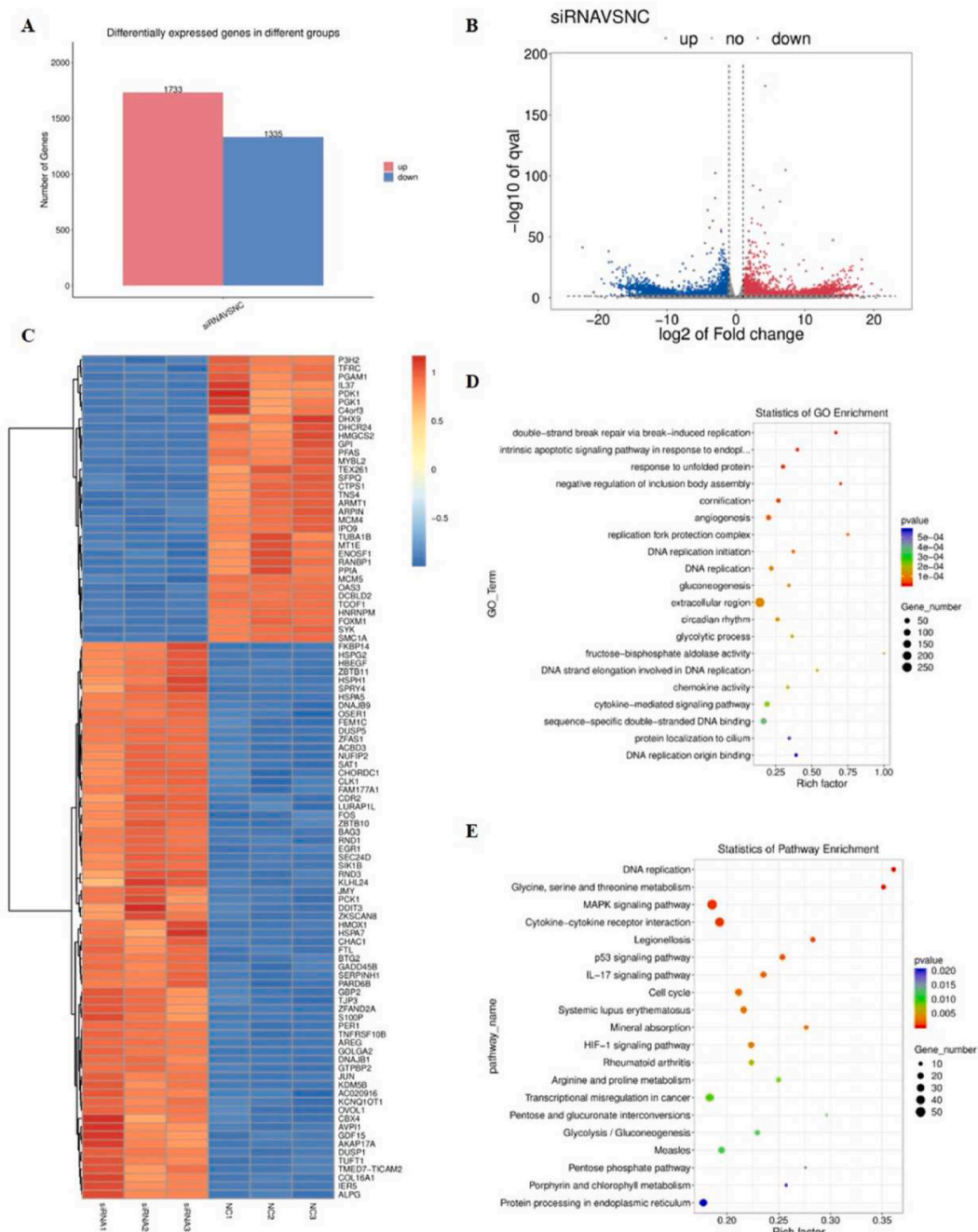**Fig. 3.** The variation of genome-wide gene expression after LINC02257 levels regulation. (**A–B**) Histogram and volcano plot showing differentially expressed genes after siRNA-mediated knockdown of LINC02257. As depicted in the figure, 1733 genes were up-regulated and 1335 genes were down-regulated in LoVo cells transfected with siRNA linc02257 relative to LoVo cells transfected with siRNA blank plasmid. (**C**) Heatmap of differential gene clustering displayed the differential genes between 3 groups of LoVo cells transfected with siRNA linc02257 and 3 groups of LoVo cells transfected with siRNA blank plasmid. FKBP14, HSPG2, and HBEGF were considerably up-regulated, while P3H2, TFRC, and PGAM1 were dramatically down-regulated. (**D**) GO Enrichment Bubble Chart. (**E**) KEGG enrichment analysis.

Though the TCGA database, we found LINC02257 is expressed at a higher level in metastatic colorectal cancer (Fig. 1A). In addition, CRC patients with higher LINC02257 expression levels had worse overall survival (Fig. 1B).

Subsequently, we analyzed the expression of LINC02257 in clinical samples and discovered that LINC02257 was upregulated in colorectal cancer, with a significant increase observed in primary colorectal cancer cases that had metastasized to the liver (Fig. 1C).

In addition, we also detected the expression of LINC02257 in various cell lines. Compared with colon epithelial cell lines (NCM460), LINC02257 expression in colorectal cancer cell lines (SW480, LoVo) was significantly upregulated. LINC02257 expression in a colorectal cancer cell line (SW620) was significantly downregulated, and LINC02257 expression in colorectal cancer cell lines (HCT116, HT29) was not significantly different (Fig. 1D). Therefore, LINC02257 is upregulated in CRC patients with CRLM and is negatively related to the prognosis of CRC patients, which indicates that LINC02257 may be closely related to the occurrence and development of CRC and liver metastasis.

2 . Up-regulated LINC02257 induces cell proliferation and migration and inhibits apoptosis in colorectal cancer cells.

To determine the biological functions of LINC02257 in colorectal cancer cells, we designed two independent siRNAs that target LINC02257. Then, we assessed the biological effect of transient LINC02257 knockdown in two CRC cell lines expressing high levels of LINC02257: SW480 and LoVo cells. siRNAs significantly decreased cell proliferation, migration, and invasion in all CRC cell lines tested (Fig. 2A–C). In contrast, overexpression of LINC02257 significantly promoted cell proliferation, migration, and invasion in HCT116 and HT29 cells, which contained no discernible increase in LINC02257 expression relative to the colon epithelial cell line.

We further investigated the impact of downregulating LINC02257 on apoptosis in CRC cells. After siRNA-mediated knockdown and overexpression (Fig. 2D), flow cytometry revealed increased apoptosis in SW480 and LoVo cells with reduced LINC02257 levels. Conversely, overexpressing LINC02257 decreased apoptosis in HCT116 and HT29 cells.

3 . The expression profile regulated by LINC02257

For explore the mechanism regulated by LINC02257, we take the difference multiple $FC \geq 2$ or $FC \leq 0.5$ (the absolute value of $\log_2 FC \geq 1$) as the change threshold and the p value < 0.05 as the standard for screening differential genes. The results showed that there were 1733 upregulated genes and 1335 downregulated genes among the differentially expressed mRNAs (Fig. 3A–B). The differential gene clustering heatmap (Fig. 3C) was used to cluster genes according to the similarity of gene expression profiles of samples. After LINC02257 was knocked out of colorectal cancer cells, the transcription of genes such as FKBP14, HSPG2, and HBEGF considerably increased, whereas the transcription of genes such as P3H2, TFRC, and PGAM1 decreased dramatically. Based on the
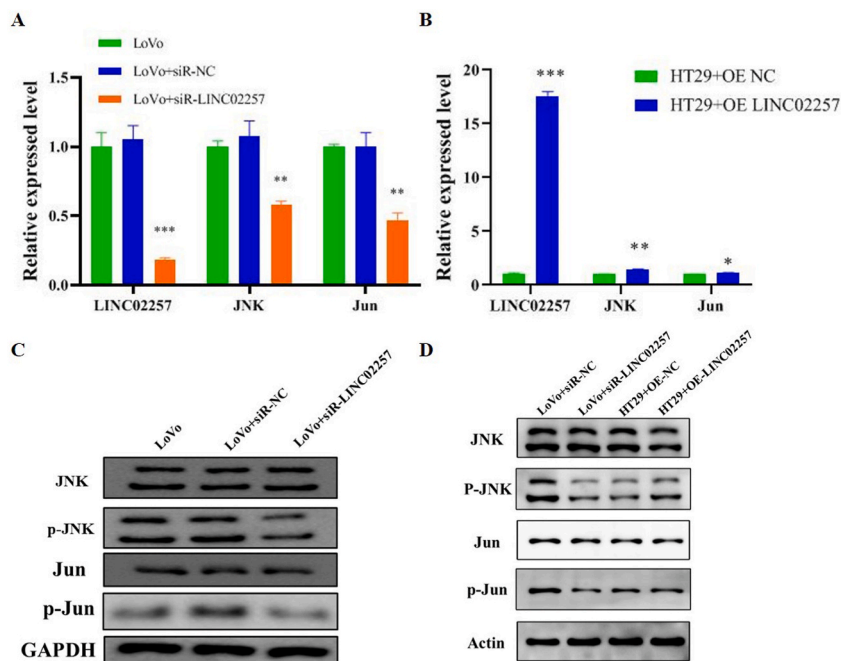

Fig. 4. LINC02257 promotes the expression of phosphorylated JNK in CRC cells. (A–B) qPCR detected MAPK pathway expression. LINC02257 knockdown in LoVo cells decreased JNK and Jun expression (P < 0.01). HT29 cells overexpressing LINC02257 had higher JNK (P < 0.01) and Jun (P < 0.05) expression than controls. (C–D) WB detected MAPK pathway expression. LINC02257 knockdown in LoVo cells decreased JNK and Jun activity while maintaining protein levels. Phosphorylated JNK increased significantly in HT29 cells overexpressing LINC02257. (*p < 0.05; **p < 0.01; ***p < 0.001).

differential genes, we also conducted GO enrichment analysis and KEGG enrichment analysis. GO enrichment analysis indicated that these different genes mostly participate in the biological processes of regulating transcription, DNA-templated expression, and signal transduction; in the molecular functions of protein binding, metal ion binding, and DNA binding; and in the cellular components of
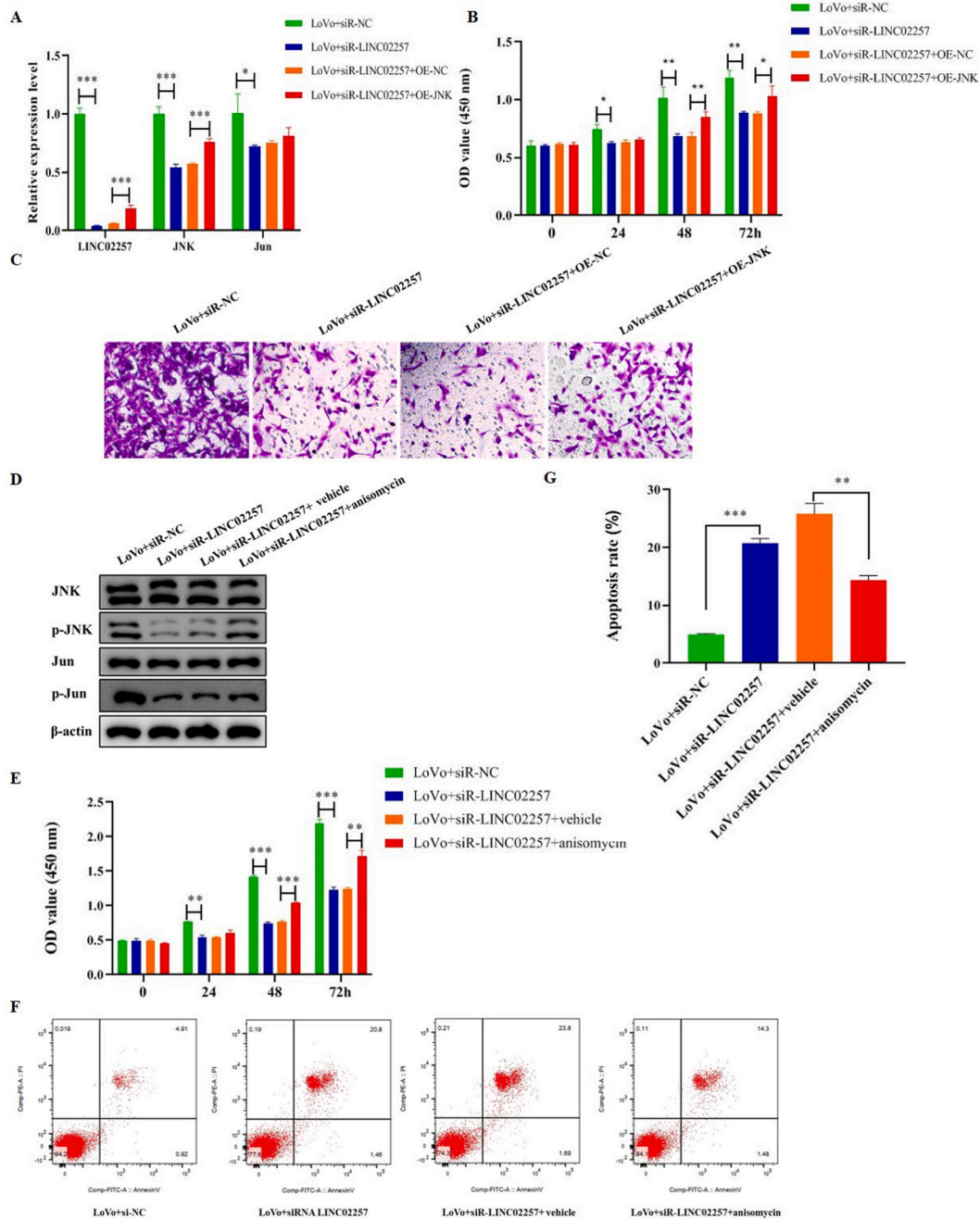

Fig. 5. LINC02257 promotes cell proliferation, migration, and inhibits apoptosis in CRC cells through the JNK pathway. (A) qPCR detected JNK-Jun expression. LINC02257 knockdown in LoVo cells decreased JNK ($p < 0.001$) and Jun expression ($p < 0.05$). LINC02257 knockdown following JNK overexpression boosted JNK expression ($p < 0.001$); however, the express of Jun was not obvious. **(B)** Cell proliferation activity was detected by CCK8. LINC02257 knockdown inhibited LoVo cell growth. LINC02257 knockdown following JNK overexpression significantly boosted cell proliferation. **(C)** Transwell assays were used to detect cell migration. LINC02257 knockdown reduced LoVo cell migration. JNK overexpression significantly increased cell migration. **(D)** WB-detected JNK-Jun expression. When LoVo cells knocked down LINC02257, p-Jnk and p-Jun decreased. P-JNK and P–C-Jun increased significantly after JNK agonist treatment. **(E)** CCK8 was used to detect cell proliferation. JNK agonist restored colorectal cancer cell proliferation after SiLINC02257 transfection. **(F–G)** Flow cytometry was used to measure cell apoptosis in each group. Compared to SiLINC02257 alone, the JNK agonist decreased LoVo cell apoptosis.
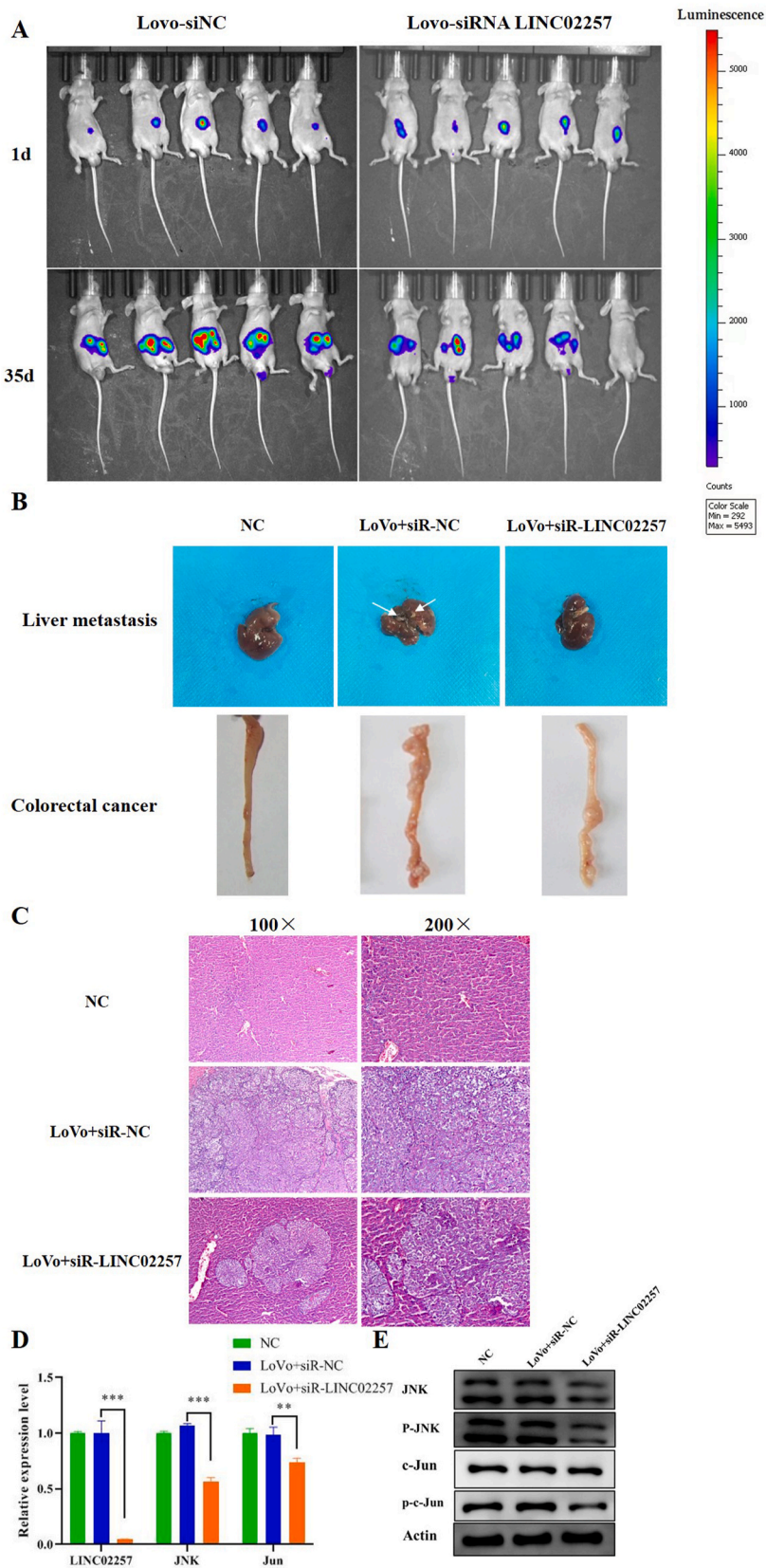
Fig. 6. LINC02257 induces the growth and metastatic spread of colorectal cancer in *vivo* **(A)** Small animal imaging technology was used to observe the tumor size and distribution of nude mice on the day and 35th day after receiving colorectal cancer cell injection. Knockdown of LINC02257 significantly inhibited liver metastasis. (B) Samples showed colorectal cancer liver metastasis. LoVo cell transplantation increased colorectal cancer foci and liver metastases. Transfected siRNA LINC02257 granulocytes reduced liver metastasis. (C) Hematoxylin-eosin (HE)-stained mouse liver histopathology. HE staining of liver tissue showed that LoVo cells had a looser arrangement, more inflammatory factors and glands, and an uneven nucleus-cytoplasm ratio. The siRNA linc02257 group showed less irregular and inflammatory cell infiltration than the LoVo group. (original magnification × 100 and 200). (D) RT−qPCR detected JNK-Jun expression in vivo. LINC02257 knockdown in LoVo cells decreased JNK and Jun expression. (E) WB detected JNK-Jun expression. LINC02257 knockdown in LoVo cells decreased JNK and c-Jun activity.

membrane, cytoplasm, and nucleus. The enrichment analysis results of GO are displayed in the form of a bubble chart by using ggplot2. The GO enrichment bubble diagram is displayed by the top 20 terms with the lowest p value, and the results are shown in the following figure (Fig. 3D). KEGG enrichment analysis showed that the differentially expressed genes were mainly enriched in DNA replication, glycine serine threonine metabolism, and the MAPK signaling pathway (Fig. 3E).

4 LINC02257 mediated the expression of phosphorylated JNK in colorectal cancer cells.

To investigate the potential LINC02257 pathways in colorectal cancer cells. We analyzed the effects of LINC02257 knockdown or up-regulation on the expression of downstream target molecules. Compared to the negative control, administration of LINC02257 siRNA to LoVo cells significantly decreased the MAPK-related signaling molecules JNK and Jun ($P < 0.01$) (Fig. 4A–B). Western blot results further confirmed that LINC02257 knockdown in LoVo cells decreased JNK and Jun activity while maintaining total protein levels (Fig. 4C). Conversely, HT29 cells stably overexpressing LINC02257 had significantly increased expression of JNK and Jun (Fig. 4D).

5 LINC02257 mediated the LoVo cells through JNK signaling

We conducted gene expression analysis from LoVo cells with either downregulation of LINC02257 or overexpression of JNK following LINC02257 downregulation. We detected JNK and Jun downregulation in LoVo LINC02257 knockdown cells compared with the negative control. However, administration of an overexpression JNK plasmid reversed the effect of siRNA LINC02257 on JNK downregulation ($P < 0.001$) (Fig. 5A). Reducing LINC02257 expression in LoVo cells led to a significant decrease in cellular proliferation and migration, as demonstrated by CCK8 and Transwell assays (Fig. 5B–C). To confirm the ability of LINC02257 regulate cellular proliferation and migration, we used an agonist assay as a second independent method. LINC02257 knockdown resulted in a significant decrease in the level of P-JNK and P-Jun (Fig. 5D), in the proliferative activity of LoVo cells (Fig. 5E), and increased the cells' apoptosis (Fig. 5F–G). The addition of JNK agonist can increase the amount of P-JNK, restore cell growth, and decrease cell apoptosis. Therefore, we demonstrated that LINC02257 impacts colorectal cancer cell proliferation, migration, and apoptosis via the JNK pathway.

6 LINC02257 induces the growth and metastatic spread of colorectal cancer in vivo

The siRNA-mediated knockdown of LINC02257 resulted in a substantial reduction in tumor volume and liver metastasis compared to the Si-NC control, indicating that LINC02257 may increase the liver metastasis of colorectal cancer (Fig. 6A). Furthermore, we observed liver samples to determine whether LINC02257 expression influences metastasis formation in vivo (Fig. 6B). The siRNA-mediated knockdown of LINC02257 led to a significant decrease in metastases in samples in comparison with LoVo stably expressing LINC02257 cells. In terms of tumor growth parameters, we observed that inflammatory cell infiltration and cells with an uneven nucleus-cytoplasm ratio were reduced after administration of siRNA LINC02257 in mouse liver tissue (Fig. 6C). RT−qPCR and WB also confirmed that the expression of JNK and Jun in the siRNA LINC02257 group was significantly reduced in the tissues compared with the controls (Fig. 6D–E).

4. Discussion

In the present study, we first identified an important novel therapeutic lncRNA, LINC02257, that could be exploited to kill malignant cells in vivo. Statistical analysis of the TCGA database revealed a strong association between the expression of LINC02257 and the incidence of CRC as well as liver metastases. Subsequent experiments demonstrated that LINC02257 expression in CRC cells enhances cell proliferation and migration while suppressing apoptosis. To elucidate the mechanisms underlying LINC02257's action, we conducted next-generation sequencing (NGS) and gene enrichment analysis. The results showed that LINC02257 influences a broad array of downstream signaling pathways, predominantly involved in DNA replication, glycine, serine, and threonine metabolism, as well as the MAPK signaling pathway. To investigate the potential LINC02257 pathways in colorectal cancer cells. We overexpressed LINC02257 in LoVo cells and discovered that the downstream MAPK-related signaling molecules JNK and Jun were associated with LINC02257 expression. It is worth noting that distinct results were observed in the analysis of JNK and Jun using RT-PCR and WB. Despite a significant reduction in JNK and Jun expression upon siR-LINC02257 as evidenced by RT-PCR, the overall protein levels of total JNK and Jun did not exhibit a remarkable change compared to phosphorylated forms (p-JNK and p-Jun) in the WB analysis. We postulate that this discrepancy may be attributed to the effect of protein phosphorylation. Based on the experimental findings, it is

reasonable to hypothesize that upon phosphorylation, the JNK and Jun proteins remain stable and are not degraded, thereby maintaining an unchanged total protein content. Alternatively, the concentration of the total protein may be sufficiently high, which could obscure any observable differences in the bands. Further studies verified that LINC02257 influences cell proliferation, migration, and apoptosis in CRC cells through the JNK pathway. To evaluate the therapeutic potential of LINC02257, we injected knocked-down LoVo cells into the serous membrane of the cecum of nude mice and observed that the administration of siRNA LINC02257 led to a significant decrease in metastases in the CRC liver metastases models. This opens up exciting new avenues of investigation for the development of precision medicine for CRLM.

It is now widely recognized that lncRNAs exert significant regulatory effects on a wide array of biological processes, including genome regulation, through various mechanisms [29,30]. It affects gene expression at epigenetic, transcriptional, and translational levels and is strongly linked to malignant tumors [12,31,32]. In cancers, lncRNA can act as oncogenes or tumor repressors. lncRNA has the potential to be utilized in numerous scenarios, such as a diagnostic marker, a prognosis marker, and a therapeutic target [33–37]. A previous study [27] has reported the oncogenic roles of LINC02257 in tumor progression. And their clinical assays [27] demonstrated that LINC02257 was an independent poor prognostic factor for both overall survival (OS) and disease-specific survival (DSS) in CRC. However, the functional mechanisms of LINC02257 in their study were not investigated in great detail. In our work, we not only functionalized LINC02257 but also demonstrated the oncogenic roles of LINC02257 in CRLM.

It is unclear why the expression of LINC02257 is elevated in CRLM tissues. Recent studies has confirmed that m^{6}A alteration has a significant role in the development, proliferation, invasion, and metastasis of CRC [38], and the regulatory function of m^{6}A modified lncRNA in colorectal cancer has also been reported. Yang X et al. [39] discovered that m^{6}A methyltransferase METTL14 might prevent colorectal cancer proliferation and metastasis by downregulating the proto-oncogene lncRNA XIST. Wu Y et al. [33] discovered that m^{6}A-induced lncRNA RP11 can cause CRC cell spread through post-translational Zeb1 overexpression. m^{6}A-modified lncRNA GAS5 can alter the progression of CRC through regulating the activation of YAP [34]. These data indicate that by altering lncRNA, m6A may play a significant role in CRC. Even though the precise regulatory mechanism is unclear, LINC02257 appears to be associated with m6A alteration in malignancies. On the basis of the preceding findings, we hypothesize that m6A methylation impacts the expression of LINC02257, hence enhancing liver metastasis in colorectal cancer. However, we did not conduct any studies to demonstrate this. It is imperative to clarify the effect of m6A methylation alteration on LINC02257 and its mechanism in CRLM.

However, there are still some limitations in this study. Firstly, given the limited number of cases who participated in our experiment, more research including a considerable number of cases is required to confirm our findings. Secondly, In this study, LINC02257's prognostic effect was only analyzed biologically, and its promoting effect on CRLM and prognostic judgment effect were not discussed in our center's cases. In addition, transcriptome analysis showed that LINC02257 may be involved in a wide range of downstream signaling pathways, and it cannot be ruled out that other mechanisms may contribute to its cancer-promoting effect, which requires further investigation.

5. Conclusion

In conclusion, this study introduces an innovative therapeutic strategy for CRLM and potentially other conditions, centered on the discovery of a unique primate-specific long non-coding RNA, LINC02257, implicated in colorectal carcinogenesis. Mechanistically, our findings indicate that LINC02257 modulates the MAPK-related JNK-Jun pathway, which plays a crucial role in CRC metastasis.

Ethics approval and consent to participate

Informed consent was obtained from all individual participants included in the study. This study was approved by the Ethics Research Board of Peking Union Medical College Hospital (PUMCH), ID: K23C1279. Patients signed informed consent regarding publishing their data.

Consent for publication

Informed consent was obtained from all individual participants included in the study. All authors gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Xiangan Wu: Writing – original draft. **Xiaokun Chen:** Writing – original draft. **Xiao Liu:** Writing – original draft. **Bao Jin:** Writing – review & editing. **Yuke Zhang:** Writing – review & editing. **Yuxin Wang:** Writing – review & editing. **Haifeng Xu:** Supervision. **Xueshuai Wan:** Supervision. **Yongchang Zheng:** Supervision. **Lai Xu:** Supervision. **Yi Xiao:** Supervision. **Zhengju Chen:** Software, Formal analysis, Data curation. **Haiwen Wang:** Software, Formal analysis, Data curation. **Yilei Mao:** Supervision. **Xin Lu:** Supervision. **Xinting Sang:** Supervision. **Lin Zhao:** Conceptualization. **Shunda Du:** Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30841.

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