# Long Non-Coding RNA NEAT1 Promotes HCC Progression via PKM2 and Exosome-Mediated Transfer

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## Abstract

# Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors with high mortality and poor prognosis. Long non-coding RNAs NEAT1 (lncRNA NEAT1) have been found to play an important role in HCC progression. However, the role and potential molecular mechanism of lncRNA NEAT1 in HCC remain largely unclear.

# Methods

The role of lncRNA NEAT1 both *in vitro* and *in vivo* was investigated, with RNA pull-down and RNA immunoprecipitation (RIP) assays being performed to determine the interaction among NEAT1 and FOXO3 and PKM2. In addition, HCC cells were treated with exosomes derived from NEAT1-overexpressing HCC cells, and then cell proliferation, migration and invasion were assessed using *in vitro* assays.

# Results

In this study, overexpression of NEAT1 promoted the proliferation, migration and invasion of HCC cells, whereas NEAT1 knockdown exhibited the opposite effects. Mechanistically, NEAT1 was found to recruit transcription factor FOXO3 to PKM2 promoter region and upregulate PKM2 expression. Meanwhile, overexpression of NEAT1 increased tumor growth and metastasis in a mouse xenograft model of HCC *in vivo* via upregulation of PKM2. Furthermore, overexpression of NEAT1 promoted exosome release from HCC cells. Exosomes secreted from NEAT1-overexpressing HCC cells promoted the proliferation, migration and invasion of HCC cells.

# Conclusion

We found that NEAT1 could promote HCC progression via upregulation of PKM2 and exosome-mediated transfer. These data indicated that NEAT1 may be a therapeutic target in HCC.

## Introduction

HCC is the third leading cause of cancer death worldwide, accounting for 90% of all primary liver cancers [1, 2]. HCC is estimated to have an incidence of 1 million new cases each year, with more than 750,000 deaths [3–5]. Great progress has been made in the treatment of HCC, but the 5-year overall survival rate of patients with HCC is still worse [6, 7]. Therefore, it is of great significance to explore the molecular targets and treatment strategies for the treatment of HCC [8].

Long non-coding RNAs (lncRNAs) are a type of non-coding RNAs with a transcriptional length of more than 200 nt [9]. It has been found that lncRNAs could bind with transcription factors to regulate the expression of its target genes [10]. In addition, lncRNAs are widely involved in various biological processes, including cell migration and invasion [11, 12]. LncRNA NEAT1 is a member of the lncRNA family, comprising two subtypes, NEAT1_1 (3.7 KB) and NEAT1_2 (23 KB) [13, 14]. Evidence has shown that NEAT1 are involved in the progression of liver cancer [15]. Li et al showed that NEAT1 could promote the proliferation and invasion of HCC cells via miR-296-5p/CNN2 Axis [16]. Zhou et al found that NEAT1 could promote the proliferation and inhibit the apoptosis of HCC cells via miRNA-22-3p/akt2 axis [17]. However, the underlying regulatory mechanism of NEAT1 has not been fully understand in HCC.

Exosomes, the membrane vesicle-like bodies containing proteins, lipids and nucleic acids, can be secreted by a variety of cells [18–20]. Exosomes have been implicated in a wide range of physiological functions, including immune regulation and intercellular communication [21–23]. It has been shown that exosome-mediated lncRNAs transfer is important in the progression of HCC [24, 25]. Zhou et al found that HCC cell-derived exosomal linc-FAM138B could alleviate the progression of HCC via targeting miR-765 [26]. In this study, our results showed that overexpression of NEAT1 could promote HCC cell migration and invasion via upregulation of PKM2. In addition, exosomes secreted from NEAT1-overexpressing HCC cells could promote HCC cell migration and invasion. These data indicated that NEAT1 may be a therapeutic target in HCC.

# Materials And Methods

### Patients and samples

A total of 30 HCC tissues and paracancerous tissues were collected from patients who underwent HCC surgery at Affiliated Drum Tower Hospital, Medical School of Nanjing University. All tissues were immediately frozen in liquid nitrogen and stored at a −80°C refrigerator. The written informed consent was ontained from each paticipant. This study was approved by the Ethics Committee of the Affiliated Drum Tower Hospital, Medical School of Nanjing University.

### Data collection

GSE6764 and GSE14520 datasets which contain the PKM2 expression data between normal tissues and HCC tissues, were downloaded from GEO database (https://www.ncbi.nlm.nih.gov/geo/).

### Cell culture

Human HCC cell lines 97H and Huh7 were donated by the medical school of Wuhan University. Human embryonic kidney 293T cells were obtained from American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, MA, USA) containing 10% FBS at 37°C in a 5% $CO_2$ incubator.

## Cell transfection

NEAT1, FOXP3 and PKM2 were silenced by specific small interfering RNAs (siRNAs), termed NEAT1 siRNA1/2 (si-NEAT1 1#, si-NEAT1 2#), FOXP3 siRNA1/2 (si-FOXP3 1#, si-FOXP3 2#), PKM2 siRNA1 (si-PKM2). The full length of NEAT1 or PKM2 was ligated into the pcDNA3.1 vectors to obtain pcDNA3.1/NEAT1 (NEAT1 OE) and pcDNA3.1/PKM2 (PKM2 OE) plasmids. These plasmids above were obtained from GenePharma (Shanghai, China). In addition, the knockdown (KO) vecotor of PKM2 (Psilencer/PKM2) were sythesized by Hanbio (Beijing, China). The plasmids were transfected into 97H and Huh7 cells using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions.

## Real time-quantitative PCR (RT-qPCR)

Total RNAs isolated from 97H and Huh7 cells were obtained with the application of the TRIpure Total RNA Extraction Reagent. Next, the EntiLink™ 1st Strand cDNA Synthesis Kit was used to synthesize cDNA. Later on, qPCR was performed using a SYBR Green Mix kit (Beyotime, Shanghai, China) on a FAST7500 Realtime PCR system (American Applied Biosystems, USA). Calculation of gene expressions was conducted on the basis of $2^{-\Delta\Delta Ct}$ method.

## Cell proliferation

The 97H and Huh7 cells were seeded onto 96-well plates at a density of $5\times10^3$ cells/well and incubated for 24, 48 and 72 h. After that, cells in each well were added with 10 μL CCK-8 (Dojindo Laboratories, Kumamoto, Japan) solutions and incubated for another 2 h, followed by the measurement of the absorbance value at the wavelength of 450 nm by a microplate reader.

## Colony formation assay

The 97H and Huh7 cells were seeded onto 6-well plates and incubated for for 2 weeks. After that, cells were fixed with 4% formaldehyde for 15 min and then stained with 1% crystal violet for 15 min. Later on, cell colonies were observed and photographed by a microscope (Leica, German).

## Transwell assays

Transfected 97H and Huh7 cells were placed on the upper chamber with 200 μL serum-free RPMI 1640 medium. Meanwile, the RPMI 1640 medium containing 12% FBS was added into the lower chamber. After 24 h of incubation, the cells migrating or invading to the lower membrane surface were fixed with 4% formaldehyde and then stained with 0.2% crystal violet solution for 10 min. Subsequently, the migrated or invaded cells were observed under the microscope. To evaluate cell invasion, the upper chamber was pre-coated with matrigel (BD, USA).

## Luciferase reporter assay

The promoter of PKM2 was inserted into the pGL6-miR-based luciferase reporter vector. 97H or Huh7 cells were co-transfected with the luciferase reporter vector and NEAT1 OE or si-NEAT1 2# plasmids. After 48 h of transfection, the luciferase activity was detected using the Dual Luciferase Reporter Assay System (Beyotime, Shanghai, China).

### RNA pull-down assay and mass spectrometry (MS) analysis

The sense and antisense NEAT1 were transcribed *in vitro* and biotinylated with the Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase (Roche). After that, the cell lysates from 97H or Huh7 cells were incubated with the purified biotinylated transcripts for 1 h at room temperature. Later on, streptavidin agarose beads were used to isolate the biotinylated-labeled RNAs and their binding protein partner. Subsequently, the retrieved proteins were resolved by gel electrophoresis, followed by the MS analysis. Meanwhile, the binding proteins were identified by western blot assay.

### RIP assay

RIP assay was performed using the EZ-Magna RIP RNA-binding protein immunoprecipitation Kit (Millipore). 97H or Huh7 cells were lysed in RIP lysis buffer. Next, the cell lysates from 97H or Huh7 cells were mixed with magnetic beads, and FOXP3 antibody or immunoglobulin G (IgG) antibody. After that, the co-precipiated RNAs were examined by RT-qPCR assay.

### Chromatin immunoprecipitation (ChIP) assay

EZ-ChIPTM Chromatin immunoprecipitation Kit (Millipore) was used to perform ChIP assay. 97H or Huh7 cells were incubated with formaldehyde for 10 min and then subjected to ultrasonic breaker treatment to break the chromatin. After that, the products were incubated with FOXP3 antibody overnight at 4°C. Next, the protein A-Sepharose magnetic beads were used to precipitate the DNA-protein complex. Later on, cross-linked protein-DNA complexes were reversed at 65°C overnight, and then purified DNA was quantified by RT-qPCR assay.

### Western blot assay

Cells were lysed using the RIPA lysis buffer (Sigma-Aldrich), and the total amount of protein was quantified using the BCA method. After that, 40 μg of protein was detached by 10% SDS-PAGE and then transferred to the PVDF membrane. Later on, the PVDF membrane was blocked in 5% skimmed milk and then incubated with the primary antibodies against FOXP3, PKM2, CD63, CD9, HSP90 and GAPDH overnight at 4°C. After incubating with the goat anti-rabbit secondary antibody at room temperature for 2 h, the blots were visualised using the ECL fluorescence detection kit (Beyotime, Shanghai, China) and captured using the Bio-Rad image analysis system. ImageJ software was used to quantify the immunoblots.

### Fluorescence *in situ* hybridization (FISH) and immunofluorescence (IF) assays

Cells were fixed with 4% formaldehyde at room temperature, and then treated with 0.5% Triton X-100. For FISH assay, hybridization was implemented with Cy3-labeled NEAT1 probe overnight. After that, cells were incubated with the secondary antibody at 4°C overnight. For IF assay, 97H or Huh7 cells were incubated with primary antibodies against FOXP3 at 4°C overnight, and then the secondary antibody at 4°C for 1h. The stained cells were observed using the confocal laser microscope (Olympus). Nuclei was counterstained using DAPi.

### Exosome extraction and identification

Exosomes were isolated from 97H or Huh7 cells by ultracentrifugation method. Briefly, the supernatant of 97H or Huh7 cells was collected and centrifuged at 300 × *g* for 10 min, 2,000 × *g* for 10 min, 10,000 × *g* for another 30 min. After that, the supernatant was ultracentrifuged at 140,000 × *g* supernatant for 70 min twice. Later on, the supernatant was removed, the pellet was obtained and resuspended in 50 μL PBS. The number and size of exosomes was analyzed using a Nanoparticle Tracking Analysis (NTA) instrument. In addition, exosomes were also identified by transmission electron microscopy (TEM) western blot and flow cytometry assays.

### Xenograft model in nude mice

Female BALB/C nude mice (4-6 week old) were obtained commercially from National Laboratory Animal Center (Beijing, China). Totally, $1\times10^7$ HCC cells transfected with pcDNA3.1 (OE NC), pcDNA3.1 NEAT1 (NEAT1 OE), pcDNA3.1 NEAT1 + PKM2 siRNA1 (NEAT1 OE + si-PKM2) plasmids were subcutaneously injected into the left flank of the nude mice. Tumor growth was recorded daily, and tumor volume was measured with a vernier caliper and calculated using the equation: (length × width$^2$)/2. The nude mice were sacrificed at day 23, the tumor tissues were removed completely.

In other experiments, HCC cells transfected with OE NC , NEAT1 OE , NEAT1 OE + si-PKM2 plasmids were injected into the mice via tail vein to observe the distant metastasis. The nude mice were sacrificed at day 23 and the number of lung metastases in these mice was determined. These study were approved by the Ethics Committee of the Affiliated Drum Tower Hospital, Medical School of Nanjing University, and animals were maintained following the guidelines of the Institutional Animal Care and Use Committee.

### Immunohistochemical

The tumor tissues were fixed in 10% formaldehyde, embedded in paraffin and then spliced into 6 μm sections. After that, paraffin sections were dewaxed using xylene. Later on, the sections were incubated the primary antibodies against PKM2 and Ki67 at 4°C overnight. Later on, the sections were incubated with biotinylated-labeled second antibody at 37°C for 30 minutes. Next, the sections were visualized with DAB solution and stained with hematoxylin. Pictures were captured under a fluorescence microscope.

### Statistics analysis

All data were repeated in triplicate. Comparisons between two groups were analyzed using the unpaired t test. One-way analysis of variance (ANOVA) and Tukey's tests were carried out for multiple group comparisons. Values are shown as the mean ± SD. Differences were considered to be statistically significant at *P<0.05.

# Results

### NEAT1 promoted the proliferation, migration and invasion of HCC cells

To clarify the role of NEAT1 in HCC cells, we transfected NEAT1-overexpressing plasmid (NEAT1 OE) into 97H and Huh7 cells (Figure 1A). Meanwhile, we used two different siRNAs (si-NEAT1 1# and si-NEAT1 2#) to downregulate NEAT1 in 97H and Huh7 cells (Figure1A). The results of CCK-8, colony formation and transwell assays showed that overexpression of NEAT1 significantly promoted the viability, proliferation, migration and invasion in 97H and Huh7 cells (Figure 1B, 1C, 1D and 1E). In contrast, downregulation of NEAT1 markedly inhibited the viability, proliferation, migration and invasion in 97H and Huh7 cells (Figure 1B, 1C, 1D and 1E). These data suggested that NEAT1 could promote HCC cell proliferation, migration and invasion.

### NEAT1 regulates the expression of PKM2 through transcription factor FOXP3

PKM2 is highly expressed in human cancers and functions as a oncogene [27-29]. We found that overexpression of NEAT1 markedly increased the mRNA and protein expression of PKM2 in 97H and Huh7 cells, while knockdown of NEAT1 exhibited the opposite effects (Figure 2A and 2B). Luciferase reporter assay showed that overexpression of NEAT1 notably increased the luciferase activity of 97H and Huh7 cells that were transfected with vector containing PKM2 promoter, suggesting that NEAT1 promoted PKM2 transcriptional activity (Figure 2C).

It has been shown that lncRNA could regulate gene transcription through transcription factor modulation [30, 31]. To further investigate the underlying mechanism by which NEAT1 promote HCC progression, we performed RNA pull down, silver staining and mass spectrometry assays to identify proteins associated with NEAT1 (Figure 2D). FOXP3 was identified as a potential binding target for NEAT1 and the interaction between NEAT1 and FOXP3 was verified by RNA pull down and RIP assays (Figure 2E, 2F and 2G). We next to investigate the region of NEAT1 responsible for the binding activaity of FOXP3 protein. RIP assay showed that 900-1200 fragment site of NEAT1 was notably recovered by FOXP3 protein, indicating that NEAT1 could bind with FOXP3 at the 900-1200 fragment site (Figure 2H). Meanwhile, the catRAPID analysis showed high affinity between NEAT1 transcript and FOXP3 protein (Figure 2I).

The results of ChIP assay indicated that overexpression of NEAT1 increased the binding of the FOXP3 protein to the PKM2 promoter; whereas, downregulation of NEAT1 decreased the binding of the FOXP3 protein to the PKM2 promoter (Figure 2J). The results of RT-qPCR indicated indicated that FOXP3 siRNA2 significantly reduced the level of FOXP3 both in cytoplasma and nucleus (Figure 2K). In addition, FOXP3 knockdown could reverse the effect of NEAT1 on the transcriptional activity of PKM2, indicating that the

FOXP3 might be required for the transcriptional regulation of NEAT1 on PKM2 (Figure 2L). Furthermore, knockdown of FOXP3 using Crispr/cas9 notably downregulated the expression of PKM2 in 97H cells (Figure 2M). The data in TCGA dataset showed a positive correlation between NEAT1 and PKM2 expression (Figure 2N). Meanwhile, the co-localization of NEAT1 and FOXP3 in nucleus further validated their interaction (Figure 2O). Collectively, NEAT1 could upregulate the expression of PKM2 through transcription factor FOXP3.

### PKM2 promotes the proliferation, invasion and migration of HCC cells

We next to explore the role of PKM2 in HCC. The data in GEO databases GSE6764 and GSE14520 showed that PKM2 was markedly increased in HCC tissues relative to normal tissues (Figure 3A and 3B). In addition, RT-qPCR and western blot assays confirmed a significant upregulation in PKM2 in HCC tissues in comparison with the normal tissues (Figure 3C, 3D and 3E). Moreover, the data in TCGA dataset showed that high levels of PKM2 correlated with poor overall survival rates in HCC patients (Figure 3F).

Furthermore, RT-qPCR assay indicated that overexpression of PKM2 notably upregulated the level of PKM2 in 97H and Huh7 cells, whereas PKM2 knockdown exhibited the opposite effects (Figure 3G). In addition, the results of CCK-8, colony formation and transwell assays showed that overexpression of PKM2 markedly promoted the viability, proliferation, migration and invasion of 97H and Huh7 cells, whereas knockdown of PKM2 exhibited the opposite effects (Figure 3H, 3I, 3J, 3K). These data suggested that PKM2 could promote HCC cell proliferation, invasion and migration.

### Downregulation of PKM2 reverses the tumor-promoting effect of NEAT1

In order to further confirm the interaction between NEAT1 and PKM2, rescue experiments were performed. As shown in Figure 4A, upregulation of NEAT1 notably increased the level of PKM2; however, that effect was reversed by si-PKM2. In addition, overexpression of NEAT1 significantly promoted the viability, proliferation, migration and invasion in 97H and Huh7 cells; however, these effects were reversed by knockdown of PKM2 (Figure 4B, 4C, 4D and 4E). Collectively, downregulation of PKM2 reversed the tumor-promoting effect of NEAT1.

### NEAT1 promotes exosome release from HCC cells

Evidence has shown that tumor-derived exosomes play a vital role in cancer development [32]. To examine the impact of NEAT1 overexpression on HCC cell-derived exosomes, exosomes were isolated from the conditioned media (CM) of 97H and Huh7 cells transfected with NEAT1 OE plasmids. As indicated in Figure 5A, exosomes harvested from NEAT1-overexpressing 97H and Huh7 cells demonstrated notably higher nanoparticle concentrations compared with that from control 97H and Huh7 cells. In addition, the vesicles were positive for exosome markers CD63, CD9 and HSP90 (Figure 5B). Moreover, TEM and NTA assays showed that exosomes isolated from the CM of NEAT1-overexpressing (Exo-NEAT1) and control (Exo-control) 97H cells were round, cup-shaped and membrane-encapsulated particles with a range of 50 to 150 nm in size (Figure 5C, 5D and 5E). Furthermore, flow cytometry assay

showed that the expression of marker proteins (CD63 and HSP90) was increased in Exo-NEAT1 groups compared with that in Exo-control group (Figure 5F, G). To sum up, NEAT1 could promote exosome release from HCC cells.

### Exosomes secreted from NEAT1-overexpressing HCC cells promotes the proliferation, migration and invasion of HCC cells

Next, we investigated the role of Exo-NEAT1 on HCC. As shown in Figure 6A, 6B, 6C, 6D and 6E, 97H or Huh7 cell-derived exosomes (97H-Exo or Huh7-Exo) markedly promote the viability, proliferation, migration and invasion of 97H or Huh7 cells. As expected, exosomes secreted from NEAT1-overexpressing 97H or Huh7 cells further promoted the proliferation, migration and invasion of 97H or Huh7 cells compared with 97H-Exo or Huh7-Exo group (Figure 6A, 6B, 6C, 6D and 6E). These data showed that exosomes secreted from NEAT1-overexpressing HCC cells could promote the proliferation, migration and invasion of HCC cells.

### NEAT1 promotes HCC cell growth and metastasis *in vivo* via upregulation of PKM2

To investigate the role of NEAT1 on tumor growth *in vivo*, we established a nude mouse xenograft model. As indicated in Figure 7A, 7B and 7C, overexpression of NEAT1 significantly increased tumor volume and tumor weight; whereas, these effects were reversed by PKM2 knockdown. In addition, the result of Ki67 IHC assay showed that overexpression of NEAT1 promoted cell proliferation in tumor tissues; however, that effect was reversed by PKM2 knockdown (Figure 7D). Moreover, upregulation of NEAT1 increased the expression of PKM2 in tumor tissues; while, that effect was reversed by PKM2 knockdown (Figure 7D). Furthermore, there were significantly increased visible lung metastatic nodules in NEAT1 group compared with control group; however, these phenomenon were reversed by PKM2 knockdown (Figure 7E and 7F). These results suggested that NEAT1 promote HCC cell growth and metastasis *in vivo* via upregulation of PKM2.

## Discussion

LncRNAs play an important role in many different diseases, including human cancers [33–36]. LncRNA NEAT1, which is located at chromosome 11q13.1, is an unusual RNA polymerase II transcript that lack introns [37]. Previously, NEAT1 has been reported to be highly expressed in various human cancers, such as gastric cancer, colorectal cancer (CRC) and HCC [38–40]. In this study, we found that overexpression of NEAT1 significantly promoted the viability, proliferation, migration and invasion of HCC cells, wheras NEAT1 knockdown exhibited the opposite effects. Wang et al found that overexpression of NEAT1 could promote HCC cell proliferation and invasion via targeting miR-613 [41]. Li et al showed that NEAT1 promoted HCC cell proliferation and invasion through miR-296-5p/CNN2 axis [16]. These data showed that NEAT1 exerted oncogenic property in HCC progression.

Recent years, PKM2 has been found to play an important role in cancer progression via metabolic or non-metabolic pathways [42]. PKM2 is highly expressed in human cancers and functions as a oncogene [27–

29]. Thus, we attempt to investigate whether NEAT1 could exert oncogenic properties in HCC by regulation of PKM2. Evidences have shown that lncRNA could regulate gene transcription through transcription factor modulation or chromatin modification, thus participating in biological processes of human cancers [30, 31]. In addition, lncRNAs could act as a hinge to recruit transcription factors to the promoter of genes [43]. In this study, we found that NEAT1 could recruit transcription factor FOXP3 to PKM2 promoter region and upregulate PKM2 expression.

Li et al found that downregulation of PKM2 suppressed the proliferation, migration, and invasion of hepatocellular carcinoma cells [44]. Bian et al showed that FEZF1-AS1 could promote CRC cell proliferation and metastasis by upregulation of PKM2 [45]. In the present study, we found that PKM2 could promote the proliferation, migration and invasion of HCC cells, whereas PKM2 knockdown exhibited the opposite effects, which were consistant with previous studies. In addition, overexpression of NEAT1 promoted the proliferation, migration and invasion in HCC cells; however, these effects were reversed by PKM2 knockdown. These data showed that NEAT1 could promote the progression of HCC via upregulation of PKM2.

The tumor microenvironment (TME), an integral part of cancer, could affect tumor growth and metastasis [46]. Exosomes are important mediators of intercellular communication within TME [47]. It has been shown that tumor cells can secrete more exosomes than normal cells [48]. Interestingly, we found that NEAT1-overexpressing HCC cells can secrete more exosomes than control HCC cells. Thus, we attempt to investigate whether exosomes from NEAT1-overexpressing HCC cells could affect the migration and invasion of HCC cells. Our results showed that exosomes secreted from NEAT1-overexpressing HCC cells could promote the migration and invasion of HCC cells. Fan et al found that cancer-associated fibroblasts-derived exosomal NEAT1 promote the endometrial cancer progression via STAT3/YKL-40 pathway [49]. These data showed that exosomes released by NEAT1-overexpressing HCC cells could promote HCC progression.

# Conclusion

In this study, we found that lncRNA NEAT1 could serve as a carcinogenic lncRNA. Mechanistically, NEAT1 could promote HCC progression via PKM2 and exosome-mediated transfer. These data indicated that NEAT1 may be a therapeutic target in HCC.

# Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and material

All data generated or analyzed during this study are included in this article.

### Competing interests

The authors declare that they have no competing interests.


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### Authors' contributions

JP, YH and CY made major contributions to the conception, design and manuscript drafting of this study. YW was responsible for data acquisition, data analysis, data interpretation and manuscript revision. XZ made substantial contributions to conception and design of the study and revised the manuscript. All authors agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.


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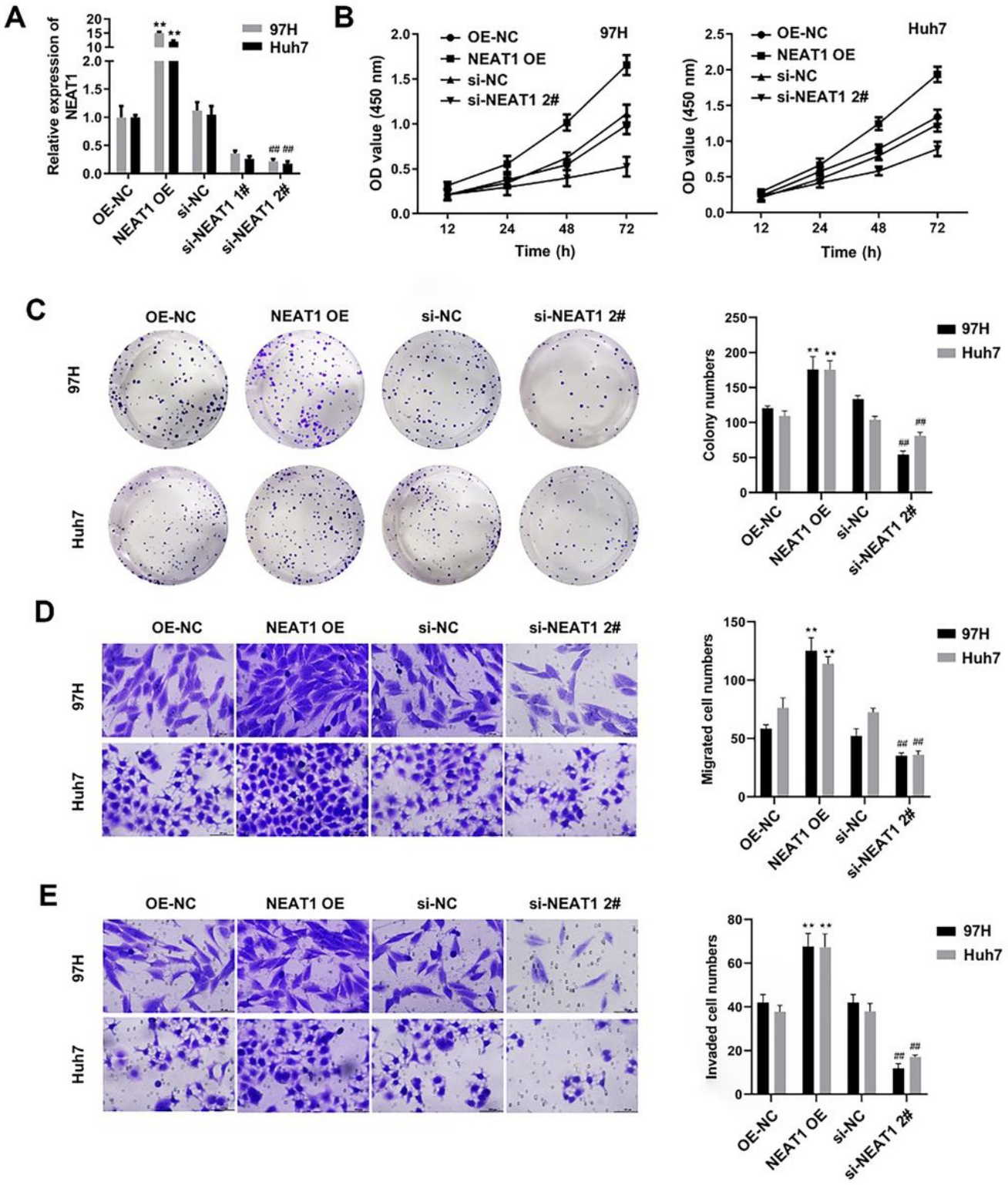
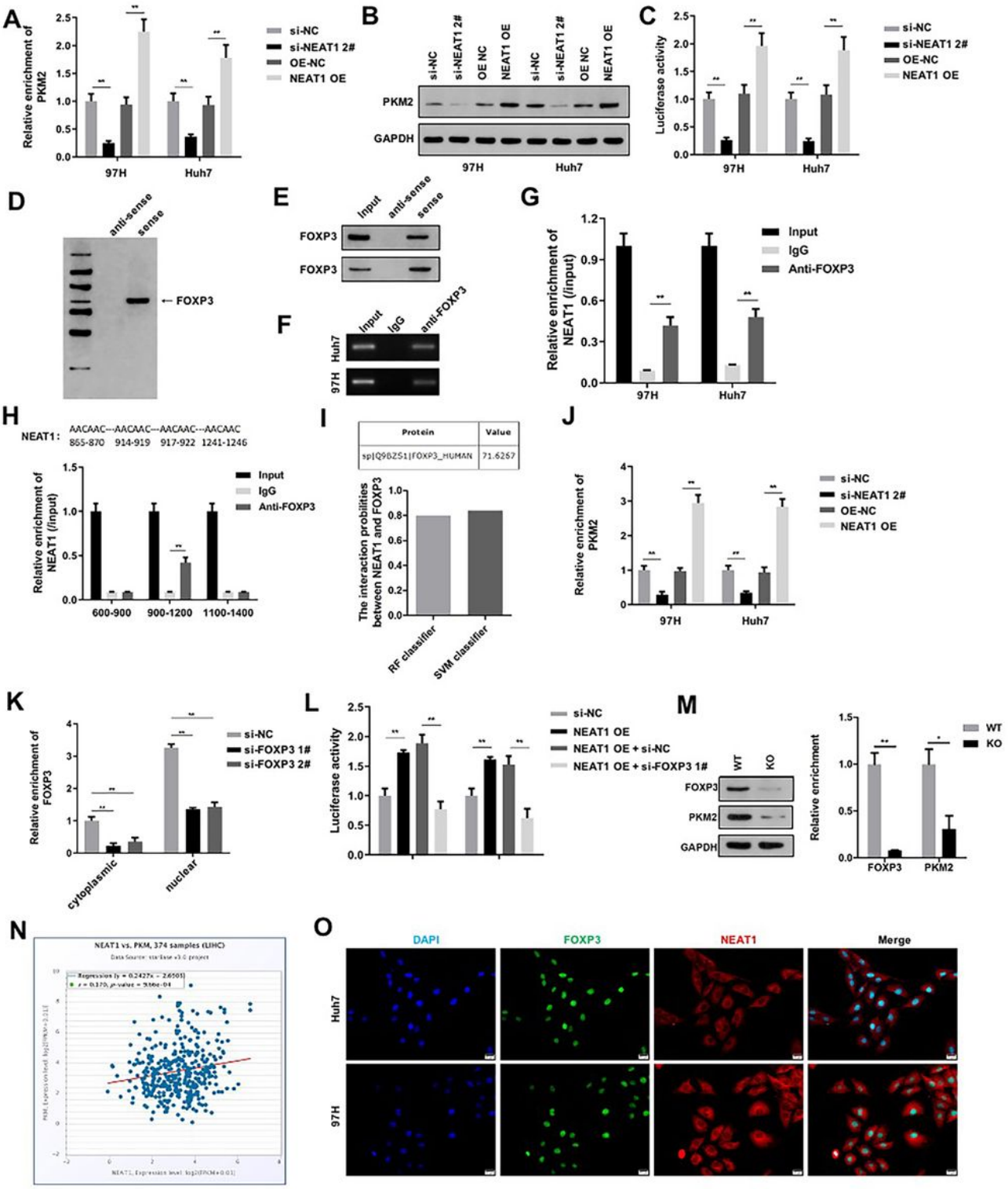
# Figures




## Figure 1

NEAT1 promotes the proliferation, migration and invasion of HCC cells. 97H and Huh7 cells were transfected with pcDNA3.1 vector (OE-NC), pcDNA3.1-NEAT1 (NEAT1 OE), siRNA NC (si-NC), NEAT1 siRNA1 (si-NEAT1 1#) or NEAT1 siRNA2 (si-NEAT1 2#) plasmids respectively. (A) RT-qPCR analysis of NEAT1 levels in 97H and Huh7 cells after transfection with the indicated plasmids. (B) CCK-8 assay was used to determine cell viability. (C) Colony formation assay was used to detect cell proliferation. (D, E)

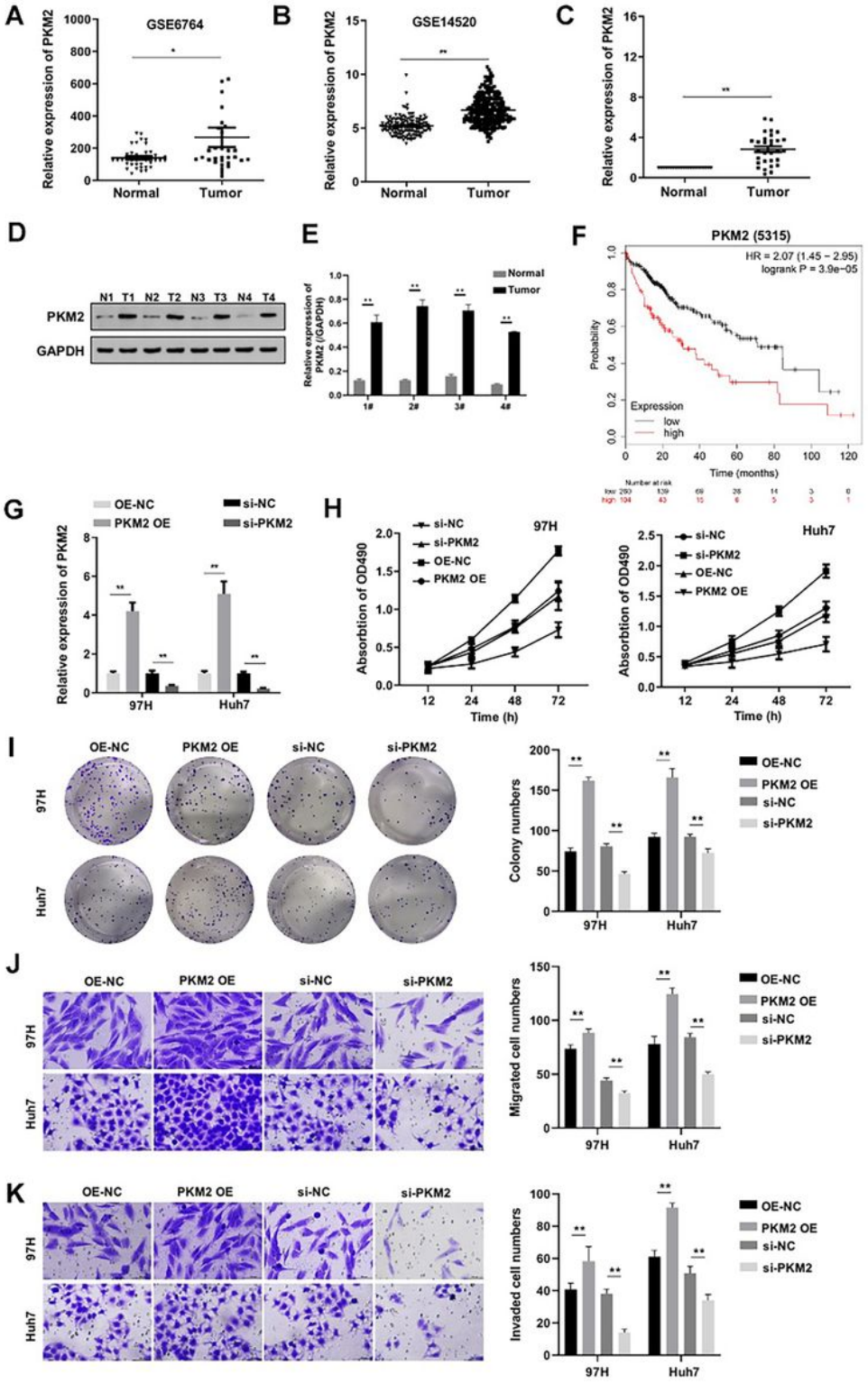
Transwell assays were performed to evaluate cell migration and invasion abilities. **P < 0.01 vs. OE-NC group; ##P < 0.01 vs. si-NC group.




## Figure 2

NEAT1 regulates the expression of PKM2 through transcription factor FOXP3. (A, B) RT-qPCR and western blot analysis of PKM2 levels in 97H and Huh7 cells after transfection with si-NEAT1 2# and NEAT1 OE plasmids. (C) Luciferase activity of psi-CHECK2-PKM2 upon transfection of si-NEAT1 2# and NEAT1 OE

plasmids in 97H and Huh7 cells. (D) The bio-NEAT1 vector was transfected into HEK293T cells, and anti-bio was used to immunoprecipitate NEAT1-binding proteins. After silver staining, NEAT1-specific bands were excised and analyzed by mass spectrometry. (E) Biotinylated NEAT1 or antisense NEAT1 probe were transcribed in vitro and incubated with 97H or Huh7 whole-cell lysates for RNA pull down assays. (F, G) RIP assay of the enrichment of FOXP3 on NEAT1 relative to IgG in 97H or Huh7 whole-cell lysates. (H) Bioinformatics predicted the binding sites of NEAT1 and FOXP3. RIP analysis determined the recovery of a portion of NEAT1 in 97H and Huh7 cells using FOXP3 antibody. (I) Bioinformatics catRAPID database predicted the combination score of NEAT1 and FOXP3. (J) ChIP assay with FOXP3 antibody showed that the binding of FOXP3 with the PKM2 promoter was regulated by NEAT1 in 97H and Huh7 cells. (K) RT-qPCR analysis of FOXP3 levels in nuclear and cytoplasmic of 97H cells after transfection with si-FOXP3 1# or si-FOXP3 2# plasmids. (L) Luciferase activity of psi-CHECK2-PKM2 upon transfection with indicated plasmids. (M) Western blot analysis of FOXP3 and PKM2 levels in 97H cells after the knockout of FOXP3 by Crispr/cas9. (N) Bioinformatics analysis showed the correlation between NEAT1 and PKM2 expression. (O) Immunofluorescence confocal colocalization experiment indicated the co-localization of NEAT1 and FOXP3 Immunofluorescence detection of FOXP3 (green) and FISH analysis of NEAT1 (red) 97H and Huh7 cells. *P < 0.05, **P < 0.01.




## Figure 3

PKM2 promotes the proliferation, invasion and migration of HCC cells. (A, B) The GSE6764 and GSE14520 datasets showed the expression of PKM2 in HCC tissues and normal controls. (C) RT-qPCR analysis of PKM2 levels in HCC tissues and normal controls. (D, E) Western blot of PKM2 expression in tumor tissues and normal controls. (F) TCGA database showed the correlation between PKM2 expression and overall survival rate in patients with HCC. (G) 97H and Huh7 cells were transfected with OE-NC, PKM2

OE, si-NC, si-PKM2 plasmids respectively. RT-qPCR analysis of PKM2 levels in 97H and Huh7 cells. (H) CCK-8 assay was used to determine cell viability. (I) Colony formation assay was used to detect cell proliferation. (J, K) Transwell assays were performed to evaluate cell migration and invasion abilities. *P < 0.05, **P < 0.01.

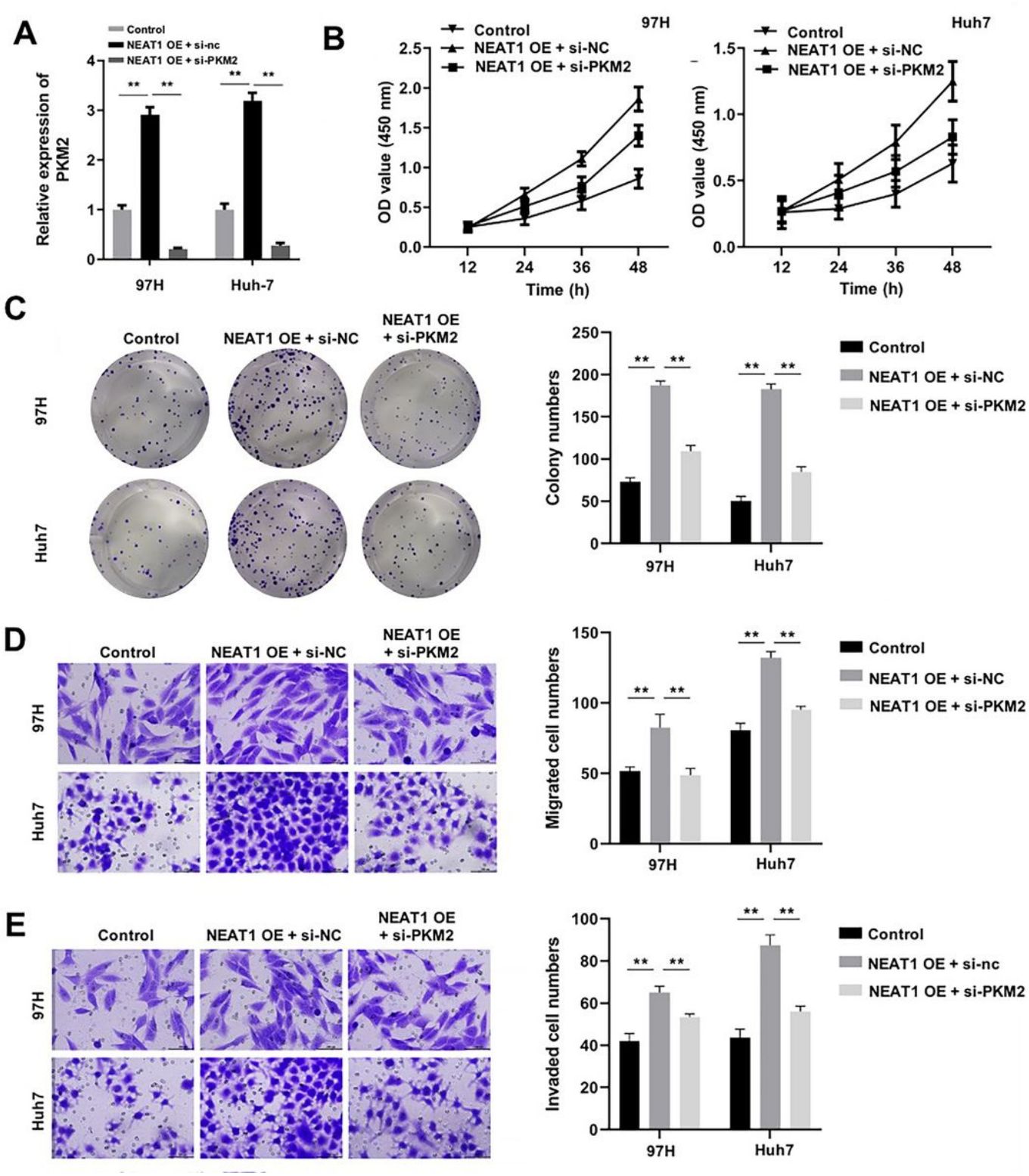
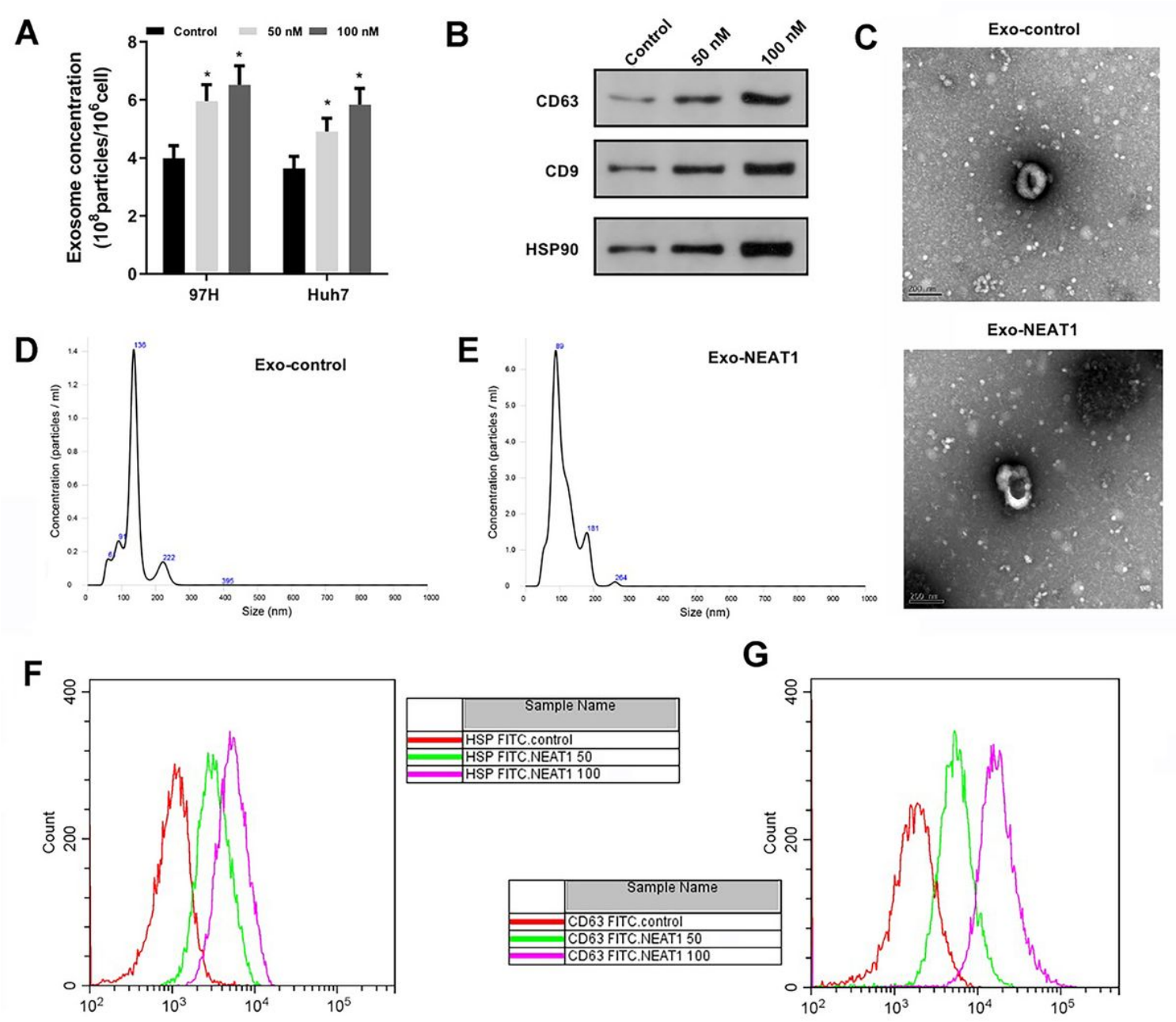



Figure 4

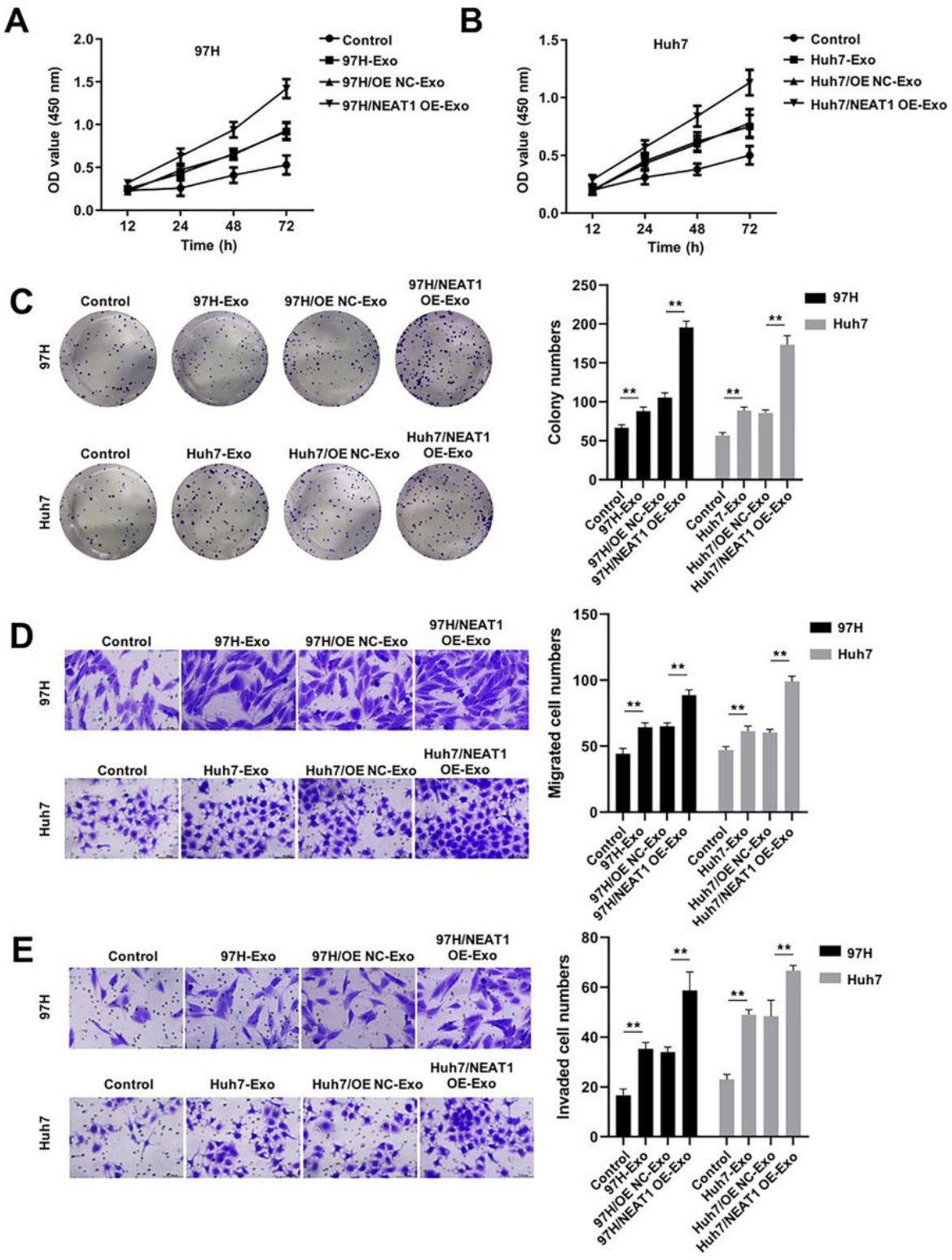
Downregulation of PKM2 reverses the tumor-promoting effect of NEAT1. 97H and Huh7 cells were transfected with NEAT1 OE + si-NC or NEAT1 OE + si-PKM2 plasmids respectively. (A) RT-qPCR analysis of PKM2 levels in 97H and Huh7 cells. (B) CCK-8 assay was used to determine cell viability. (C) Colony formation assay was used to detect cell proliferation. (D, E) Transwell assays were performed to evaluate cell migration and invasion abilities. **P < 0.01.




## Figure 5

NEAT1 promotes exosome release from HCC cells. 97H and Huh7 cells were transfected with pcDNA3.1-NEAT1 (0, 50 or 100 nM). (A) The exosome concentration was detected by NTA. (B) Western blot analysis of CD63, CD9 and HSP90 proteins in exosomes derived from transfected 97H cells. (C) TEM images of exosomes secreted by transfected 97H cells. (D, E) NTA was used to analyze the size distributions and

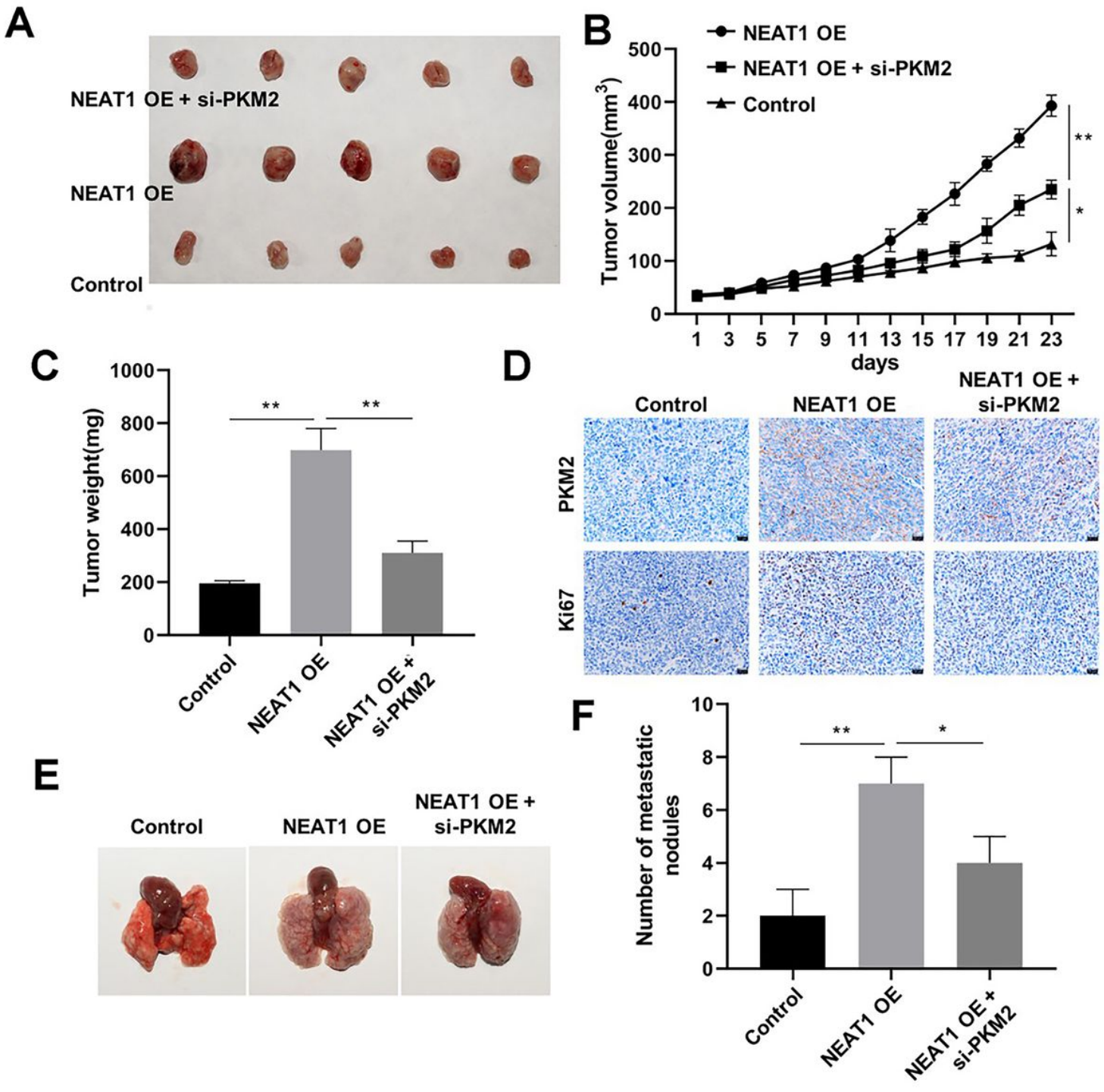
number of exosomes. (F, G) The exosome surface markers CD63 and HSP90 were identified by flow cytometry. *P < 0.05 vs. control group.




## Figure 6

Exosomes secreted from NEAT1-overexpressing HCC cells promotes the proliferation, migration and invasion of HCC cells. 97H and Huh7 cells were incubated with exosomes derived from 97H and Huh7 cells (97H-Exo, Huh7-Exo) or 97H and Huh7 cells after transfection with pcDNA3.1 vector (97H/OE-NC-

Exo, Huh7/OE-NC-Exo) and pcDNA3.1-NEAT1 (97H/NEAT1 OE-Exo, Huh7/NEAT1 OE-Exo). (A, B) CCK-8 assay was used to determine cell viability. (C) Colony formation assay was used to detect cell proliferation. (D, E) Transwell assays were performed to evaluate cell migration and invasion abilities. **P < 0.01.




## Figure 7

NEAT1 promotes HCC cell growth and metastasis in vivo via upregulation of PKM2. (A) Representative image of xenograft tumors. (B, C) The tumor volume and weight were shown. (D) IHC analysis of the

expression of Ki67 and PKM2 in tumor tissues. (E, F) Number of metastatic nodules in the lung tissues from the nude mice. *P < 0.05, **P < 0.01.