

lncRNA STEAP3-AS1 Modulates Cell Cycle Progression via Affecting CDKN1C Expression through STEAP3 in Colon Cancer

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Previous studies have reported that long noncoding RNAs (lncRNAs) have acted as new players during tumorigenesis. Metallothionein also plays an important role in tumor progression. It is mainly considered to be involved in the process of cell proliferation, oxidative stress, and multidrug resistance. However, the potential involvement of metallothionein-related lncRNAs in colon cancer remains poorly understood. In our study, we found that MT1M affected the expression of lncRNA STEAP3-AS1. STEAP3-AS1 is located in physical contiguity with STEAP3 and notably increased in colon cancer tissues and cell lines. STEAP3-AS1 expression was negatively associated with the expression of STEAP3. High levels of STEAP3-AS1 were associated with poor overall survival in colon cancer patients. In *in vitro* assays, STEAP3-AS1 knockdown could inhibit colon cancer cell proliferation and migration and arrest colon cancer cells at the G₀-G₁ phase. In tumorigenicity assays, STEAP3-AS1 knockdown could strongly inhibit tumor growth. Mechanistic investigations demonstrated that STEAP3-AS1 downregulation could increase the expression of cyclin-dependent kinase inhibitor 1C (CDKN1C) by STEAP3 upregulation. Overall, we identify the underlying role of MT1M-related lncRNA STEAP3-AS1 in colon cancer progression, which provides a novel strategy for colon cancer therapy.

INTRODUCTION

Colon cancer is the third most common malignant tumor worldwide. In the United States, it is the second leading cause of death, responsible for 8% of diagnosed cancers and 8% of cancer deaths.¹ The incidence rates were 30%–40% higher in men than in women. Owing to multi-modality therapy, the incidence and mortality rate of colon cancer have steadily decreased in recent years. However, the prognosis of colon cancer patients is still not satisfactory as a result of tumor recurrence and metastasis.² Thus, it is crucial to find clinically useful tumor markers that can be helpful for providing therapeutic strategies of colon cancer.

Long noncoding RNAs (lncRNAs) are usually defined as a type of transcripts of greater than 200 nt in length and lacking an open

reading frame of significant length (less than 100 aa).³ Originally, lncRNAs were regarded as “noise” in genome transcription and had no biological function. However, more and more studies have reported that lncRNAs act as new players during tumorigenesis by gene regulation in the transcriptional and post-transcriptional levels. lncRNAs are often dysregulated in cancer and emerge as oncogenes or tumor suppressors.^{4,5} They are widely involved in various signaling pathways, and their dysregulations are closely related to tumorigenesis.

Metallothionein (MT) comprises a multigene family with high content of cysteine residues. It consists of four isoforms, designated MT1 to MT4.⁶ In general, metallothionein is proposed as a key player in the homeostasis of zinc and copper and the detoxification process of cadmium and mercury.⁷ As an antioxidant, metallothionein can effectively eliminate the hydroxyl radical in the redox reaction.⁸ In rapidly increasing and highly metabolized tumor tissues, overexpression of metallothionein may promote tumor cell proliferation.⁹ Metallothionein has been shown to participate in the cell cycle process, and knockdown of metallothionein 2A inhibited the cell cycle from the G₁ to S phase in breast cancer.¹⁰ Metallothionein could regulate apoptosis-related proteins via interacting with zinc and iron.¹¹ Conversely, metallothionein II(a) downregulation could induce prostate cancer and ovarian cancer cell apoptosis.¹² The tumor necrosis factor (TNF)-induced oxidative stress in hepatocellular cancer could be inhibited by autophagy of metallothionein.¹³ Additionally, metallothionein is reported to be closely correlated with tumor cell drug resistance. Thus, metallothionein may affect tumor cell proliferation, apoptosis, oxidative stress, and drug resistance in cancer occurrence and progression.

Received 24 February 2020; accepted 15 June 2020;
<https://doi.org/10.1016/j.omtn.2020.06.011>.

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In humans, MT2, MT3, and MT4 proteins are encoded by a single gene. Further expansion of the MT1 gene in humans has occurred in the primate lineage, resulting in a total of 13 paralogs (MT1A to MT1J, MT1L, MT1M, and MT1X). The known active MT1 genes are MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1M, and MT1X. The rest of these paralogs (MT1C, MT1D, MT1I, MT1J, and MT1L) are pseudogenes that have been predicted to lose their protein-coding capacity through such molecular events as point or frameshift mutations.^{6,14,15} The loss of invariant cysteines is the most frequent event, accounting for pseudogenization of five MT1 genes.⁶ There was a premature stop codon (Cys5Tyr, Cys15Tyr, Cys24Tyr, Cys26X, Cys37Tyr, Cys41X, Cys50X, and Cys60Arg) in MT1JP, MT1CP, MT1DP, and MT1LP. Three non-cysteine codons encode for an aromatic residue (Gly11Phe, Ser18Phe, and Ser35Phe) covering MT1IP and MT1CP. Also, a 1-bp deletion was at the C-terminal domain in MT1IP. Because cysteine replacement either with tyrosine or with a stop codon contains only a single nucleotide substitution, these residues are expected to often result in metallothionein pseudogenization. Pseudogenes have been viewed as non-functional relics littering the human genome. Recently, it has become apparent that numerous pseudogenes are transcribed into lncRNAs and perform their diverse biological functions. MT1JP is a member of the metallothionein family and, as one of pseudogenes, it is transcribed into lncRNA, which plays a biological function in transcriptional or post-transcriptional regulation of gene expression. It was reported that MT1JP functioned as a competing endogenous RNA (ceRNA) to competitively bind to miR-92a-3p and regulated the expression of FBXW7 in gastric cancer.¹⁶ Another metallothionein MT1DP was found to be involved in the cytotoxicity process of heavy metal cadmium. MT1DP can bind to RhoC protein and increase its expression and lead to the death of hepatocytes. Considering that the pseudogenes in the metallothionein family themselves are lncRNAs, these lncRNAs may affect the real metallothionein at the transcriptional or post-transcriptional level with regard to cellular defense and cytotoxicity. MT1DP served as a competitive endogenous RNA and competed for miR-24 with MT1H.¹⁷ Otherwise, the transcription factor RunX2, together with YAP, was involved in the occurrence of liver cancer by inhibiting the expression of tumor suppressor lncRNA MT1DP via affecting FoxA1.¹⁸ However, it is rarely known whether other members of metallothionein can be regarded as lncRNAs or whether metallothionein is correlated with lncRNA in tumorigenesis.

New insights have been gained into the effects of lncRNAs in cancer by screening out significantly differential lncRNAs via rapid high-throughput sequencing methods and connecting them with special biological processes, such as tumor cells proliferation, migration, apoptosis, and metastasis.¹⁹ Presently, reports have linked lncRNAs with colon cancer. lncRNA CCAT2 (colon cancer-associated transcript 2) activated Wnt signaling via upregulation of MYC, miR-17-5p, and miR-20a in colon cancer metastasis.²⁰ lncRNA UICLM was upregulated in colon cancer liver metastasis tissues and regulated ZEB2 expression by endogenously competing with miR-215.²¹ Conversely, lncRNA CPS1-IT1 was downregulated in colon cancer

tissues, and overexpression of lncRNA CPS1-IT1 significantly inhibited colon cancer cell proliferation, invasion, epithelial-mesenchymal transition, and metastasis.²² Thus, lncRNAs could play important roles in colon cancer progression. However, the number of lncRNAs associated with colon cancer is limited and further study is urgently needed.

In previous research, we found that dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin-related protein (DC-SIGNR), one type of C-type lectin, increased the expression of metallothioneins (including MT1M, MT1B, MT1G, MT1H, MT1X, and MT1F) in colon cancer progression, and we suspected that metallothioneins may be associated with colon cancer.²³ Among these metallothioneins, MT1M was dysregulated more obviously than others. Additionally, emerging evidence has shown that MT1M was an important part in the cellular and pathological processes.²⁴ As a major member of the metallothionein family, high expression of MT1M is related to pancreatic ductal adenocarcinoma, breast cancer, and colorectal carcinoma.^{25–27} MT1M and its related lncRNAs LINC01589 and LINC00960 showed profoundly increased expression in pancreatic cancer cells in comparison with normal cells. The integration of MT1M and the related lncRNAs could be useful in the future for predicting patient survival and condition with regard to pancreatic cancer. In breast cancer, MT1M and coexpressed lncRNAs AL139280.1 and AP000851.1 were significantly related to overall survival. In colorectal cancer, MT1M was a highly expressed isoform in tumor epithelia. It is reported that metallothionein overexpression is an independent prognosticator for relatively poor survival of patients. In primary colon cancer, metallothionein expression was significantly correlated with lymph node metastasis, suggesting that metallothionein may modulate the tumor metastatic process.²⁸ However, the relationship between MT1M and lncRNA in colon cancer is unclear.

In this study, we first used lncRNA microarray to analyze MT1M-related lncRNAs and found lncRNA STEAP3-AS1, an unknown anti-sense lncRNA. We further studied the biological function of lncRNA STEAP3-AS1 and explored its molecular mechanism in colon cancer.

RESULTS

lncRNA Expression Profile in Colon Cancer Cells after MT1M Downregulation

lncRNAs are dysregulated in a large number of cancers, indicating that they are closely associated with cancer development, progression, and metastasis. The expression level of MT1M was notably silenced by small interfering RNA (siRNA) in LoVo cells, and the impact of MT1M knockdown efficiency was detected by real-time PCR. The expression levels of MT1M in two MT1M short hairpin RNA (shRNA) cell lines were both lower compared with control shRNA cells (Figure 1A). Additionally, the MT1M level in shRNA2 cells was clearly lower than that in control shRNA cells. Thus, we selected the MT1M shRNA2 interference sequence for further study. We profiled the expression of lncRNAs in MT1M shRNA and control shRNA cells by LncPath human cancer array. A total of 43 dysregulated lncRNAs were detected (Figure 1B). Gene Ontology (GO) analysis

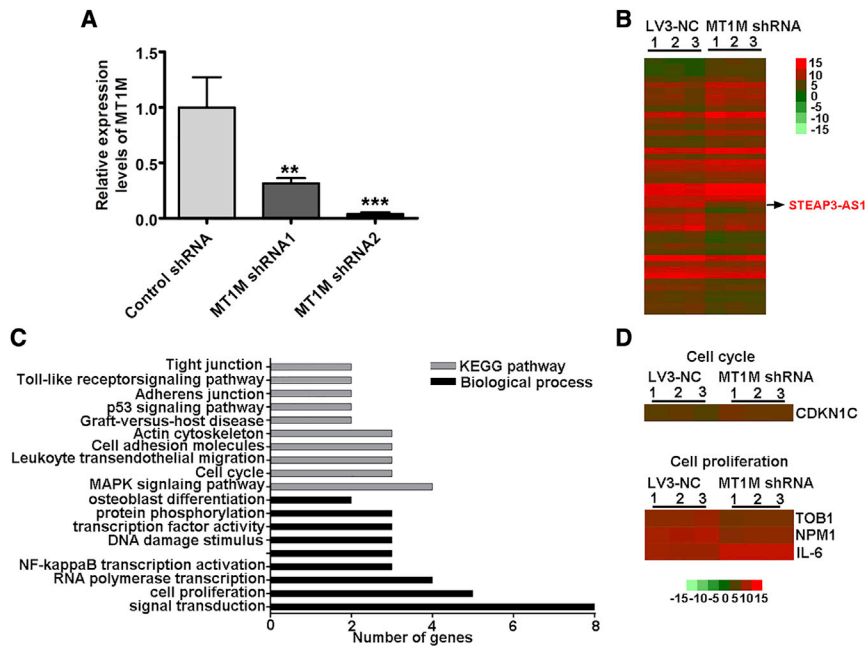


Figure 1. MT1M Dysregulates a Series of Tumor-Associated lncRNAs in LoVo Cells

(A) The knockdown efficiency of MT1M is shown in LoVo cells. (B) Hierarchical clustering was performed to show the distinguishable lncRNAs and showed the relationships among the expression levels of samples. High relative expression is indicated by red, and low relative expression is indicated by green. (C) Gene Oncology and KEGG pathway analysis of lncRNAs associated with coding genes. (D) Hierarchical clustering shows different genes associated the process of cell cycle and cell proliferation.

was performed on the lncRNA-associated mRNAs with terms under Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and biological processes (Figure 1C). With MT1M knockdown, it caused a series of biological processes, mainly including signal transduction, cell proliferation, and cell cycle. The statistical expressions of genes for cell cycle and cell proliferation are shown in Figure 1D. These data indicate that a series of lncRNAs are aberrantly expressed in MT1M knockdown colon cancer cells.

lncRNA STEAP3-AS1 Was Upregulated in Colon Cancer Tissue

We listed the top 10 dysregulated lncRNAs between the MT1M shRNA group and the control group (Figure 2A). To validate the microarray data, we selected four lncRNAs from the differentially expressed lncRNAs and validated the expression levels by real-time PCR. As shown in the results, the expression trend of those genes was consistent with microarray results (Figure 2B). To identify the role of MT1M-regulated lncRNA in tumorigenesis, we selected STEAP3-AS1, which was downregulated most prominently among those lncRNAs in the microarray above, for further study. We analyzed its expression level in the public dataset The Cancer Genome Atlas (TCGA) from 453 colon cancer samples and 41 non-tumorous samples. Results showed that STEAP3-AS1 was annotated in TCGA, and the results showed that STEAP3-AS1 was significantly elevated in tumor versus normal pairs (Figure 2C). Correlations between the clinicopathological parameters of colon cancer patients and STEAP3-AS1 levels are listed in Table 1. STEAP3-AS1 expression was statistically related to age. However, there were no differences in STEAP3-AS1 levels with sex, stage, and tumor, node, and metastasis (TNM). Additionally, we measured the correlation expression pattern of STEAP3-AS1 and STEAP3 in colon cancer tissues using TCGA dataset. We found that STEAP3-AS1 expression was signifi-

cant negatively associated with STEAP3 expression ($r = -0.1442$, $p = 0.0086$) (Figure 2D). We also analyzed the association of STEAP3-AS1 with patient survival and primary tumors. Survival analysis was performed using Kaplan-Meier curve and the log-rank test to estimate the survival probability. Result identified that the patients with high STEAP3-AS1 expression (RPKM [reads per kilobase transcript per million mapped reads] value ≥ 0.05510) tended to have worse overall survival compared with patients with low STEAP3-AS expression (Figure 2E). Then, we validated these data with RNAs extracted from tumor tissues and their matched normal tissues of 17 patients by quantitative real-time PCR. Results showed that the expression of STEAP3-AS1 was upregulated in colon cancer tissue compared with that in the matched non-tumor tissues (Figure 2F; Table S2). These results demonstrate that the expression of STEAP3-AS1 is elevated in colon cancer tissues.

Knockdown of lncRNA STEAP3-AS1 Decreased Colon Cancer Cell Proliferation and Migration

To investigate the influence of lncRNA STEAP3-AS1 on the biological function of colon cancer cells, we used quantitative real time PCR to detect the relative expression of STEAP3-AS1 in five colon cancer cell lines (LoVo, HCT-116, SW480, SW620, and LS174T) and one human intestinal epithelial cell line (HIEC). Result showed that the expression of lncRNA STEAP3-AS1 was upregulated in five colon cancer cell lines compared with that in HIEC cells (Figure 3A). lncRNA STEAP3-AS1 was expressed at much higher levels in LoVo and HCT-116 cells. Then, we selected the above two cell lines for further research and knocked down STEAP3-AS1 expression by using lentivirus transfection. The knockdown efficiency was verified by qPCR. Results implied that those two lentivirus STEAP3-AS1 shRNAs could significantly suppress the expression of STEAP3-AS1 in LoVo and HCT-116 cells (Figures 3B and 3C). To determine the role of STEAP3-AS1, we sought to explore its loss of function in colon cancer carcinogenesis. First, we used MTT assay to observe its effect on cell proliferation. Result showed that STEAP3-AS1 knockdown inhibited LoVo and HCT-116 cells proliferation compared with negative control cells (Figure 3D). Additionally, in colony formation assay, we also confirmed that the number of STEAP3-AS1-silenced cells was

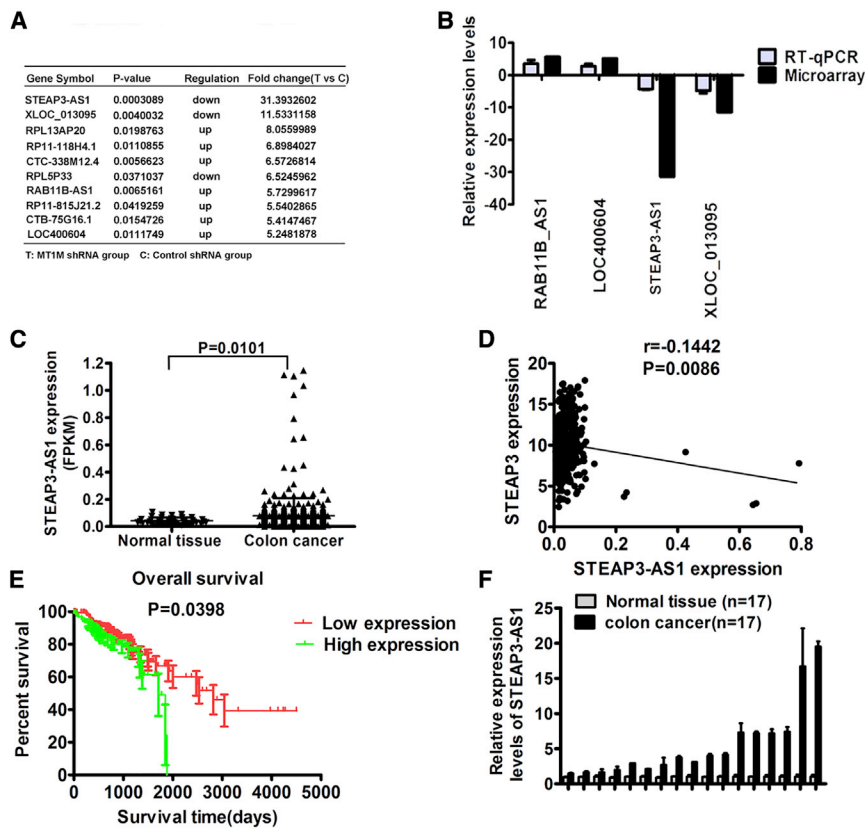


Figure 2. The Expression of lncRNA STEAP3-AS1 Is Elevated in Colon Cancer Tissues

(A) The top 10 genes were significantly upregulated or downregulated in LoVo cells after MT1M knockdown. (B) Real-time PCR demonstrated four gene trends that were consistent with microarray results. (C) RPKM value is represented for the expression level of STEAP3-AS1 in TCGA database. The expression of STEAP3-AS1 was elevated in 453 colon cancer tissues versus 41 normal tissues. (D) Correlation between the expression of STEAP3-AS1 and the expression of STEAP3 in the colon cancer data in TCGA. (E) Kaplan-Meier analysis of STEAP3-AS1 expression in overall survival in the colon cancer dataset. (F) STEAP3-AS1 expression in human colon cancer and normal tissue by real-time PCR analysis.

in cells treated with STEAP3-AS1 shRNA transfection (Figure 4D). Cyclin and acetyl-H3 are very important cellular markers in the process of the cell cycle. Cyclin E2, one member of cyclin, forms a complex and functions as a regulatory factor of CDK2. Additionally, histones are basic nuclear proteins that are important for the nucleosome structure of the chromosomal fiber in eukaryotes. Thus, we added the related western blot data as shown in Figure 4. Results showed that the expression of cyclin E2 and acetyl-H3 were also decreased in colon cancer cells when STEAP3-AS1 was knocked down.

These results demonstrate that STEAP3-AS1 downregulation inhibits colon cancer cell cycle progression *in vitro*.

lncRNA STEAP3-AS1 Knockdown Inhibited Tumor Growth *In Vivo*

To assess whether knockdown of lncRNA STEAP3-AS1 affects tumor growth *in vivo*, LoVo cells transfected with STEAP3-AS1 shRNA1 or STEAP3-AS1 shRNA2 and control shRNA were subcutaneously injected into the two flanks of nude mice. Consistent with *in vitro* results, tumor growth in the two STEAP3-AS1 shRNA groups was obviously slower than that in the control shRNA group (Figures 5A and 5B). Tumor size was calculated every 4 days. All mice were killed and tumors were dissected out 24 days after transplantation. The tumor growth rate was slower in the STEAP3-AS1 shRNA-transfected mice compared with control shRNA-transfected mice (Figure 5C). Additionally, the average tumor weight in the STEAP3-AS1 shRNA group was lower than that in the control shRNA group (Figure 5D). We observed the histological changes in two groups by H&E staining and immunostaining staining of CK20, CK7, CDK4, and STEAP3. The tumors were exclusively positive for CK20 and negative for CK7. Additionally, knockdown of STEAP3-AS1 could significantly reduce the expression of CDK4 and increase its neighboring gene STEAP3 (Figure 5E). These data confirm that knockdown of lncRNA STEAP3-AS1 may inhibit colon cancer tumorigenesis *in vivo*.

decreased compared with that of control shRNA cells (Figure 3E). Then, we assessed the influence of STEAP3-AS1 on cell migration by transwell migration assay (Figure 3F). As shown in the results, the numbers of the migration cells that went through the transwell membrane were significantly reduced following the delivery of STEAP3-AS1 silencing with transfection of two different shRNAs. Similarly, in a wound-healing assay, two STEAP3-AS1 knockdown cell lines showed a much slower recovery compared with control cells (Figure 3G). These results demonstrate that STEAP3-AS1 knockdown inhibits colon cancer cell proliferation and migration *in vitro*.

lncRNA STEAP3-AS1 Downregulation Suppressed Cells Cycle Progression

To assess whether STEAP3-AS1 downregulation affected cell cycle progression, the cell cycle was detected. Using flow cytometry, we observed that cells transfected with STEAP3-AS1 shRNA could induce cell-cycle arrest at the G₀-G₁ phase (Figure 4A). Additionally, we repeated the experiment by 1-day fetal bovine serum (FBS) starvation and justified the data in the cell cycle assay again. Actually, upon transfection of two shRNAs, the percentage of cells in the G₁ phase significantly increased with lncRNA STEAP3-AS1 knockdown in both cell lines after 1-day FBS starvation (Figures 4B and 4C). Western blot results showed that protein levels of the key checkpoint factors in the G₀-to-G₁ phase transition including CDK2, and the G-to-S phase transition including CDK4, were significantly downregulated

Table 1. Clinical Data for STEAP3-AS1 in Colon Cancer Based on TCGA

Clinicopathological Features	No.	p Value
Tissues		
Normal colon	41	0.0477
Colon cancer	453	
Age		
≥ 60 y	329	0.0314
<60 y	124	
Sex		
Female	214	0.5013
Male	239	
Stage		
I+II	264	0.1389
III+IV	189	
T (tumor)		
T1+T2	86	0.7429
T3+T4	367	
N (node)		
Absent	272	0.3844
Present	181	
M (metastasis)		
Absent	351	0.5622
Present	65	

lncRNA STEAP3-AS1 Regulated STEAP3 to Modulate CDKN1C in Colon Cancer Cells

Recent studies indicated that antisense lncRNA can affect the expression of its nearby protein-coding genes. Noticeably, lncRNA STEAP3-AS1 is located at the antisense chain of the cognate sense gene STEAP3 (Figure 6A). As shown in Figure 6B, we used the LncPro database to predict the interaction of STEAP3-AS1 and STEAP3. Results showed that the predicted score between the two molecules was 80.2355. It was demonstrated that lncRNA STEAP3-AS1 might have intense interaction with STEAP3. There were also many binding sites between STEAP3 and STEAP3-AS1 predicted by the catRAPID website (Figure 6C). The online prediction website analysis revealed binding sites between STEAP3-AS1 and STEAP3. Next, using an RNA immunoprecipitation (RIP) assay, we found that STEAP3-AS1 was precipitated with anti-STEAP3 antibodies in LoVo and HCT-116 cells (Figure 6D). The above result supported that lncRNA STEAP3-AS1 could specifically bind to the STEAP3 gene. Additionally, the mRNA and protein levels of STEAP3 were significantly increased after transfection of STEAP3-AS1 shRNAs in LoVo and HCT-116 cells (Figures 6E and 6F). It is necessary to clone the expression of lncRNA STEAP3-AS1 to detect the expression of STEAP3. Therefore, we have established the overexpression plasmid of STEAP3-AS1. We transfected the STEAP3-AS1 overexpression plasmid into colon cancer cells and observed the expression of STEAP3 after transfection. Results showed that the expression of STEAP3 was lower in STEAP3-AS1 overexpressed cells compared

with control cells in protein levels (Figure 6G). Thus, STEAP3-AS1 overexpression can downregulate the expression of STEAP3. Additionally, CDKN1C (which is a related gene of STEAP3-AS1, as shown in microarray data) was also upregulated in STEAP3-AS1 shRNA cells in mRNA and protein levels (Figures 6H and 6I). The expression of CDKN1C was decreased after STEAP3-AS1 overexpression in colon cancer cells (Figure 6J). For further study, we used a STRING 10 algorithm for constructing a protein-protein interaction network between STEAP3 and CDKN1C. Results showed that the two molecules have some exact connections in some ways (Figure 6K). Additionally, it was reported that STEAP3 might mediate the downstream response to p53, and we have demonstrated that STEAP3-AS1 regulated the expression of STEAP3. Thus, we inferred that there was some interaction between STEAP3-AS1 and p53. We therefore detected the protein expression of p53 after STEAP3-AS1 downregulation or upregulation in colon cancer. The results showed that STEAP3-AS1 downregulation could significantly increase the expression of p53. Conversely, p53 expression was decreased in STEAP3-AS1 overexpressed cells compared with control cells. To investigate the relationship between STEAP3 and CDKN1C, we interfered STEAP3 by siRNA and STEAP3 expression plasmid in LoVo and HCT-116 STEAP3-AS1 knockdown cells, respectively. Results showed that STEAP3 was downregulated after STEAP3 siRNA transfection in STEAP3-AS1 shRNA cells, and STEAP3 downregulation in the STEAP3-AS1 shRNA cells decreased the expression of CDKN1C (Figure 6L). Additionally, STEAP3 overexpression upregulated the expression of CDKN1C in STEAP3-AS1 shRNA-transfected cells (Figure 6M). We also transfected the STEAP3 overexpression plasmid into LoVo STEAP3-AS1 overexpressed cells. We found that the expression levels of STEAP3 and CDKN1C were both increased. Taken together, these data imply that lncRNA STEAP3-AS1 knockdown may increase CDKN1C by modulating the expression of its sense gene STEAP3.

DISCUSSION

Metallothionein has been demonstrated as a key modulator in tumor development and progression. Recently, it was reported that metallothionein is strongly related to the lncRNA regulatory mechanism.^{17,18} In this study, we focused on the capacity of MT1M to induce the expression of lncRNAs in colon cancer cells and found that MT1M is specifically associated with one lncRNA named STEAP3-AS1. The expression of STEAP3-AS1 was increased both in colon cancer tissues and cells. Furthermore, we have explored that silenced STEAP3-AS1 could inhibit colon cancer cell proliferation, migration, and cell cycle by regulation of CDKN1C through STEAP3. Thus, these observations together demonstrate that STEAP3-AS1 may be an important non-protein factor in colon cancer progression.

Metallothionein is a multigene family consisting of several homologous genes. It contains four main isoforms (MT1, MT2, MT13, MT4). Owing to cysteine replacement with tyrosine or a stop codon, it contributes to metallothionein pseudogenization.⁶ Thus, some of genes are known as pseudogenes that cannot encode metallothionein proteins, such as MT1DP, MT1JP, and MT1P3.²⁴ As they are

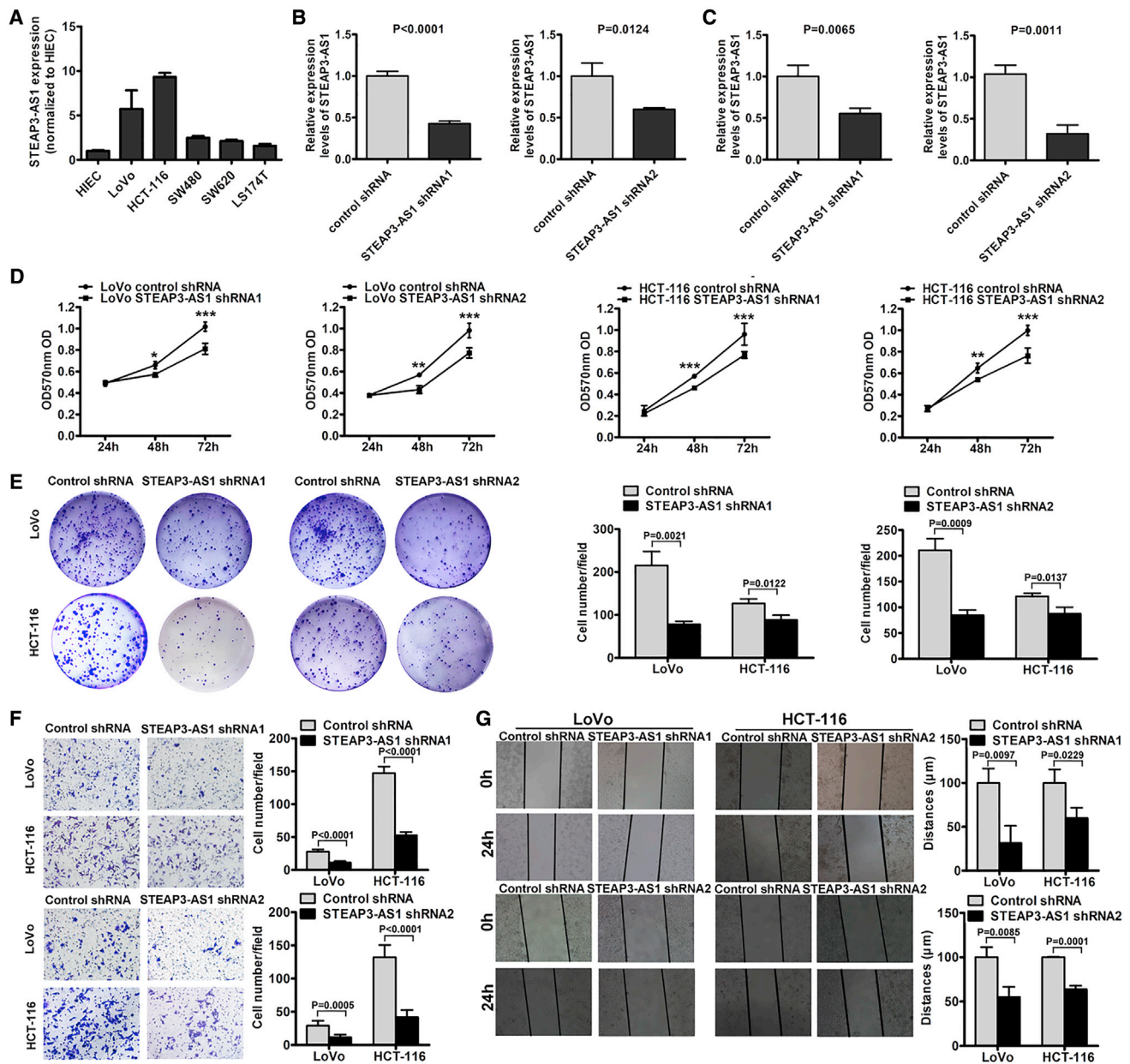


Figure 3. Knockdown of lncRNA STEAP3-AS1 Expression Inhibits Colon Cancer Cell Growth and Migration *In Vitro*

(A) lncRNA STEAP3-AS1 was increased in colon cancer cells. It was upregulated in colon cancer cells compared with normal HIEC cells. (B and C) STEAP3-AS1 expression was knocked down by two shRNAs targeting STEAP3-AS1 in (B) LoVo and (C) HCT-116 cells. (D) Downregulation of STEAP3-AS1 significantly inhibited LoVo and HCT-116 cell growth using an MTT assay. (E) The STEAP3-AS1 shRNA cell colony formation number was significantly decreased compared with that of control shRNA cells. (F) Compared with control shRNA cells, the number of two STEAP3-AS1 shRNA cell lines which went through from polycarbonate membrane was statistically decreased. (G) The migration rate of two STEAP3-AS1 shRNA group cell lines decreased compared with that of control cells. The relative cell migrations of the two colon cancer cell lines at 24 h are shown.

generally considered noncoding and nonfunctional, pseudogenes are usually ignored. Recently, several studies supported functional roles for some metallothionein pseudogenes as lncRNAs in many biological processes.^{16–18} Thus, the members of metallothionein could take effect as lncRNAs in cancer development. Except for

those metallothioneins, whether other metallothioneins could be lncRNAs or correlated with lncRNA regulation in cancer is far from being fully known. In our study, we first found that MT1M, which was not a pseudogene, regulated the expression of lncRNA STEAP3-AS1 and that STEAP3-AS1 played an important role in

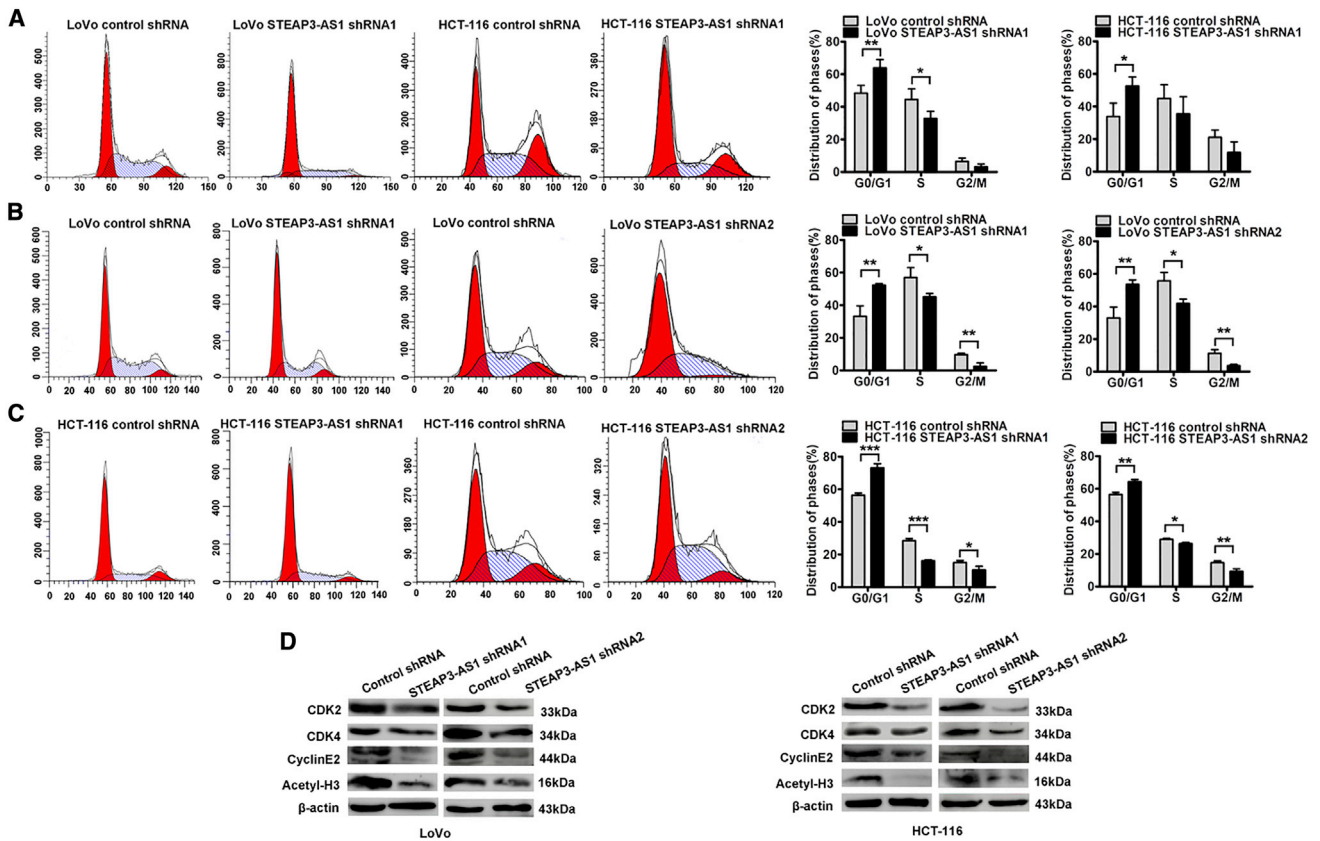


Figure 4. IncRNA STEAP3-AS1 Downregulation Affected Colon Cancer Cell Progression

(A) The flow cytometry assay shows that knockdown of IncRNA STEAP3-AS1 arrested cells at the G_0 - G_1 phase. (B and C) The flow cytometry assay demonstrated that (B) LoVo and (C) HCT-116 cells transfected with two shRNAs had cell cycle arrest at the G_0 - G_1 phase in comparison with control cells after 1-day FBS starvation. (D) The cell cycle STEAP3-AS1 downregulation decreased the expression of CDK2, CDK4, cyclin E2, and acetyl-H3. The error bars in all graphs represent SD, and the results were quantified from three experiments.

colon cancer occurrence. It is novel that metallothionein may interact with lncRNAs, and our investigations might provide an extraordinary understanding of biological processes in which metallothioneins are involved.

Antisense lncRNAs, one type of lncRNAs, are reverse complements of their sense counterparts. In recent studies, antisense lncRNAs have been valued for their highly specific effect on their neighboring genes.^{29,30,31} The pattern of sense-antisense interaction is divided into three basic types: head to head (divergent), tail to tail (convergent), and fully overlapping.²⁹ They could have effects on the genes nearby by two types of regulation: discordant or concordant. Hybridizing with their sense transcript, antisense lncRNA could result in RNA masking, steric inhibition, transcriptional interference, or methylation.³² Generally, this is based on the knockdown of antisense transcripts and the expression of cognate sense gene detection. In the discordant manner, knockdown of the antisense transcript could increase the expression of the corresponding sense gene, as in the agonist-activator way.^{29,33} In the case of concordant regulation, antisense knockdown leads to the synergistic reduction of the sense tran-

script.³⁴ In our study, IncRNA STEAP3-AS1 is the antisense transcript of STEAP3, and, of significance, our findings clearly showed that the interaction of two molecules might be discordant. Knockdown of IncRNA STEAP3-AS1 could elevate the expression of STEAP3 on both the mRNA and protein levels. STEAP3, also known as TSAP6 (tumor suppressor-activated pathway 6), is a member of the human 6-transmembrane epithelial antigens the of prostate family.³⁵ It can reduce iron and function as an iron transporter.³⁶ STEAP3 could be involved in a wide range of biological processes, such as endocytosis and exocytosis regulation, cell proliferation, cell cycle, and apoptosis.^{35,37} It was reported to be a candidate prognostic marker of metastatic high-grade serous carcinoma.³⁸ In colorectal cancer, the expression of STEAP3 was significantly increased in tumor tissues compared with normal colon mucosa.³⁹ We have demonstrated that STEAP3-AS1 may regulate the expression of STEAP3 and in some ways act as an important role in colon cancer.

Furthermore, we explored the potential regulatory capacity of IncRNA STEAP3-AS1. Accordingly, CDKN1C may partly contribute to STEAP3-AS1-mediated expression of STEAP3. This conclusion is

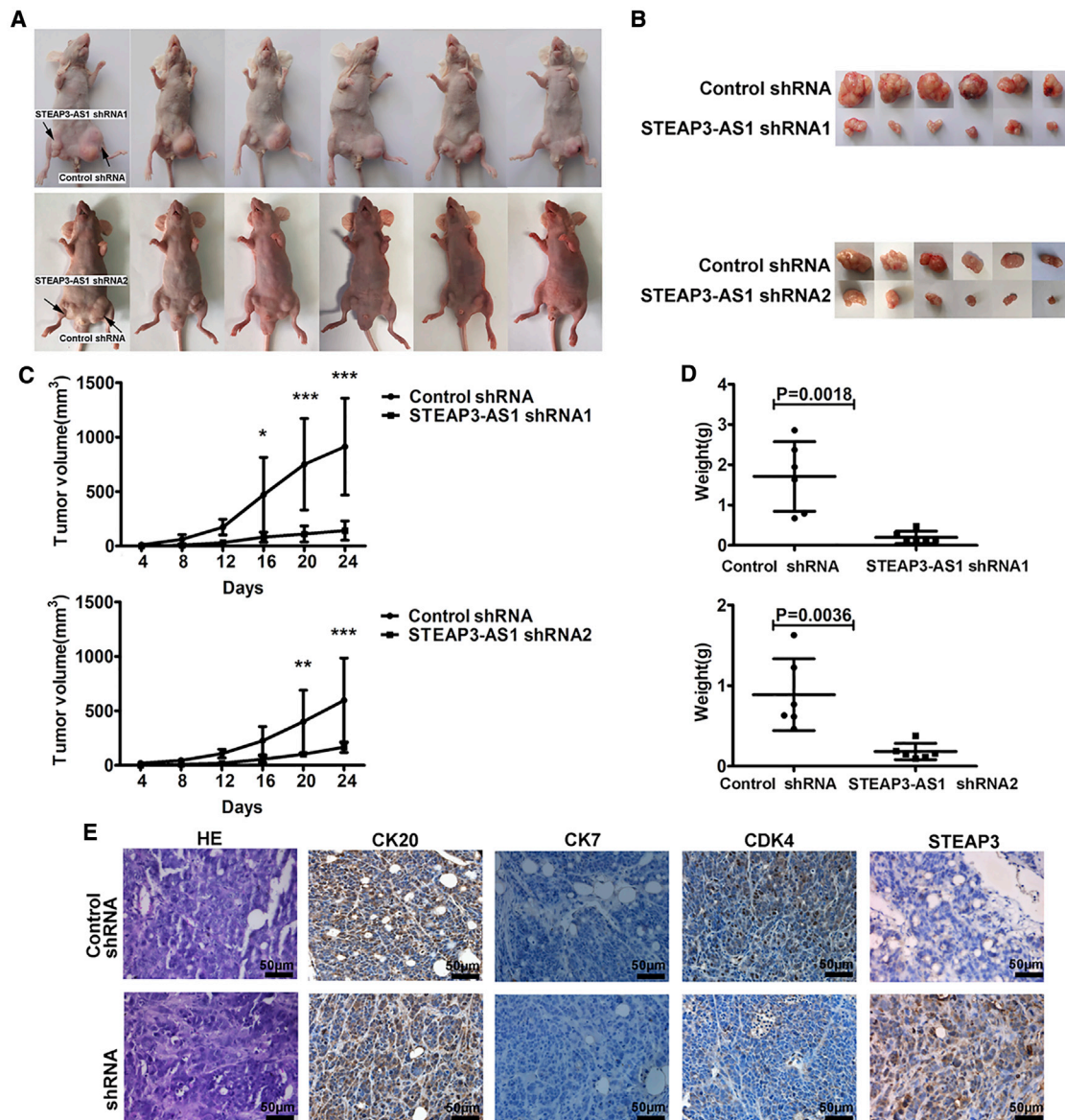
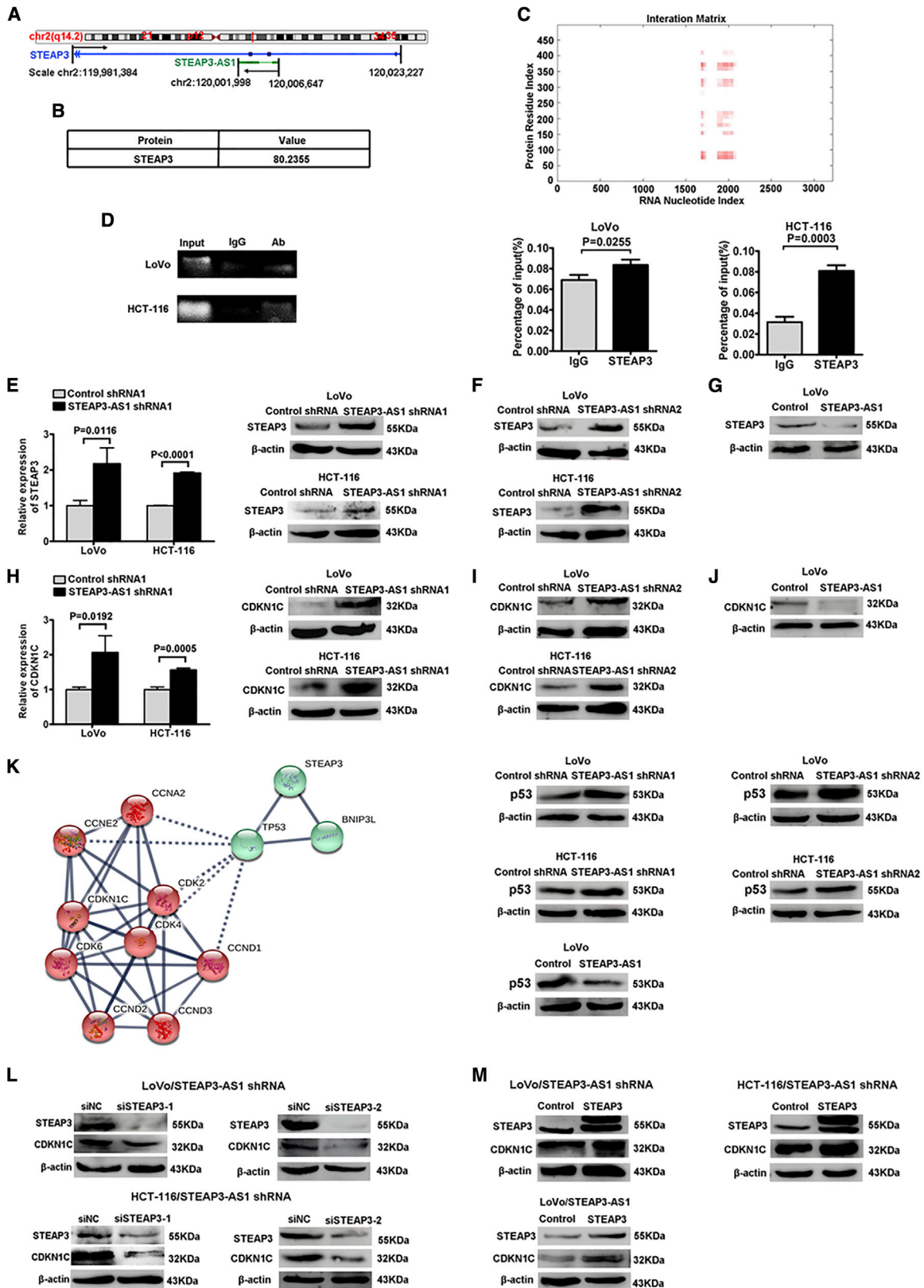


Figure 5. IncRNA STEAP3-AS1 Knockdown Inhibits Tumor Growth *In Vivo*

(A and B) Knockdown of IncRNA STEAP3-AS1 expression significantly inhibited tumor growth of LoVo cells in the nude mouse model. (A) The tumors were photographed with an in vivo imaging system. (B) The harvested transplanted tumors. (C and D) The tumor volume (C) and weight (D) of mice injected by STEAP3-AS1 shRNA cells was smaller than that of control shRNA cells. (E) Representative images of H&E, CK20, CK7, CDK4, and STEAP3 staining of tumor tissues. The error bars in each graph represent SD, and the results are shown from three independent experiments. Each bar represents the mean \pm SD from three independent experiments.

based on several phenomena. STEAP3-AS1 knockdown upregulated the expression of CDKN1C in colon cancer cells. Conversely, STEAP3-AS1 overexpression decreased CDKN1C expression. Additionally, we transfected siRNAs which was targeted the region of its sense gene STEAP3 and observed the expressions of STEAP3 and CDKN1C. After STEAP3 knockdown, the expression of CDKN1C was also reduced. STEAP3 overexpression could upregulate CDKN1C expression. CDKN1C, a cyclin-dependent kinase inhibitor (CKI) belonging to the CIP/KIP family, is predicted to be related to

STEAP3-AS1. Recombinant CDKN1C potentially inhibits the expression of cyclin complexes, including cyclin D1-CDK4, cyclin A-CDK2, and cyclin B1-CDC2.⁴⁰ It is also essential to the upregulation of CDKN1C in human hematopoietic cells for transforming growth factor β (TGF- β)-induced cell cycle arrest.⁴¹ B-Myb partially competed with CDKN1C to induce G₁ arrest in human osteosarcoma cells.⁴² Thus, CDKN1C is an important inhibitor in the cell cycle process. Additionally, it is reported that CDKN1C has been endowed with some hallmarks of cancer, including invasion apoptosis,



(legend on next page)

differentiation, angiogenesis, and metastasis.^{43,44} CDKN1C was significantly decreased in hepatocellular carcinoma tissues, and CDKN1C downregulation mediated by Jab1/Csn5 accelerated the growth and invasion of hepatocellular carcinoma cells *in vitro* and *in vivo*.⁴⁵ In gastric cancer cell lines, Shin et al.⁴⁶ found that the general mechanism for inactivation of CDKN1C seemed due to the formation of an inactive chromatin through histone deacetylation. The expression of CDKN1C also decreased dramatically in colorectal carcinomas compared with normal tissue.⁴⁷

Furthermore, potential interaction with STEAP3 and CDKN1C were constructed by the STRING 10 database. Results showed that both of these molecules might be related to p53. One cluster may occur through p53, STEAP3, and BNIP3L. The other cluster may connect p53 and CDKN1C, potentially via CDK2, CDK4, CDK6, CCND1, CCND3, CCND2, CCNA2, and CCNE2. We have also demonstrated that downregulation of STEAP3-AS1 could decrease the expression of CDK2 and CDK4. Supporting these, Passer et al.³⁷ reported that TSAP6 could be downstream of p53 and affect the cell apoptosis and cell-cycle progression. It is sufficient to cause the secretion of exosomes through STEAP3 transcription by p53.⁴⁸ For CDKN1C, its loss could be attributable to hyperactivation of p53 at the DN3–DN4 transition.^{49,50} Also, it was reported that in quercetin- and cisplatin-treated cells, the expression of CDKN1C, CCNA2, CCND2, CCND3, CCNE1, and CDK2 could be simultaneously elevated.⁵¹ Thus, we suspected there might be some interactions between STEAP3 and CDKN1C, and further studies are needed.

MATERIALS AND METHODS

Cell Culture and Nude Mice

Human colon cancer cell lines LoVo, HCT-116, SW480, SW620, and LS174T and the human intestinal epithelial cell line HIEC were used in this study. Cells were routinely cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. 6- to 8-week-old nude mice were purchased from Dalian Medical University. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Dalian Medical University.

Plasmid

The two shRNA sequences for knockdown of lncRNA STEAP3-AS1 were as follows: shRNA1, 5'-GCACCTTTAAACTGTCCTACA-3'; shRNA2, 5'-GGGAACAAGCTGAACACAACA-3'. The siRNAs targeting STEAP3 were as follows: siRNA1, 5'-AAGUUGUAGGCAUAGAAGCAGGCUUCUAUGCCUACAACUUCG-3'; siRNA2,

5'-GAGUUCAGCUUCGUUCAGUTTACUGAACGAAGCUGAACUCTT-3'.

lncRNA Microarray Analysis

The Arraystar LncPath human cancer array is designed for global human lncRNAs and protein-coding transcripts. lncRNA microarray analysis simultaneously profiles the expression of 2,829 lncRNAs and 1,906 of their protein-coding gene targets related to human cancer. Samples were derived from LoVo cells, which were transfected with lentivirus vectors containing MT1M shRNA or negative control shRNA. Differentially expressed lncRNAs with statistical significance were confirmed. The dysregulated lncRNAs were identified using a threshold of fold change > 2.0 and an adjusted *P*-value < 0.05.

TCGA Dataset

The RPKM expression value of lncRNA STEAP3-AS1 in TCGA database was downloaded. These data contained 457 colon cancer tissues and 41 normal tissues.

Quantitative Real-Time PCR

Total RNA was extracted from the cultured cells using RNAiso Plus (TaKaRa, China) according to the manufacturer's instruction. Quantitative real-time PCR was performed to detect STEAP3-AS1, CDKN1C, STEAP3, and GAPDH (internal control) using SuperReal PreMix Plus (SYBR Green) (Tiangen Biotech, China). The results were normalized to GAPDH to analyze relative genes expression using the 2^{-ΔΔCT} method. Information for primers is listed in Table S1. All quantitative real-time PCR experiments were performed in triplicate.

MTT Assay

The proliferation of lncRNA STEAP3-AS1 was examined by methyl thiazolyl tetrazolium (MTT) (Sigma, USA) assay. Briefly, cells were cultured in 96-well plates at a density of 5 × 10³ cells per well for 24, 48, and 72 h. Then, 20 μL of MTT reagent (5 mg/mL) was added to each well and incubated at 37°C and 5% CO₂ in a humidified incubator for 4 h, followed by removing medium and adding 200 μL of dimethyl sulfoxide (BioSharp, China) to each well and shaken on a rotary platform for 10 min to dissolve formazan crystals. A microplate spectrophotometer (Bio-Rad, USA) was used to measure the optical density of each well at 570 nm.

Transwell Migration

For the migration assay, cells were digested and diluted. Then, a total of 4.5 × 10⁵ cells in 200 μL of serum-free medium were placed in the upper chamber of a 8-μm pore size 24-well transwell plate (Corning

Figure 6. lncRNA STEAP3-AS1 May Regulate the Expression of CDKN1C by Modulating STEAP3

(A) The graphic shows that STEAP3 is the neighboring gene of lncRNA STEAP3-AS1. (B and C) Binding sites between STEAP3-AS1 and STEAP3 predicted by (B) LncPro database and (C) catRAPID website. (D) Relative STEAP3-AS1 levels detected by RIP assay. (E–G) Expression of STEAP3 after STEAP3-AS1 knockdown with STEAP3-AS1 (E) shRNA1 and (F) shRNA2 or (G) overexpression in colon cancer cells by real-time PCR and western blot analysis. (H–J) Effect of silencing by STEAP3-AS1 (H) shRNA1 and (I) shRNA2 or (J) overexpressing STEAP3-AS1 on CDKN1C expression by real-time PCR and western blot analysis. (K) Expression of p53 after STEAP3-AS1 downregulation or upregulation in colon cancer cells. (L) Western analysis of CDKN1C expression after STEAP3-AS1 and STEAP3 knockdown in two cell lines. (M) Effect of overexpressing STEAP3 on CDKN1C expression in STEAP3-AS1 shRNA cells and STEAP3-AS1 overexpressed cells. Data are shown as mean ± SD; repeated three times.

Life Sciences, NY, USA), and 600 μ L of RPMI 1640 medium containing 20% FBS was added into the lower chamber. After 37°C with 5% CO₂ incubation for 24 h, the cells in the upper surface of the membrane that had not migrated were removed with cotton swabs, while the migrated cells on the lower surface of the membrane were fixed in water-free methanol for 15 min and stained with 0.5% crystal violet for 30 min. The number of cells migrating through the membrane was counted by using a light microscope (Olympus, Tokyo, Japan).

Cell Cycle Analysis

Cells were harvested and fixed in 70% ethyl alcohol. After 24 h, cells were resuspended by RNase A and propidium iodide (KeyGen Biotech, China). The stained cells were performed by a FACSCalibur instrument (BD Biosciences, USA).

Tumorigenicity Assays in Nude Mice

Suspensions of LoVo control shRNA or LoVo STEAP3-AS1 shRNA cells (2×10^6) in 500 μ L of PBS were injected subcutaneously into groins of the same mouse. Tumor volumes were measured and calculated according to the following formula: $(\text{length} \times \text{width}^2) \times 0.5$.

LncPro Dataset

LncPro is a website for predicting the interaction between RNA and protein. The lncRNA and protein sequences are coded into vectors by using information of secondary structure, hydrogen-bonding propensities, and van der Waals interaction. The score can be the measurement of interactions between RNA-protein pairs. Also, it is suggested that there exists a potential interaction between RNA and protein when the predicted score is above 50.

Western Blot Analysis

Proteins were extracted with a whole-cell lysis assay (KeyGen Biotech, China). Protein concentrations were measured using a bicinchoninic acid (BCA) kit (Beyotime, China). Primary antibodies were used against CDK2 (1:500, Proteintech, China), CDK4 (1:1,000, Proteintech, China), cyclin E2 (1:500, Proteintech, China), acetyl-H3 (1:1,000, Solarbio, China), STEAP3 (1:150, Bioworld, China), CDKN1C (1:1,000, Elabscience, China), and p53 (1:2,000, Proteintech, China), and β -actin was used as a control (1:1,000, Elabscience, China). Protein bands signals were visualized using WesternBright enhanced chemiluminescence (ECL) (Advansta, USA) and analyzed using ImageQuant LAS 500.

Protein-Protein Interaction Network

A STRING 10 algorithm was used for protein-protein interaction analysis. All associations were selected for highest confidence (>90%). Clustering was exhibited by a Markov cluster (MCL) algorithm. Continuous lines stand for connections with clusters, and dashed lines represent connections among clusters.

Statistical Analysis

The data are presented as the mean \pm SD as indicated. Experimental results between two groups were compared using an unpaired two-tailed t test. TCGA database analysis was done by a Mann-Whitney

U test. For multiple groups, a one-way ANOVA was used. For all tests, a p value of <0.05 was statistically significant. The analysis was performed by GraphPad Prism (GraphPad, USA).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2020.06.011>.

AUTHOR CONTRIBUTIONS

H.N. and X.L. were responsible for performing experiments, acquisition of data, analysis, and drafting the manuscript. X.Z. and Z.C. provided and collected the clinical data. Y.X., Y.S., J.C., and X.S. provided technical support. Y.Z. and S.R. were responsible for designing the experiments and study supervision. All authors read and approved the final manuscript.

CONFLICTS OF INTERESTS

The authors declare no competing interests.

ACKNOWLEDGMENTS

This work was supported by grants from the Chinese National Science Foundation Projects, Liaoning (31470800, 81372669, and 31270867) and the Science and Technology Planning Project of Liaoning Province, China (2019-BS-142).

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