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ORIGINAL RESEARCH

Long noncoding RNA NEAT1 promotes cell proliferation and invasion by regulating hnRNP A2 expression in hepatocellular carcinoma cells

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Abstract: Growing evidence demonstrates that long noncoding RNAs (lncRNAs) are involved in the progression of various cancers, including hepatocellular carcinoma (HCC). The role of nuclear-enriched abundant transcript 1 (*NEAT1*), an essential lncRNA for the formation of nuclear body paraspeckles, has not been fully explored in HCC. We aimed to determine the expression, roles and functional mechanisms of *NEAT1* in the proliferation and invasion of HCC. Based on real-time polymerase chain reaction data, we suggest that *NEAT1* is upregulated in HCC tissues compared with noncancerous liver tissues. The knockdown of *NEAT1* altered global gene expression patterns and reduced HCC cell proliferation, invasion and migration. RNA immunoprecipitation and RNA pull-down assays confirmed that U2AF65 binds to *NEAT1*. Furthermore, the study indicated that *NEAT1* regulated *hnRNP A2* expression and that this regulation may be associated with the *NEAT1*–U2AF65 protein complex. Thus, the *NEAT1*-*hnRNP A2* regulation mechanism promotes HCC pathogenesis and may provide a potential target for the prognosis and treatment of HCC.

Keywords: long noncoding RNA, *NEAT1*, RNA-binding protein, HCC

Introduction

Long noncoding RNAs (lncRNAs) are a new class of noncoding RNAs that are longer than 200 nucleotides. These RNAs have been found to be frequently dysregulated in various diseases and play an essential role in imprinting control, cell differentiation, immune responses, human diseases, tumorigenesis and other biologic processes.[1–3] Understanding the lncRNAs that function in various diseases and their precise molecular mechanisms will be critical for exploring potential strategies for early diagnosis and therapy.[4] Recent studies have indicated that lncRNA–protein complexes can give rise to unique transcriptional programs, which result in different functions based on the interactions between the bound protein and RNA.[5–7] Genetic studies have proven that a significant number of lncRNAs are associated with hepatocellular carcinoma (HCC) and that the interaction between lncRNAs and RNA-binding proteins is involved in HCC tumorigenicity.[8,9]

The lncRNA nuclear-enriched abundant transcript 1 (lncRNA-*NEAT1*) is transcribed from the familial tumor syndrome multiple endocrine neoplasia type 1 locus on chromosome 11 and lacks any introns.[10,11] *NEAT1* is retained in the nucleus, where it forms the core structural component of paraspeckle suborganelles and acts as a transcriptional regulator for numerous genes, including the genes involved in cancer progression.[12] Aberrant *NEAT1* expression has been reported in several human malignancies, including

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leukemia,[13] glioma,[14] non-small cell lung cancer,[15] prostate cancer,[16] breast cancer[17] and ovarian carcinoma.[18] *NEAT1* plays important roles in tumorigenesis; specifically, it is involved in the cooperation of multiple paraspeckle-localized RNA-binding proteins, including splicing factor family proteins,[19] and it regulates messenger RNA (mRNA) export[12] and controls target gene transcription by protein sequestration into paraspeckles.[20] A recent study analyzing multiple samples indicated that the expression level of *NEAT1* strongly correlates with the number of tumor nodes, metastasis and TNM stage in HCC patients.[21] However, the role of *NEAT1* as a driver of this regulation and its function in these molecular mechanisms remains unclear in HCC.

In this study, we found that *NEAT1* was upregulated in HCC tissues and cell lines. Furthermore, the upregulation of *NEAT1* promoted cell proliferation, migration and invasion in HCC cell lines. To focus on the function and potential molecular mechanism of *NEAT1* in HCC, we profiled its gene expression pattern by gene sequencing in a *NEAT1*-knockdown HepG2 cell line and verified by reverse transcription quantitative polymerase chain reaction (RT-qPCR) that heterogeneous nuclear ribonucleoprotein A2 (*hnRNP A2*) and *IQGAP1* were downregulated and signal transducer and activator of transcription 1 (*STAT1*), oncostatin M receptor (*OSMR*) and insulin-like growth factor binding protein 3 (*IGFBP3*) were upregulated. Based on RNA–protein interaction data obtained from starbase 2.0, RNA immunoprecipitation (RIP) and RNA pull-down assays, we verified that U2 small nuclear RNA auxiliary factor 2 (U2AF65) protein binds to *NEAT1*. Furthermore, we confirmed that *NEAT1* functioned by regulating *hnRNP A2* expression, which might be associated with the *NEAT1*–U2AF65 protein complex.

Materials and methods

Patients and cell lines

Twelve clinical tumor samples were collected from surgical resections of liver tumors conducted at the Department of Hepatobiliary Surgery of the The Calmette Affiliated Hospital of Kunming Medical University, The First Hospital of Kunming, Kunming, Yunnan, People's Republic of China). Adjacent normal tissues, which are defined as normal in the results, were obtained 2 cm distal from the HCC tissue. These tissues were divided into two groups according to the 2003 American Joint Committee on Cancer TNM classification: grades I–II HCC group (n=5) and grades III–IV HCC group (n=7). All patients provided written informed consent, and ethical consent for this study was granted by the Committee for Ethical Review of Research Involving Human Subjects of the The Calmette Affiliated Hospital of Kunming Medical University, The First Hospital of Kunming. The HCC cell lines HepG2, SMMC-7721 and HCCLM3 were purchased from American Type Culture Collection (Manassas, VA, USA). All HCC cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin.

Cell transfection

All constructs used to knock down *NEAT1* were purchased from RiboBio (People's Republic of China). After 24 h of culture, the HepG2 and SMMC-7721 cells were transfected using Opti-MEM I and Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) for 6 h at approximately 70% confluence according to the manufacturer's instructions. The effect of knockdown was detected by RT-qPCR 48 h after transfection. *NEAT1* was stably knocked down by selection with gentamicin (G418; Thermo Fisher Scientific) as described previously.[14] Cells were stably transduced with retroviral vectors expressing T7-tagged *hnRNP A2* complementary DNA (cDNA) to overexpress *hnRNP A2* as described previously.[22] The following small interfering RNAs (si-RNAs) were used to knock down expression:

Si-*NEAT1*: Target: GCCTCCGGTCATACTAGTT
Forward: 5′-GCCUCCGGUCAUACUAGUU dTdT-3′
Reverse: 3′-dTdT CGGAGGCCAGUAUGAUCAA-5′
Si-*U2AF65*: Target: CCAACTACCTGAACGATGA
Forward: 5′-CCAACUACCUGAACGAUGA dTdT-3′
Reverse: 3′-dTdT GGUUGAUGGACUUGCUACU-5′
Si-NC: Forward: 5′-UUCUCCGAACGUGUCACG UTT-3′
Reverse: 5′-ACGUGACACGUUCGGAGAATT-3′.

RNA extraction and RT-qPCR

Total RNA was extracted from the cell lines using TRIzol reagent. RNA was then transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and a reaction volume of 20 µL. Real-time polymerase chain reactions (real-time PCRs) were conducted in a 20 µL reaction volume on a Thermo Fisher Scientific 7500 using an SYBR Green mix. The expression of each RNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and fold changes in expression were calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers were used in this study: *NEAT1*: Forward: GUCUGUGUGGAAGGAGGAATT
Reverse: UUCCUCCUUCCACACAGACTT
hnRNP A2: Forward: CAGCAACCTTCTAACTA CGGTCC

Reverse: CTGCCTCCTGGACCATAGTTTC
GAPDH: Forward: GAACGGGAAGCTCACTGG
Reverse: GCCTGCTTCACCACCTTCT-3′
IQGAP1: Forward: GGTTATCACCCTCATTCGTTC
Reverse: TTTCCTCTTGGAGTGCTGTCT
STAT1: Forward: CAGAAATGTGAAGGA CAAGGTT
Reverse: GATAGGGTCATGTTCGTAGGTG
OSMR: Forward: TGCTTCTCCTGCTTCTGTAATA
Reverse: TGCTTCTCCTGCTTCTGTAATA
IGFBP3: Forward: AGCTCCAGGAAATGCTAGTGA
Reverse: AGGCTGCCCATACTTATCCAC.

Cell proliferation assay (carboxyfluorescein diacetate, succinimidyl ester-labeled assay and cell counting kit-8 assay)

A total of 2×10^5 HepG2 or SMMC-7721 cells labeled with 2 μM carboxyfluorescein diacetate, succinimidyl ester (CFSE) were seeded in six-well plates 48 h after si-*NEAT1* or si-NC treatment. The cells were collected, and the CFSE intensity was detected by flow cytometry to measure cell proliferation at 0 and 72 h after seeding. Forty-eight hours after the si-*NEAT1* or si-NC treatment, the cells were seeded in 96-well plates at a density of 2,000 cells per well. A cell counting kit-8 (CCK-8) assay was used to assess cell viability at 0, 12, 24 and 48 h after seeding. The absorbance was measured at 450 nm using an automatic microplate reader.

Analysis of invasiveness and mobility

The invasive and migratory potentials were assessed with an in vitro Transwell (EMD Millipore, Billerica, MA, USA) assay as previously described.[23] Briefly, a total of 2×10^5 HepG2 or SMMC-7721 cells suspended in 200 μL of serum-free DMEM were seeded in the upper chamber, whose porous membrane was coated with Matrigel (BD Biosciences; 200 μg/mL, 100 μL) for the Transwell invasion assay; the membrane remained uncoated for the migration assay. Subsequently, serum was added to the lower chamber (final concentration: 15%) as a chemoattractant. After migration for 24 h or invasion for 48 h, cells that had penetrated the filters were fixed in dry methanol, stained with 0.1% crystal violet and then photographed. The cells on the filters in each group were detached by trypsin. The cells were then counted using a hemocytometer.

RNA sequencing and data analysis

HepG2 cells were transfected with si-*NEAT1* or si-NC in duplicate. Forty-eight hours after transfection, total RNA was isolated from the cells using TRIzol (Thermo Fisher Scientific) according to the manufacturer's protocol. RNA purity was assessed using an ND-1000 Nanodrop instrument. The A260:A280 and A260:A230 ratios of each RNA sample exceeded 1.8 and 2.0, respectively. RNA integrity was evaluated using an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA), and the RNA integrity number evaluation of each sample exceeded 7.0. Briefly, mRNAs were isolated from the total RNA and fragmented to a size of approximately 200 bp. Subsequently, second strand of cDNA was synthesized, followed by adaptor ligation and enrichment with a low cycle according to the instructions of the TruSeq® RNA LT/HT Sample Prep Kit (Illumina, San Diego, CA, USA). The purified library products were evaluated using the Agilent 2200 TapeStation and Qubit® 2.0 (Thermo Fisher Scientific). The samples were then diluted to 10 pM for cluster generation in situ on the HiSeq 2500 pair-end flow cell, followed by sequencing (2×100 bp) on HiSeq 2500. The transformed data of the two groups were compared using Welch's *t*-test. The threshold for up- and downregulated genes was defined as a fold change >2.0 and a *P*-value <0.05. Geneontology and Kyoto Encyclopedia of Genes and Genomes analyses were employed to determine the roles of these differentially expressed mRNAs.

RNA transcription in vitro

The target fragments of RNAs were transcribed using the MEGAscript Kit (Ambion). The DNA templates used for transcription were cloned using a TOPO TA Cloning Kit (Invitrogen). The following primers were used for the RT-qPCR:

Fragment 1 of *NEAT1*: Forward: CTGAGTTAGAT GAGACGAGGGG; Reverse: CTGGCATGGACA AGTTGAAGA
Fragment 2 of *NEAT1*: Forward: CCTAGCATGT TTGACAGGCG; Reverse: AATGCTAGGACTC ACACTGGC
Fragment 3 of *NEAT1*: Forward: CTGTATTCAGGA GGCTACCATT; Reverse: AACGCCCCAAGTTAT TTCATC
Fragment 4 of *NEAT1*: Forward: AAGGTGGGGAAG ACTGAAGAA; Reverse: AGGAACAAATCCAGA AGAGCC
Fragment 5 of *NEAT1*: Forward: AGCCAAGACTAG AGGGGAAAC; Reverse: ACAACAGCATACCC GAGACTAC.

RNA pulldown

An RNA pull-down analysis was performed as previously described.[7] Briefly, biotinylated *NEAT1* or RNA fragments

were incubated with cell protein extracts (8 μg), which were then targeted with streptavidin beads and washed. The bound proteins were resolved by gel electrophoresis. The specific bands were excised and identified by Western blotting. Additional details are described in the figure legends.

RNA immunoprecipitation

RIP experiments were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (EMD Millipore) according to the manufacturer's instructions. Additional details are described in the figure legends.

Electrophoretic mobility shift assay (EMSA)

EMSA experiments were performed using a Light Shift Chemiluminescent RNA EMSA Kit. The target RNAs were labeled with the Pierce™ RNA 3′ End Biotinylation Kit (Thermo Fisher Scientific). A total of 20 μL containing cell extracts, labeled RNA and unlabeled RNA were mixed as described in the figure legends. The masses of these systems were resolved using 6% native polyacrylamide gel electrophoresis. Then, they were transmembraned and photographed by charge coupled device camera using chemiDoc XRS (Bio Rad).

In vivo tumor xenograft studies

The stably transfected cell lines were generated as described previously.[24] The axillary fossae of male athymic nude mice aged 4–6 weeks were then bilaterally inoculated with 2×10^6 stably overexpressing si-*NEAT1* HepG2 cells or control cells per site. Tumor size was monitored by measuring the length and width with calipers, and the volumes were calculated with the following formula: (L × W2) ×0.5, where L is the length and W is the width of each tumor. The mice used in this experiment were purchased from Kunming Medical University and maintained under specific pathogen-free conditions at Kunming Medical University in Kunming, Yunnan, People's Republic of China. All mouse studies were performed in compliance with the Institutional Animal Care and Use Committee policies and the guidelines of Kunming Medical University.

Statistical analysis

All data are presented as the mean ± standard deviation (SD). The data are displayed in graphs, and significance of differences was assessed using GraphPad 5.0 software. Flow cytometry data were analyzed with Flowjo software.

Results

NEAT1 is upregulated in HCC tissues and cell lines

The expression level of *NEAT1* was analyzed in 12 HCC tissues, three adjacent normal tissues, and the HepG2, SMMC-7721 and HCCLM3 cell lines using RT-qPCR. HCC tissues and cell lines expressed higher levels of *NEAT1*, compared with adjacent normal tissues (Figure 1A).

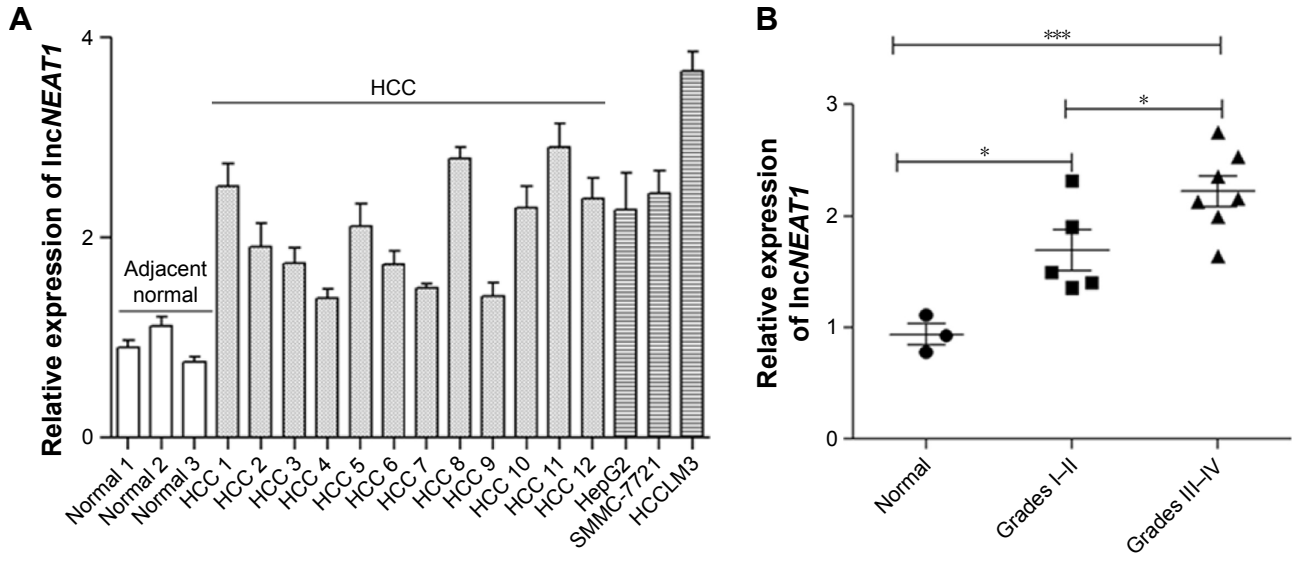

Figure 1 The expression of *NEAT1* is upregulated in HCC tissues and cell lines.

Notes: (**A**) Quantitative analysis of the expression levels of *NEAT1* in samples normalized to *GAPDH* according to the $2^{-\Delta\Delta Ct}$ method. Three adjacent normal tissues, 12 HCC tissues and the HCC cell lines HepG2, SMMC-7721 and HCCLM3 were analyzed. (**B**) The statistical graph shows the quantitative analysis of the expression levels of *NEAT1* in adjacent normal tissues, low-grade HCC (I–II) and high-grade HCC (III–IV). The significance of differences in each group was measured with a one-way ANOVA followed by Dunnett's post hoc multiple comparison test. *$P<0.05$, ***$P<0.001$.

Abbreviations: ANOVA, analysis of variance; HCC, hepatocellular carcinoma; *GAPDH*, lyceraldehyde 3-phosphate dehydrogenase; *NEAT1*, nuclear-enriched abundant transcript 1.

Furthermore, the 12 HCC tissues, as shown in Figure 1A, were classified as low-grade HCC (I–II) and high-grade HCC (III–IV) according to the TNM stage, which was determined by the pathologic diagnosis and the clinical status. The data indicated that *NEAT1* expression directly correlated with the pathologic grade of HCC (Figure 1B).

Knockdown of *NEAT1* inhibited the proliferation, migration and invasion of HCC cells

To assess the function of *NEAT1* in HCC cells, the cells were transfected with small interfering RNA (siRNA) against *NEAT1*, si-*NEAT1*, and si-NC was used as a control. The efficacy of siRNA knockdown was measured in HCC cell lines (approximately 70% decrease in HepG2 cells and 60% decrease in SMMC-7721 cells; Figure 2A). Proliferation was assessed with CFSE labeling and a CCK-8 assay, and these experiments indicated that the knockdown of *NEAT1* significantly inhibited the proliferation of HepG2 and SMMC-7721 cells, compared with the si-NC group ($P<0.01$; Figure 2B, C). Transwell assays were used to detect the effect of si-*NEAT1* on the invasiveness and migration of HCC cells. As shown in Figure 3A, the knockdown of *NEAT1* reduced the invasiveness and migration of HepG2 and SMMC-7721

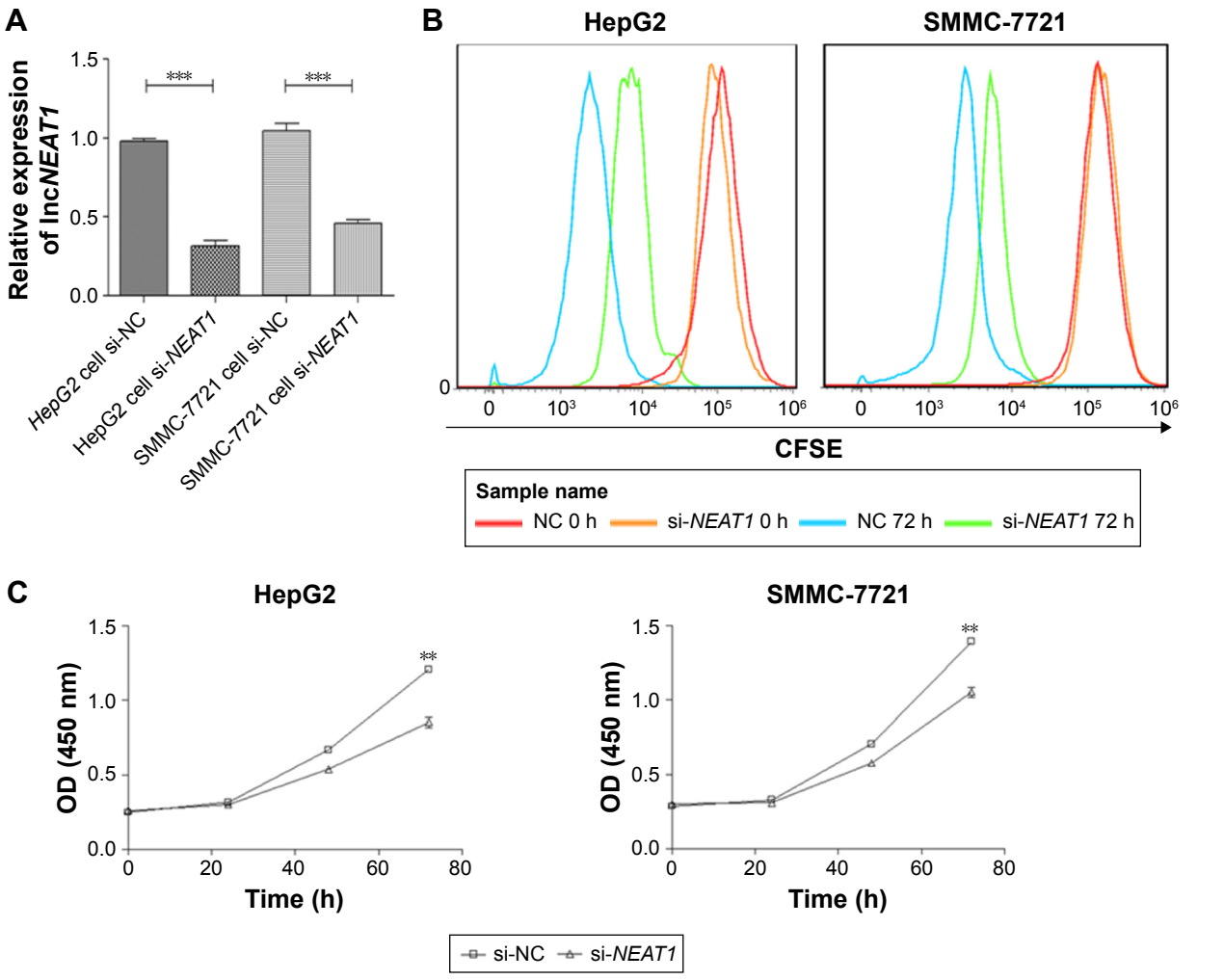

Figure 2 Knocking down NEAT1 inhibited the proliferation of the hepatocellular carcinoma cell lines HepG2 and SMMC-7721.

Notes: (**A**) HepG2 and SMMC-7721 cells were treated with 100 nM si-NEAT1 or si-NC for 6 h. Total RNA was extracted 48 h later, and the expression levels of *NEAT1* in each group were quantitatively analyzed and normalized to the *GAPDH* expression according to the $2^{-\Delta\Delta Ct}$ method. The significance of differences between groups was measured with a paired-sample *t*-test. ***$P<0.001$. (**B**) A total of 2×10^5 HepG2 or SMMC-7721 cells labeled with 2 μM CFSE were seeded in six-well plates 48 h after si-*NEAT1* or si-NC treatment. The cells were collected, and the CFSE intensity was detected by flow cytometry to measure cell proliferation 0 and 72 h after seeding. The data represent at least three independent experiments. (**C**) A total of 2×10^3 HepG2 and SMMC-7721 cells were seeded in a 96-well plate 48 h after si-NEAT1 or si-NC treatment. Ten microliters of CCK-8 was then added to each well 0, 24, 48 and 72 h after seeding and the plate was incubated for 1 h at 37°C. The cell viability was then assessed based on the OD450 value using an automatic microplate reader. Data are presented as the mean ± SD from three independent experiments. The significance of differences between groups was measured with a two-way ANOVA. **$P<0.01$.

Abbreviations: ANOVA, analysis of variance; CCK-8, cell counting kit-8; CFSE, cell tracer: carboxyfluorescein diacetate, succinimidyl ester; *GAPDH*, lyceraldehyde 3-phosphate dehydrogenase; *hnRNP A2*, heterogeneous nuclear ribonucleoprotein A2; NC, normal control; *NEAT1*, nuclear-enriched abundant transcript 1; OD, optical density; SD, standard deviation; si, small intefering.

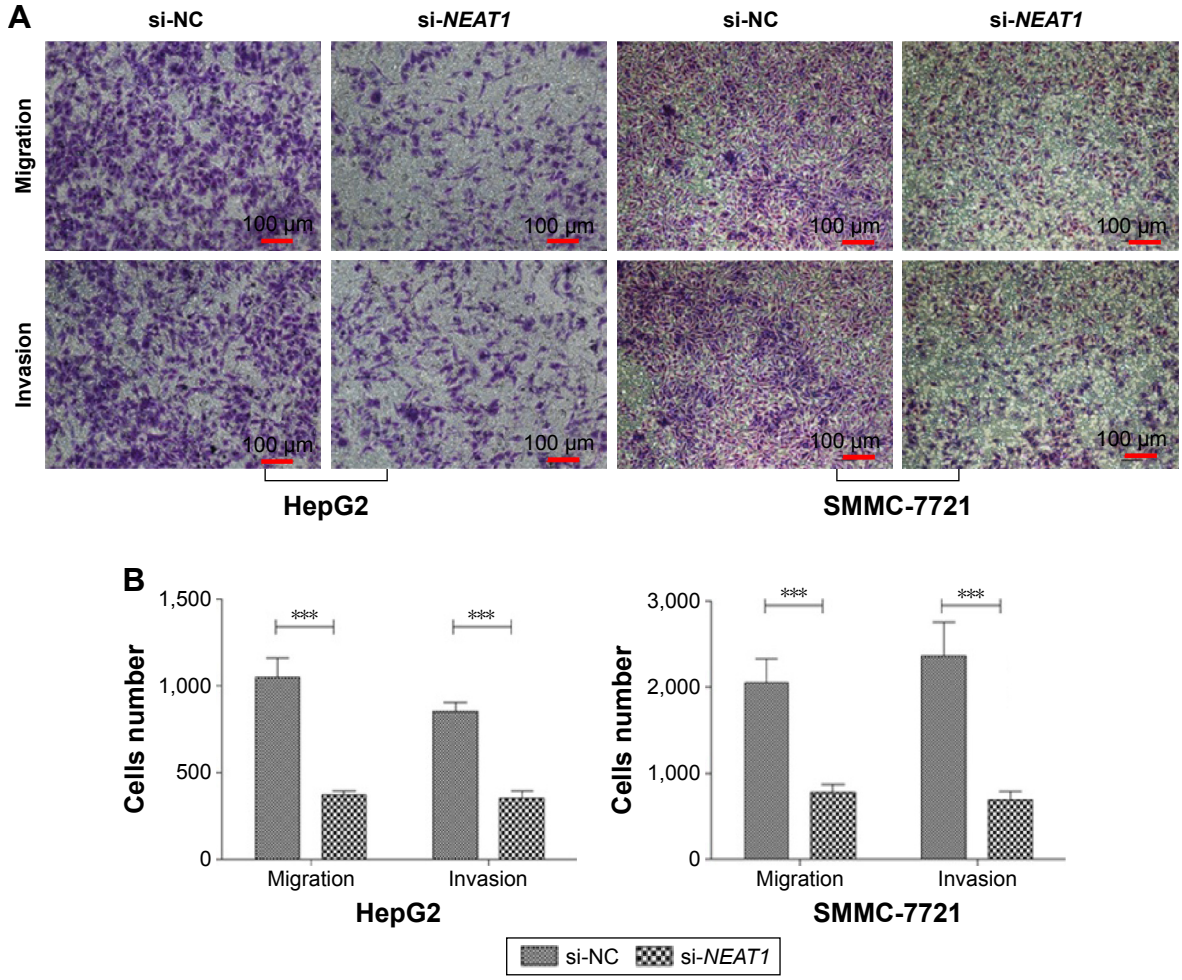

Figure 3 Knocking down *NEAT1* inhibited the migration and invasion of the hepatocellular carcinoma cell lines HepG2 and SMMC-7721.

Notes: (**A**) A total of 2×10^5 HepG2 and SMMC-7721 cells treated with si-*NEAT1* or si-NC were suspended in 200 μL of serum-free DMEM in the upper chamber, which contained a porous membrane coated with Matrigel for the Transwell invasion assay; the membrane was uncoated for the migration assay. Subsequently, 15% serum was added to the lower chamber as a chemoattractant. After migration for 24 h or invasion for 48 h, cells that had penetrated the filters were fixed in dry methanol, stained with 0.1% crystal violet and then photographed. The scale bar represents 100 μm. (**B**) In each group, cells on the filter were detached with trypsin, and the number of cells was counted and is presented in the statistical graph. Data are presented as mean ± SD from three independent experiments. Significant differences from each group were measured by paired sample *t*-test. ***$P<0.001$.

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; *hnRNP A2*, heterogeneous nuclear ribonucleoprotein A2; NC, normal control; *NEAT1*, nuclear-enriched abundant transcript 1; SD, standard deviation; si, small intefering.

cells compared with the si-NC group, as evidenced by crystal violet staining (Figure 3A). Moreover, significantly fewer cells detached from the Transwell filters in the si-*NEAT1* group than in the si-NC group ($P<0.001$; Figure 3B).

Knockdown of NEAT1 altered global gene expression patterns in HCC cells

To study the molecular mechanism of *NEAT1* knockdown, global transcriptional changes in HepG2 cells occurring 48 h after *NEAT1*-siRNA treatment were assessed by gene sequencing. Overall, 377 genes were differentially expressed in *NEAT1* knockdown cells (including 229 upregulated and 148 downregulated genes, fold change >2.0 and *P*-value <0.05). A gene ontology analysis showed that many differentially expressed genes are involved in the biologic processes and molecular functions relevant to cancer pathogenesis, such as cytokine-mediated signaling pathways, the activation of mitogen-activated protein kinase (MAPK) activity, the janus kinase (JAK)–signal transducers and activators of transcription (STAT) cascade (which is involved in the growth hormone signaling pathway), RNA splicing, the regulation of gene expression, RNA binding, cell proliferation and growth (Figure 4A). The associated biologic processes and molecular functions as shown in Figure 4A were the top ten variations after *NEAT1* knockdown. The Kyoto Encyclopedia of Genes and Genomes pathway analysis identified variations in the tumor necrosis factor (TNF) signaling pathway, p53 signaling pathway, transcriptional

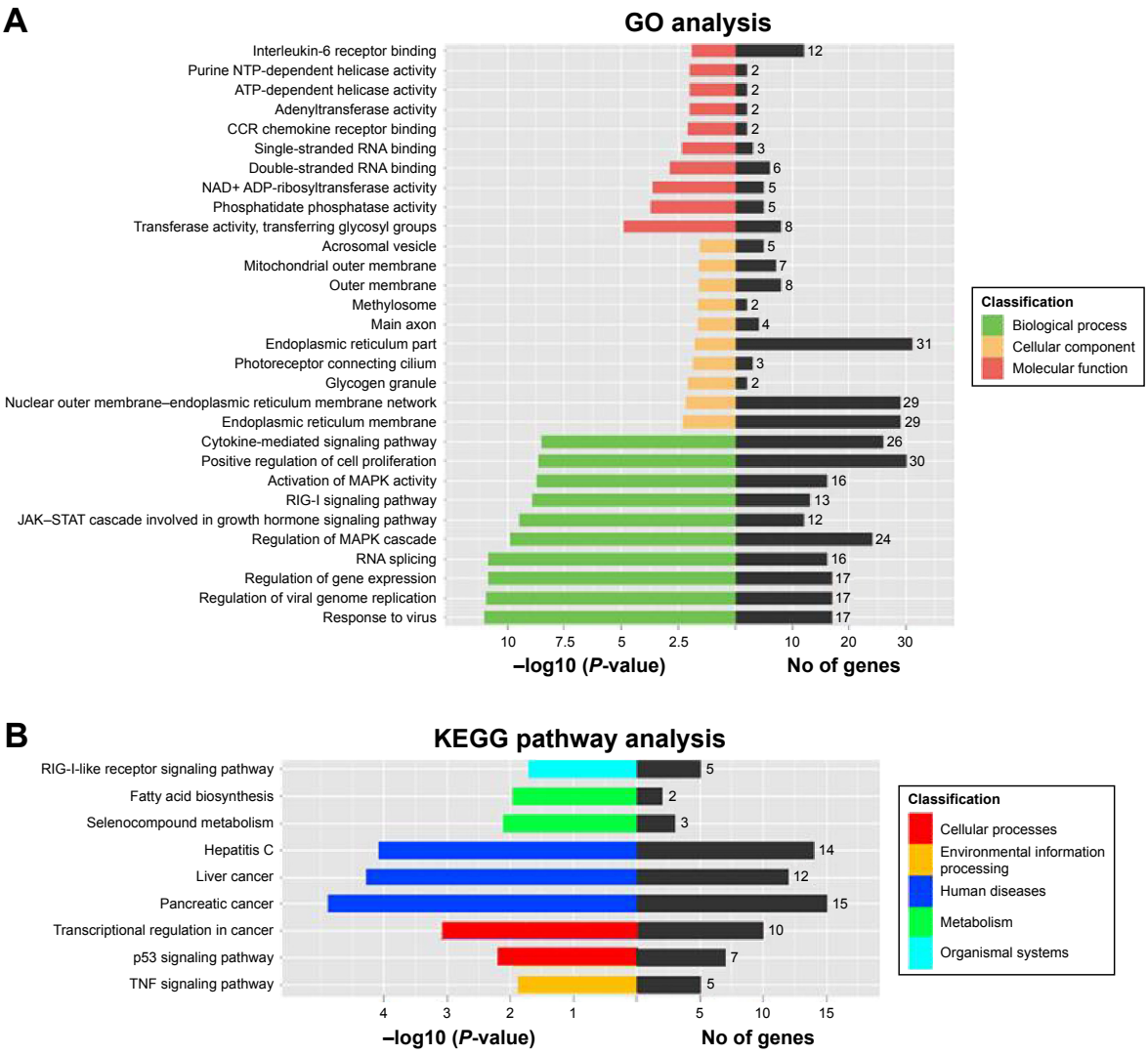

Figure 4 Knockdown of *NEAT1* altered global gene expression patterns in HCC cells.

Notes: HepG2 cells were treated with si-*NEAT1* for 6 h, and RNA was extracted 48 h later. Gene sequencing assays were performed to screen for global transcriptional changes. (**A**) Hits for different genes and associated biologic processes and molecular function are presented based on the GO analysis of NEAT1 knockdown gene sequencing data. (**B**) Hits for different genes and associated pathways are presented based on the KEGG pathway analysis of NEAT1 knockdown gene sequencing data.

Abbreviations: CCR; C chemokine receptor; GO, gene ontology; HCC, hepatocellular carcinoma; hnRNP A2, heterogeneous nuclear ribonucleoprotein A2; KEGG, Kyoto Encyclopedia of Genes and Genomes; NC, normal control; *NEAT1*, nuclear-enriched abundant transcript 1; NTP, nucleoside triphosphate; si, small interfering.

regulation and RIG-I-like receptor signaling pathway after *NEAT1* knockdown (Figure 4B). Based on the bioinformatics analysis and the fact that the overexpression of *NEAT1* in HCC tissues and cell lines promoted HCC cell proliferation and invasion, we selected 13 genes, including six upregulated and seven downregulated genes, that are involved in the biologic processes relevant to cancer pathogenesis and have been reported as potential tumor suppressors or oncogenes in HCC (Table 1).[8,25–35] Among these genes, we confirmed the downregulation of *hnRNP A2* and *IQGAP1* and the upregulation of *STAT1*, *OSMR* and *IGFBP3* in HepG2 cells using RT-qPCR assays (Figure 5).

The association of *NEAT1* and U2AF65

Several studies have found that many lncRNAs are involved in the molecular regulation pathways by interactions with RNA-binding proteins.[5] Therefore, we hypothesized that *NEAT1* might affect cellular functions in a similar manner. We screened starbase 2.0 protein–lncRNA interaction data and found that U2AF65 protein has the highest clip read number associated with *NEAT1*. RIP assays were performed with U2AF65 antibody and nonspecific antibody (IgG control; Figure 6A) using extracts from HepG2 and SMMC-7721 cells to verify associated RNA enrichment. As shown in Figure 6B and C, *NEAT1* enrichment (but not

GAPDH mRNA enrichment) was observed in the U2AF65 antibody group, compared with the IgG control group. Primers against genes that were aberrantly expressed (Figure 5) were also used to detect enrichment using U2AF65 antibody. This experiment showed that mRNA *hnRNP A2* was significantly enriched using U2AF65 antibody (Figure 6B, C). However, a RIP assay using *hnRNP A2* antibody did not result in *NEAT1* enrichment. We further performed an RNA pull-down assay using full-length NEAT1 (3′ end biotinylated) with extracts from HepG2 and SMMC-7721 cells to identify associated proteins. RNA-associated proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 6D), and bands associated with lncRNA-LET at the U2AF65, β-actin and *hnRNP A2* locations (highlights 1, 2 and 3 in Figure 6D) were subjected to Western blot assays. Western blot assays using U2AF65, β-actin and *hnRNP A2* antibody identified a full-length NEAT1 band associated with U2AF65 protein, but not β-actin or *hnRNP A2* protein (Figure 6E). Several studies showed that some lncRNAs bind to RNA-binding proteins by a secondary structural element or a specific fragment.[36] *NEAT1* and U2AF65 interreaction data in starbase 2.0 showed target sites clip read numbers that were mostly located at the 3′ end and 5′ end of 3,750 bp NEAT1. Therefore, five RNA fragments (500–1,000 bp) of NEAT1 transcribed in vitro were 3′ end biotinylated and used in RNA pull-down and Western blot assays with U2AF65 antibody. As shown in Figure 6F, U2AF65 protein was associated with fragments 2 and 5 of NEAT1, as detected by Western blot assays. The biotinylated RNA fragment 2 of *NEAT1* was used in a subsequent EMSA because the signal intensity of this protein was higher than that of fragment 5 (Figure 6F).

Table 1 Selected transcriptional changes

Selected gene name	Fold changes	*P*-value
FASN (fatty acid synthase)	0.352775678	0.000588873
TXNRD1 (thioredoxin reductase 1)	0.421050498	0.009009959
AR (androgen receptor)	0.443871707	0.005314096
SRXN1 (sulfiredoxin 1)	0.451058201	0.007842611
hnRNP A2 (heterogeneous nuclear ribonucleoprotein A2/B1)	0.478395062	0.016578563
FUS (FUS RNA-binding protein)	0.483988604	0.011259985
IQGAP1 (IQ motif containing GTPase activating protein 1)	0.498584211	0.014995828
DDX58 (DEXD/H-box helicase 58)	4.630358625	0.000000012
HERC5 (HECT and RLD domain containing E3 ubiquitin protein ligase 5)	3.336812332	0.000000271
STAT1 (signal transducer and activator of transcription 1)	3.668795232	0.004716635
OSMR (oncostatin M receptor)	2.154985755	0.007187685
NAMPT (nicotinamide phosphoribosyltransferase)	2.079423868	0.011377416
IGFBP3 (insulin-like growth factor binding protein 3)	2.007540475	0.014632835

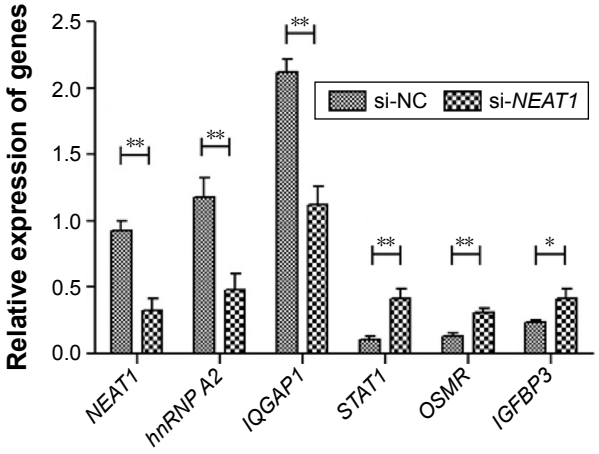

Figure 5 Knockdown of NEAT1-regulated genes which were related with HCC progression.

Notes: The expression levels of genes selected for further validation were normalized to the expression of *GAPDH* according to the $2^{-\Delta\Delta Ct}$ method. The significance of differences between groups was assessed with a paired-sample *t*-test. $^{*}P<0.05$, $^{**}P<0.01$. The data shown represent three independent experiments.

Abbreviations: *GAPDH*, lyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; *hnRNP A2*, heterogeneous nuclear ribonucleoprotein A2; *NEAT1*, nuclear-enriched abundant transcript 1; si, small interfering.

NEAT1 regulated *hnRNP A2* expression through U2AF65

U2AF65 is an essential splicing factor of polypyrimidine tract pre-mRNA[37] and also known as a competitor of the hnRNP protein family in the regulation of transcript function.[38] However, the role of U2AF65 in *NEAT1-hnRNP A2* regulation remains unknown. A Western blot assay and immunohistochemistry assays indicated that knocking down lncRNA *NEAT1* downregulated *hnRNP A2* protein, but not U2AF65 protein expression in HepG2 cells (Figure 7A). According to RIP and RNA pull-down assays, *NEAT1* did not directly bind to *hnRNP A2* protein. Moreover, EMSA showed that hnRNP mRNA competed with *NEAT1* fragment 2 to bind proteins in HepG2 cell extracts (Figure 7B). We hypothesized that *NEAT1* regulates *hnRNP A2* expression through the *NEAT1*–U2AF65 complex. Therefore, U2AF65 was knocked down and *hnRNP A2* expression was detected by Western blot and immunohistochemistry assays. Western blot and immunohistochemistry assays indicated that knocking down U2AF65 also downregulated *hnRNP A2*. According to these data, U2AF65 may participate in *NEAT1-hnRNP A2* regulation (Figure 7C, D).

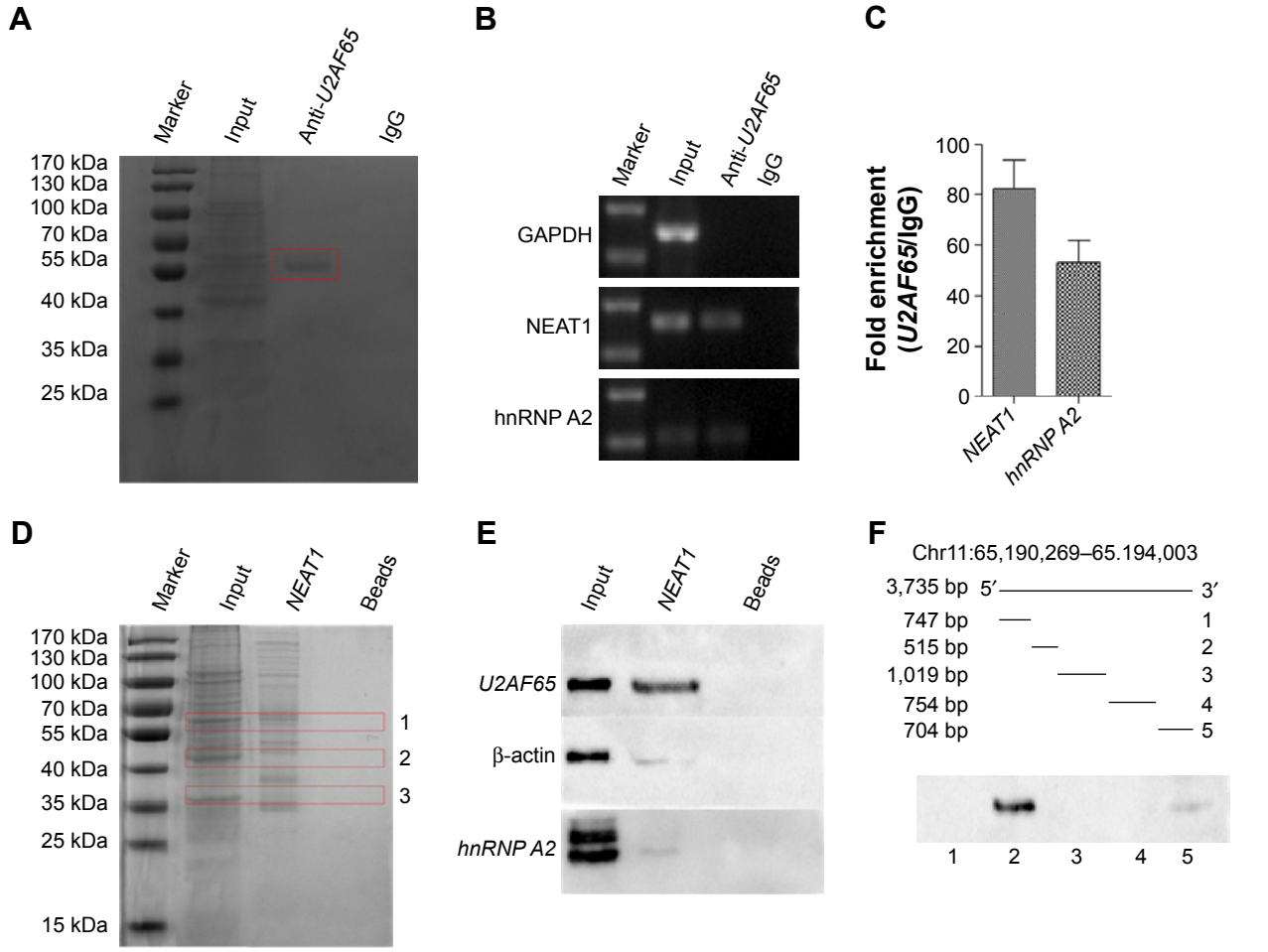

Figure 6 U2AF65 binds to *NEAT1* and mRNA *hnRNP A2*.

Notes: (**A**) RIP experiments were performed using the U2AF65 antibody and IgG control antibody. HepG2 or SMMC-7721 cell lysate (input control) was incubated with beads coated with U2AF65 antibody or IgG control antibody for 6 h at 4°C. Proteins eluted from the beads with SDS in each group were subjected to gel electrophoresis. The gel was stained with Coomassie blue and photographed. The highlighted region represents U2AF65 protein. (**B**) Beads attached to an antibody–protein–RNA complex were incubated with proteinase K at 55°C for 30 min, and the RNA was extracted by the TRIzol:chloroform method. RT-qPCR was then performed using specific primers to detect lncRNA-*NEAT1*, *hnRNP A2* or *GAPDH*. The RT-qPCR product of each group was subjected to electrophoresis and photographed. (**C**) The quantitative expression levels of *NEAT1* and *hnRNP A2* were normalized to *GAPDH* expression in each group according to the $2^{-\Delta\Delta Ct}$ method. RIP enrichment was assessed based on RNA associated with the U2AF65 protein, which was compared with the IgG control group. The fold enrichment of *NEAT1* and *hnRNP A2* in the anti-U2AF65 group is presented in the statistical graph. The data represent the average and standard deviation of three independent experiments. (**D**) Full-length *NEAT1* was biotinylated and targeted with streptavidin beads and then incubated with whole HepG2 extracts (input control) for 1 h at 4°C and washed. Streptavidin beads without biotinylated RNA are shown as a control. Associated protein eluted from the beads was subjected to gel electrophoresis, stained with Coomassie blue and photographed. (**E**) The highlighted regions were subjected to Western blotting using *U2AF65* antibody for region 1, β-actin antibody for region 2 and hnRNP A2 antibody for region 3. The bands in each group were visualized by exposure for 20 s under a CCD camera. (**F**) RNAs corresponding to the different fragments (1–5) of *NEAT1* were biotinylated and incubated with HepG2 whole cell extracts, targeted with streptavidin beads and washed. Bound U2AF65 protein was detected by Western blotting (n=3).

Abbreviations: CCD, charge coupled device; *GAPDH*, lyceraldehyde 3-phosphate dehydrogenase; *hnRNP A2*, heterogeneous nuclear ribonucleoprotein A2; IgG, immuglobulin G; *NEAT1*, nuclear-enriched abundant transcript 1; RIP, RNA immunoprecipitation; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SDS, sodium dodecyl sulfate; U2AF65, U2 small nuclear RNA auxiliary factor 2.

Overexpression of *hnRNP A2* rescued the proliferation and invasion in HCC cells that express low levels of NEAT1

hnRNP A2 is an essential splicing factor that promotes HCC proliferation and invasion[39] and activates alternative splicing switch that downregulates a dominant-negative isoform of A-Raf, which leads to activation of the Raf-MEK-ERK pathway and cellular transformation.[22] Our data indicated that *NEAT1* participates in *hnRNP A2* regulation. To verify that *NEAT1* functions by *hnRNP A2* regulation, *hnRNP A2* was overexpressed in *NEAT1* knockdown cells using a retrovirus that encodes *hnRNP A2* (Figure 8A), as described previously.[22] Control cells were transfected with an empty vector (lv-control). Subsequent proliferation and invasion assays indicated that the overexpression of *hnRNP A2* promoted the proliferation and invasion of *NEAT1* knockdown

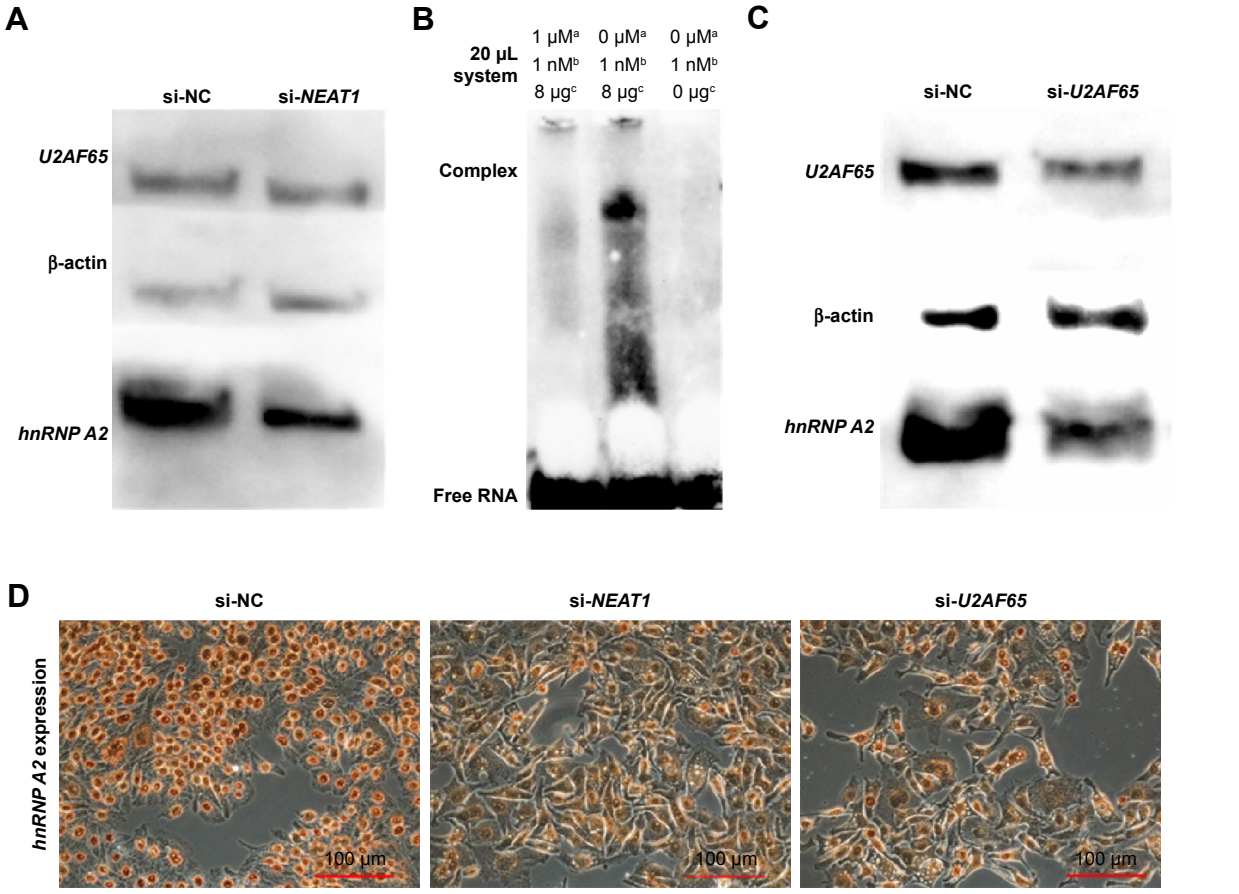

Figure 7 Knocking down *NEAT1* and U2AF65 downregulated *hnRNP A2* expression.

Notes: (**A**) *NEAT1* was knocked down in HepG2 cells using si-*NEAT1* or si-NC (control). HepG2 cell extracts were obtained 48 h after transfection. The extracts from each group were subjected to Western blotting to detect U2AF65, *hnRNP A2* and β-actin. (**B**) An EMSA competition experiment was performed using 1 nM of the biotinylated lncRNA-*NEAT1* fragment 2, a 1,000-fold molar excess of unlabeled *hnRNP A2* mRNA and whole HepG2 extracts in a total volume of 20 μL. Different ratios of biotinylated RNA, protein and unlabeled RNA were incubated. The masses of the 20 μL system were resolved using 6% native polyacrylamide gel electrophoresis, and the proteins were then transferred to a nylon membrane and crosslinked. Representative images obtained with a CCD camera are shown. [a]Unlabeled *hnRNP A2* mRNA; [b]Biotin lncRNA-*NEAT1* fragment 2; [c]HepG2 extract. (**C**) *U2AF65* mRNA was knocked down in HepG2 cells using si-*U2AF65* or si-NC (control). HepG2 cell extracts were obtained 48 h after transfection. The extracts from each group were subjected to Western blotting to detect U2AF65, *hnRNP A2* and β-actin. (**D**) Slides of HepG2 cells were incubated with *hnRNP A2* antibody at 4°C for 12 h after si-*NEAT1*, si-U2AF65 or si-NC treatment, and the slides were then incubated with secondary HRP-antibody, colored with a DAB kit and photographed. The scale bar represents 100 μm.

Abbreviations: CCD, charge coupled device; DAB, diaminobenzidine; EMSA, electrophoretic mobility shift assay; *hnRNP A2*, heterogeneous nuclear ribonucleoprotein A2; HRP, horseradish peroxidase; mRNA, messenger RNA; NC, normal control; *NEAT1*, nuclear-enriched abundant transcript 1; si, small interfering; U2AF65, U2 small nuclear RNA auxiliary factor 2.

cells, compared with the lv-control group (Figure 8B–D). These data suggest that *NEAT1* promoted HCC cell proliferation and invasion by regulating *hnRNP A2*.

Knockdown of *NEAT1* reduced HepG2 cell growth and downregulated *hnRNP A2* expression in vivo

HepG2 cells were transfected with si-*NEAT1* and expressed lower level of *NEAT1* than the control cell line. Then the hnRNP A2 expression level was tested to explore *NEAT1*-*hnRNP A2* regulation in vivo. Cells of either line were bilaterally injected into the axillary fossa of male athymic nude mice, and the tumor growth activity and *hnRNP A2* expression were measured. As shown in Figure 9A, the average volume of tumors derived from the si-*NEAT1* group 14 days after xenograft transplantation was significantly smaller than that of the control group (n=4 animals per group, $P<0.001$). Furthermore, total RNA was also extracted from the tumors in each group to measure the expression level of *hnRNP A2* by RT-qPCR. The results revealed that the expression of *hnRNP A2* mRNA was significantly lower in the si-*NEAT1* group than in the control group ($P<0.001$; Figure 9B). Furthermore, Western blot assays indicated that the protein expression of *hnRNP A2* was decreased in the si-*NEAT1* group compared with the control group (Figure 9C); a representative immunohistochemistry image is shown in Figure 9D.

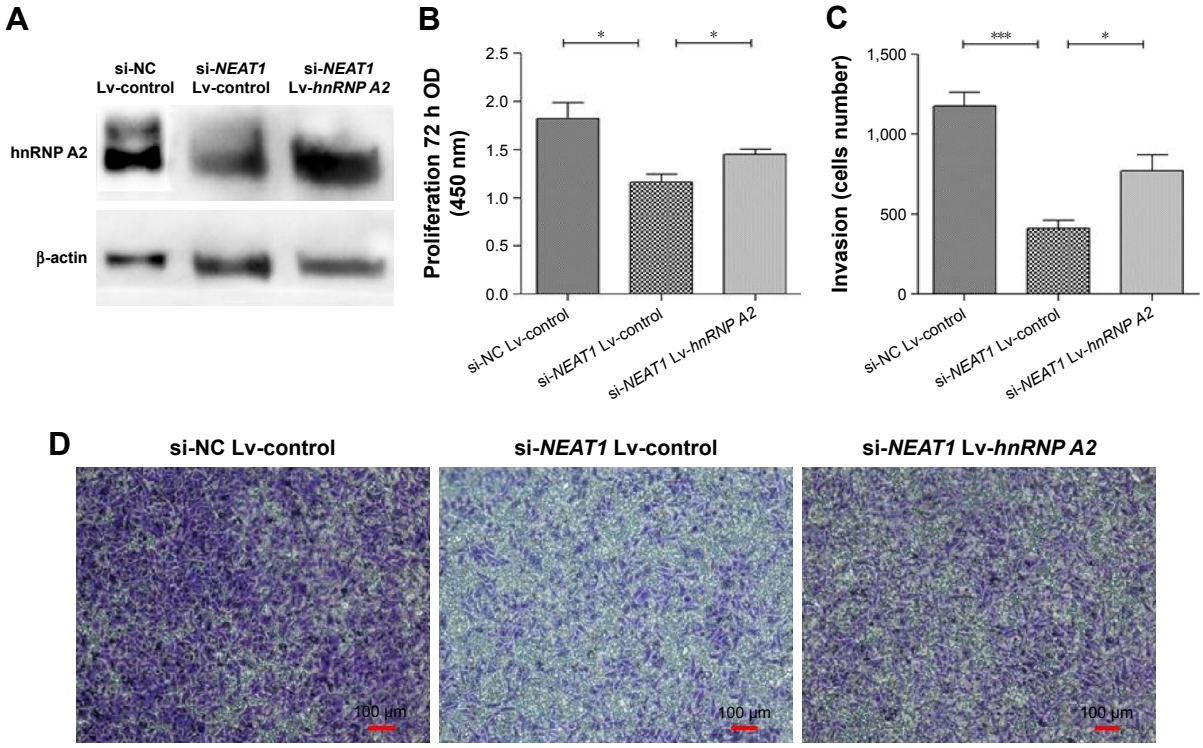

Figure 8 The overexpression of *hnRNP A2* rescued the proliferation and invasion of HCC cells expressing low levels of lncRNA-*NEAT1*.

Notes: (**A**) NEAT1 was knocked down in HepG2 cells using si-*NEAT1* or si-NC (control). The overexpression of hnRNP A2 in cells transfected with retroviruses encoding empty vector or *hnRNP A2* was analyzed by Western blotting for hnRNP A2. β-actin was used as a loading control. (**B**) The cells from each group described in **A** were seeded in 96-well plates and cultured with 100 μL of DMEM containing 10% serum for 72 h. Ten microliters of CCK-8 was added to each well and incubated for 1 h at 37°C. The cell viability was then assessed based on the OD450 value using an automatic microplate reader. Number (**C**) and representative images (**D**) of invasive cells per filter in each group described in **A**. Transwell assays were performed according to the method described in Figure 2D. The significance of differences between groups was assessed with a one-way ANOVA followed by Dunnett's multiple comparison post hoc test. *$P<0.05$, ***$P<0.01$.

Abbreviations: ANOVA, analysis of variance; CCK-8, cell counting kit-8; DMEM, Dulbecco's Modified Eagle's Medium; HCC, hepatocellular carcinoma; *hnRNP A2*, heterogeneous nuclear ribonucleoprotein A2; lncRNA, long noncoding RNAs; NC, normal control; *NEAT1*, nuclear-enriched abundant transcript 1; OD, optical density; si, small interfering.

Discussion

According to a previous study, *NEAT1* expression is higher in cancers than in normal tissues, including leukemia, glioma, non-small cell lung cancer, prostate cancer, breast cancer, ovarian carcinoma and HCC. *NEAT1* expression was associated with metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), which is a well-known oncogene because it regulates the alternative splicing of endogenous target genes involved in cancers, including HCC.[21] The biologic mechanisms of *NEAT1* regulation in cancer have gradually been revealed in recent studies. In prostate cancer, *NEAT1* is involved in the modulation of oncogenic growth by altering the epigenetic landscape.[18] Induced by hypoxia in hypoxia-inducible factors transcriptional pathways, *NEAT1* also plays an important role in promoting proliferation and reducing apoptosis, which contributes to tumorigenesis.[40] In glioma, *NEAT1* regulates glioma cell proliferation, invasion and migration through the miR-449b-5p/c-Met axis.[14] Moreover, a study of HCC patients indicated that the expression level of *NEAT1* strongly correlated with tumorigenesis and metastasis, including the number of tumor nodes, metastasis, portal vein tumor embolus, vascular invasion, tumor capsular infiltration and TNM stage.[22] However, the specific functions, regulatory roles and biologic mechanisms of *NEAT1* in HCC remained unclear. We verified that *NEAT1* is upregulated in HCC tissues and cell lines and affects HCC (Figure 1). Further study indicated that *NEAT1* promotes cell proliferation, migration and invasion in the HCC cell lines HepG2 and SMMC-7721 (Figures 2 and 3). RNA sequencing and data analyses indicated that *NEAT1* plays important roles in the regulation of pathways and genes in HCC cells (Figure 4). We also verified that *hnRNP A2* and *IQGAP1* were downregulated and that *STAT1*, *OSMR* and *IGFBP3* were upregulated in HCC cells (Figure 5).

LncRNA–protein complexes have been shown to initiate unique transcriptional programs that result in various functions by different combinations of RNA-binding protein interactions.[5] Specific hypothesis-driven studies confirmed this theory in HCC. LncRNA-LET, a tumor suppressor, binds to NF90, to affect hypoxia-inducible factor-1a mRNA

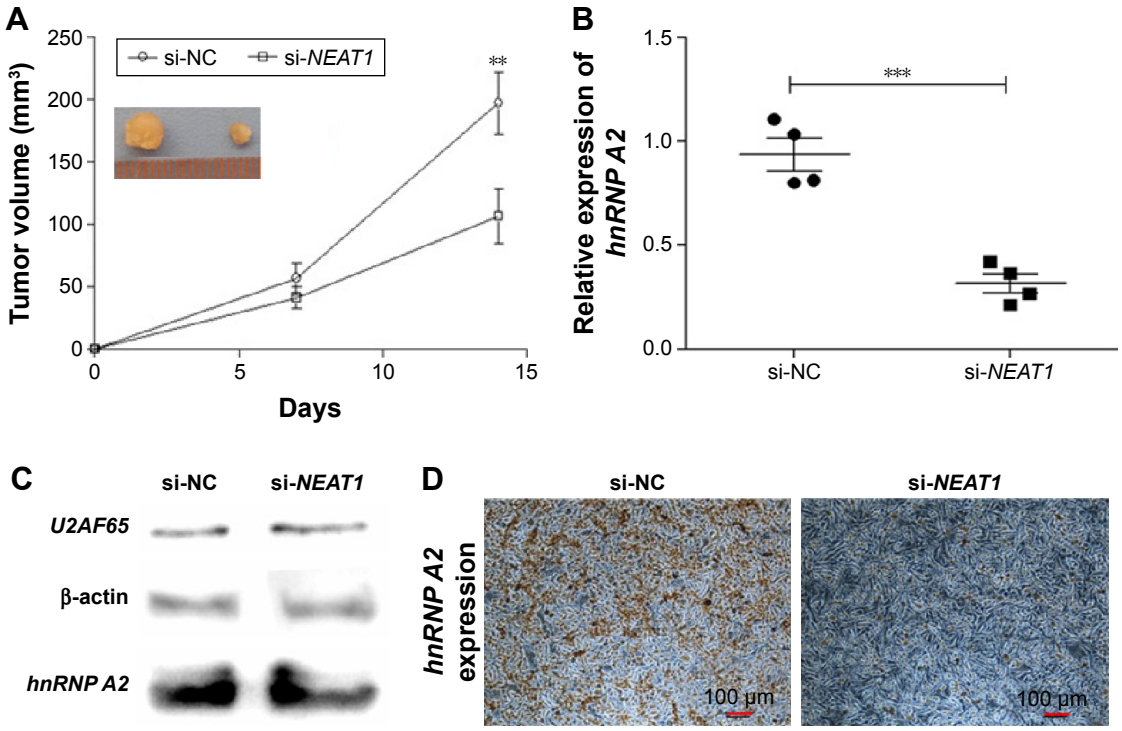

Figure 9 Knocking down *NEAT1* inhibited HepG2 cell growth and downregulated hnRNP A2 expression in vivo.
Notes: (**A**) The axillary fossae of male athymic nude mice aged 4–6 weeks were bilaterally inoculated with 2×10^6 cells/0.1 mL per site. HepG2 cells were transfected with si-*NEAT1* and expressed a lower level of *NEAT1* than the control group. Tumors were taken and size was monitored 7 and 14 days later (n=4). $^{**}P<0.01$. (**B**) Total RNA was extracted from the tumors 14 days later and the expression levels of *hnRNP A2* in each group were quantitatively analyzed by normalizing them to the *GAPDH* level according to the $2^{-\Delta\Delta Ct}$ method (n=3). $^{***}P<0.001$. (**C**) The tumor extracts from each group were subjected to a Western blot analysis to detect U2AF65, *hnRNP A2* and β-actin. (**D**) A representative IHC image obtained using hnRNP A2 antibody as the primary antibody is shown. The scale bar represents 100 μm.
Abbreviations: *GAPDH*, lyceraldehyde 3-phosphate dehydrogenase; hnRNP A2, heterogeneous nuclear ribonucleoprotein A2; IHC, immunohistochemistry; NC, normal control; *NEAT1*, nuclear-enriched abundant transcript 1; si, small interfering; U2AF65, U2 small nuclear RNA auxiliary factor 2.

accumulation and stability under hypoxic conditions in HCC.[7] LncRNA-MEG3,[41] lncRNA Rs10680577,[42] LncRNA-HEIH[43] and HOTTIP[44] function through lncRNA–protein complexes according to reports. Because *NEAT1* plays important roles in promoting the proliferation and invasion of HCC cells and is involved in gene regulation, we screened the RNA–protein interaction data in starbase 2.0. Specifically, we searched for proteins that were either up- or downregulated in *NEAT1* knockdown cells (Figure 3C) and determined whether they directly bound to *NEAT1*. However, the starbase 2.0 data identified few possible interactions between *NEAT1* and the proteins we selected based on the clip read number. Therefore, we hypothesized that *NEAT1* functions by gene regulation, which might be associated with the *NEAT1*–RNA-binding protein complex. We screened the RNA–protein interaction data in starbase 2.0 and found that U2AF65, polypyrimidine tract-binding protein (PTB), eukaryotic initiation factor 4A-III (eIF4AIII) and fused in sarcoma (FUS) might interact with *NEAT1*. Using RIP and RNA pull-down assays, we found that *NEAT1* binds to U2AF65 protein and that the binding target might be located in *NEAT1* fragments 2 (chr11: 65, 191, 001–65, 191, 516) and 5 (chr11: 65, 193, 299–65, 194, 003) in vitro (Figure 6). Furthermore, a RIP assay showed that *hnRNP A2* mRNA was enriched by U2AF65 protein (Figure 6). This finding suggested that U2AF65 interacts with *hnRNP A2* mRNA, which was downregulated in *NEAT1*-knockdown HCC cells. *hnRNP A2* was reported to be an essential splicing factor that promotes cell proliferation and invasion and correlates with poor outcome in HCC patients.[22,39] Specifically, this factor activates the Raf-MEK-ERK pathway, which results in cellular transformation in HCC.[40] A previous study indicated that lncRNA functions through a specific region which was the combination target of RNA–protein.[8] Because U2AF65 protein interacts with *NEAT1* and *hnRNP A2* mRNA, the *NEAT1*–U2AF65–*hnRNP A2* complex was further explored in HCC cells. Our RNA pull-down data suggested that *NEAT1* directly binds to U2AF65 protein, but not *hnRNP A2* protein (Figure 6). Knocking down *NEAT1* or U2AF65 reduced the downregulation of hnRNP A2 transcript and protein. An EMSA showed *NEAT1* fragment 2 (chr11: 65, 191, 001–65, 191, 516), which specifically interacts with

U2AF65, competes with *hnRNP A2* mRNA to bind proteins in vitro. Our future studies will focus on the specific region that regulates the function of *NEAT1* in HCC. Our updated data obtained using recombinant protein will confirm that *NEAT1* competes with *hnRNP A2* to bind U2AF65 protein. Furthermore, overexpression assays will be used to confirm that *NEAT1* fragment 2 (chr11: 65, 191, 001–65, 191, 516) and the 515 nt region regulate *hnRNP A2* and tumorigenesis in HCC. Taken together, our data indicate that *NEAT1* regulates *hnRNP A2* expression in HCC cells, and this regulation might be associated with the *NEAT1*–U2AF65 protein complex.

NEAT1 has been previously described to function by regulating the epigenetic landscape.[14,18,21] As demonstrated above, knocking down *NEAT1* decreases *hnRNP A2* expression, which is strongly correlated with HCC tumorigenesis. However, whether *NEAT1* promotes HCC cell proliferation and invasion by regulating *hnRNP A2* regulation remains to be verified. To explore the correlation between *NEAT1* and *hnRNP A2* in HCC tumorigenesis, we compared the proliferative and invasive potential of si-*NEAT1*-transfected and si-*NEAT1* and *hnRNP A2*-cotransfected cells. Our data indicated that the overexpression of *hnRNP A2* rescued the proliferation and invasion inhibited by *NEAT1* knockdown in HCC cells (Figure 8). Because *NEAT1* was also found to regulate hnRNP A2 expression, this finding suggested that *NEAT1* might promote cell proliferation and invasion by regulating *hnRNP A2*.

Analyses of clinical pathologic data, in vitro assays based on knockdown/knockout or overexpression models in cell lines and in vivo xenograft models have confirmed that lncRNAs regulate tumorigenesis and metastasis.[36] Therefore, we compared the tumor growth of *NEAT1*-knockdown cells and control cells in athymic nude mice. Our data indicated that knocking down *NEAT1* inhibited tumor growth in vivo. Interestingly, *hnRNP A2* expression was downregulated in the tumors of the *NEAT1* knockdown group. These data suggested that *NEAT1* promotes tumor growth and affects *hnRNP A2* in vivo.

In summary, this work suggests that the lncRNA *NEAT1* is an oncogene and is strongly correlated with altered *hnRNP A2* expression. Thus, *NEAT1*-*hnRNP A2* regulation might be a potential mechanism of HCC progression.

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Disclosure

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