Original Research

p62 Promotes Malignancy of Hepatocellular Carcinoma by Regulating the Secretion of Exosomes and the Localization of β-Catenin

Weibang Yang[1,2,†], Jinxing Wei[2,†], Lihong Lv[3,†], Jiyan Xie[4], Ang Li[2], Zhihua Zheng[5], Wenfeng Zhuo[2], Peiqing Liu[5,*], Jun Min[2,*]

[1]Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 510120 Guangzhou, Guangdong, China
[2]Department of Hepatobiliary Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 510120 Guangzhou, Guangdong, China
[3]Clinical Trial Institution of Pharmaceuticals, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 510120 Guangzhou, Guangdong, China
[4]Department of Gastrointestinal Surgery, The Eighth Affiliated Hospital of Sun Yat-sen University, 518033 Shenzhen, Guangdong, China
[5]Laboratory of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-sen University, 510006 Guangzhou, Guangdong, China
*Correspondence: minjun@mail.sysu.edu.cn (Jun Min); liupq@mail.sysu.edu.cn (Peiqing Liu)
[†]These authors contributed equally.
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Abstract

Background: p62 is a multi-domain protein and participates in a variety of cellular biological activities. p62 is also related to tumor malignancy. However, the underlying molecular mechanism of p62 regulating the progression of hepatocellular carcinoma (HCC) remains unclear. **Methods**: The expression levels of p62 in HCC tissues and adjacent non-tumor tissues were confirmed using the TCGA dataset and immunohistochemistry. Stable p62-overexpressing HepG2 cells and p62-knockdown MHCC97H cells were established with lentiviral vectors. Cell proliferation, migration, and invasion assays were carried out to investigate the role of p62 in HCC cells and HCC-derived exosomes. The relationship between p62 and β-catenin was investigated by immunofluorescence and co-immunoprecipitation assays. Male nude mice (BALB/c-nu/nu) were used to establish the xenograft tumors. **Results**: We found that p62 was significantly upregulated in HCC, and a high level of p62 indicated the promotion of malignancy including cell proliferation, migration, and invasion. Exosomes derived from p62-overexpressing HepG2 also demonstrated the ability to promote tumor malignancy. Immunofluorescence and co-immunoprecipitation assays indicated that p62 interacts with β-catenin and regulates the localization of β-catenin to affect the intercellular junction. p62 also promotes tumor growth of HCC and down-regulates the expression of β-catenin *in vivo*. **Conclusions**: The results of this study concluded that p62 promotes the malignancy of HCC by regulating the secretion of exosomes and the localization of β-catenin. These findings may provide new ideas for the diagnosis and treatment of HCC.

Keywords: p62; β-catenin; hepatocellular carcinoma (HCC); exosomes; malignancy

1. Introduction

Hepatocellular carcinoma (HCC) is a neoplastic disease with high morbidity and mortality all over the world, especially in Asia and Africa [1]. Although there are many therapeutic options for HCC, long-term survival prognosis remains poor [2]. The metastatic property of tumor is one of the main causes of death in patients with HCC, so it is necessary to learn more about the mechanism of HCC metastasis [3].

As a classic autophagy receptor, protein p62 is encoded by the gene SQSTM1 [4]. It is also a multi-domain protein that selectively interacts with different signaling mediators to regulate selective autophagy, metabolic reprogramming, and antioxidant reactions [5]. The function of p62 in different cancers remains controversial, with evidence suggesting that p62 is promoted in many kinds of cancer including colorectal cancer, prostate cancer, breast cancer, and others [6–10]. High p62 expression is relevant to aggressive clinical features, highlighting the potential of

p62 as an important target for anticancer therapy [11,12]. However, the detailed mechanisms of p62-mediated cancer invasion and metastasis have not been fully elucidated.

Exosome is a kind of extracellular vesicle with a size range of 30–150 nm and secreted by most species [13]. It can carry proteins, lipids, and various RNAs for cell-to-cell transport [14,15]. Tumor cells may cooperate with other cells by releasing exosomes and transferring oncogenic molecules to the recipient cells [16]. In recent years, increasing evidence has indicated that exosome may participate in the process of cancer invasion and metastasis, indicated that it can be used as a biomarker for the diagnosis of cancers [17–19]. However, the role of exosome in the metastasis of cancer and the mechanisms related to the regulation of selective sorting of exosomal cargos remain largely unknown.

β-catenin serves as a part of the adherens junction complex with E-cadherin and acts as a transcription coactivator of Wnt target gene expression [20]. It is abnor-

mally activated in many kinds of cancer and involved in regulating carcinogenesis and epithelial-mesenchymal transition of cancers [21,22]. Epithelial-mesenchymal transition (EMT) is considered as an important step in cancer metastasis. Mesenchymal proteins N-cadherin and Vimentin are increased while epithelial marker E-cadherin is reduced during EMT. Transcription factors including Snail, Twist1, and ZEB1 control this switch, and the expression of these genes varies according to the signals mediating EMT [23,24]. Based on the localization of β-catenin, it shows a dual regulative effect on the process of EMT [25]. However, the mechanism of delicately controlling the two different roles of β-catenin is not very clear.

The purpose of this study was to elucidate the molecular mechanism and function of p62 in HCC, and the results ultimately showed that p62 was significantly upregulated in HCC tissues and metastatic HCC cell lines. Overexpressing p62 can significantly promote the oncogenic behavior including cell growth, mobility, and invasiveness of HCC by regulating the localization of β-catenin and secretion of exosomes *in vitro*. These findings may provide new ideas for the diagnosis and treatment of HCC.

2. Materials and Methods

2.1 The Cancer Genome Atlas (TCGA) Database

The data of gene expression, together with the corresponding clinical data of hepatocellular carcinoma and normal liver tissue samples, were downloaded from the TCGA database (http://ualcan.path.uab.edu/analysis.html).

2.2 Cell Lines and Cell Culture

The HepG2 cell line was obtained from the American Type Culture Collection (ATCC, USA). SNU182, SNU387, Hep3B, and Li-7 were provided by Stem Cell Bank, Chinese Academy of Sciences (China). Huh7 cell line was purchased from the Japanese Collection of Research Bioresources (Japan). MHCC97H cell line was provided by CELLCOOK Biological Technology (China). All HCC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. The FBS used for exosome isolation was depleted of extracellular vesicles by ultracentrifugation for 12 h at 120,000 $\times$g at 4 °C (Optima L-100XP, Beckman, USA). The co-culture assay refers to incubate recipient cells with 10 μg indicated exosomes for 24 h.

2.3 Western Blotting Assay

The HCC cells and isolated exosomes were harvested and lysed in RIPA buffer (Beyotime, China) supplemented with a protease-inhibitor cocktail (SIGMA, USA). BCA assay was used to measure the protein concentration. Equal amounts of lysates were loaded and separated on 9% SDS-PAGE gels. Then, the proteins were electrotransferred to polyvinylidene difluoride membranes (PVDF), blocked in 5% skim milk at 37 °C for 60 min, and incubated with primary antibodies at 4 °C overnight. After incubating with secondary antibodies for 60 min at room temperature and washing again, the membranes were visualized by an enhanced chemiluminescence system (Image Quant, GE, USA). The experiment was repeated three times. Antibody information was listed as follows: p62 (Catalog: #88588), β-catenin (Catalog: #8480), E-cadherin (Catalog: #5296), Vimentin (Catalog: #5741), Snail (Catalog: #3879), Twist 1 (Catalog: #46702), Alix (Catalog: #92880), β-actin (Catalog: #4970), GAPDH (Catalog: #2118), goat anti-mouse IgG (Catalog: #7076), and goat anti-rabbit IgG (Catalog: #7074) (Cell Signaling Technology, USA, 1:1000); Tsg101 (Catalog: A1692), Wnt5a (Catalog: A19133), and Wnt3a (Catalog: A0642), CD63 (Catalog: A5271), and HSP90 (Catalog: A5027) (ABclonal Technology, China, 1:1000); GSK3β (Catalog: 22104-1-AP), phospho-GSK3β^{Ser9} (Catalog: 67558-1-Ig), and active GSK-3β^{Try216} (Catalog: 29125-1-AP) (Proteintech, USA, 1:1000).

2.4 RNA Isolation and Quantitative RT-PCR

TRIZOL Reagent (SIGMA, USA) was used for the RNA extraction in accordance with the manufacturer's instruction. Reverse transcription was conducted using Prime Script RT Master Mix (TOYOBO, Japan) in accordance with the standard protocol. SYBR Premix EX Taq TMII (TOYOBO, Japan) was used for RT-PCR. The differences in relative expression levels between groups were analyzed using the $2^{-\Delta\Delta Ct}$ method. β-actin was used as the internal control. The primer sequences used for RT-PCR were shown in **Supplementary Table 1**.

2.5 Clinical Tissues and Immunohistochemistry (IHC) Analysis

We analyzed samples from 81 HCC patients who underwent hepatectomy at Sun Yat-sen Memorial Hospital, Sun Yat-sen University (Guangzhou, China), between January 2018 and November 2019. The ethical approval was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital [approval number: 2017(107)], and informed consent was obtained from each patient. Immunohistochemistry analysis was used to detect the expression of p62 in paraffin embedded adjacent non-tumor tissues and HCC tissues. The protocol was performed according to previously described [26].

2.6 Establishment of Stable Cell Lines

The EGFP:T2A:Puro-U6>hSQSTM1 knockdown lentiviral vector for SQSTM1/p62, the mCherry:T2A:Puro-EF1A>hSQSTM1 overexpressing lentiviral vector for SQSTM1/p62, and the control lentiviral vector were provided by Vector Builder (Guangzhou, China). The shRNAs targeting p62 were designated as described

in **Supplementary Table 2**. Lentivirus infection and puromycin selection were used to establish the stable p62-overexpressing HepG2 cells and p62-knockdown MHCC97H cells.

2.7 Cell Proliferation Assay

CCK-8 assay was used to detect the cell proliferation. Cells were seeded in 96-well plates at a density of 5×10^3 cells in 100 μL of culture medium. After 24, 48, 72, and 96 h, 100 μL of CCK-8 reagent (MCE, USA) was added into each well following by 60 min incubation at 37 °C. OD value of each well was detected using a microplate reader (Thermo, USA) at a wavelength of 450 nm. The experiment was repeated three times.

2.8 Wound Healing Assay

A scratch wound was created using a 1 mm wide sterile pipette tip after cells reached 80–90% confluence in 6-well plate. The detached cells were removed by washing with PBS three times, and then serum-free culture medium was added into each well. A microscope was used to capture the images at 0, 24 and 48 h at 100 $\times$ magnification and the wound edge was detected.

2.9 Migration and Invasion Assays

A Transwell Chamber (Corning, USA) was used for cell migration and invasion assays. For the migration assay, 700 μL of culture medium containing 20% FBS was added to the lower chamber. After that, 1×10^5 cells were seeded into the upper chamber in 200 μL of serum-free medium and incubated for 48 h. Similar procedure was repeated for the invasion assay. 50 μL of a mixture of serum-free DMEM and Matrigel (1:10, BD Biosciences, USA) was added to the upper chambers before the cells were seeded. Then, the cells remaining on the upper surface of the membrane were removed, and the cells that crossed through the 8 μm sized pores were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Finally, calculated the number of migrated/invaded cells with a microscope in five random fields of view at 200 $\times$ magnification.

2.10 Immunofluorescence

Cells were grown on glass dishes for 24 h, and then treated with 0.3% Triton X-100 for 15 min after being fixed in 4% paraformaldehyde for 15 min at room temperature. After that, 10% goat serum in PBS was used to block the cells for 60 min at room temperature. After the incubation with primary antibodies and Alexa-conjugated secondary antibody (Cell Signaling Technology, USA), the cells were stained with DAPI for 5 min and visualized on a confocal microscope (Olympus LV3000, Japan).

2.11 Exosome Isolation and Identification

Cells were seeded in 15ml vesicle-depleted medium on 10 cm dishes for 2–3 days. The culture media were harvested and centrifuged at 300 g for 10 min and then 2000 g for 20 min. After centrifugation at 10,000 g for 30 min at 4 °C, the supernatant was further filtered through a 0.22 μm filter (Pall Corporation, USA) to remove large vesicles. Next, appropriate volume of ExoQuick-TC Exosome Precipitation Solution (System Biosciences, USA) was added to the filtered culture medium and incubated at 4 °C overnight. After centrifugation at 1500 g for 30 min, exosome pellets were resuspended in PBS for further analysis or stored at –80 °C. Nanoparticle Tracking Analysis Instrument (Particle Metrix, Germany) and Transmission Electron Microscopy (JEM-1400, JEOL, Japan) were used to identify the morphology and concentration of the exosomes. 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (N-Rh-PE) was used to deal with the parent cells in accordance with the manufacturer's instruction, and then the exosomes derived from these cells were labeled with red fluorescence.

2.12 Animal Experiments

The animal experiments were carried out with approval by the Ethics Committee of Sun Yat-sen University. Male athymic nude mice (BALB/c-nu/nu, 6 weeks old) were obtained from the Animal Center of Sun Yat-sen University. HepG2-Control and HepG2-p62-OE xenograft tumors were established by subcutaneous injection of cells (5×10^6 cells in 100 μL of PBS) into the right armpit in each group of five nude mice. Tumor volume was quantified using a caliper and calculated as $0.5 \times$ width^2 $\times$ length and were recorded every three days until the tumor volume reached 1000 mm^3. After 24 days, the mice were sacrificed and their tumors were excised, imaged, and weighed.

2.13 Statistical Analysis

All statistical analyses were performed using SPSS version 20.0 (IBM, Chicago, Illinois, USA) and GraphPad Prism 7 (GraphPad Software, San Diego, California, USA). Statistical differences were evaluated using a student's t-test and the data were presented as the mean $\pm$ SD. The results represent a total of three independent experiments. The value of p less than 0.05 was considered as statistically significant.

3. Results

3.1 p62 is Highly Expressed in HCC Tissues and Indicates Poor Prognosis in Human HCC

The TCGA dataset showed that the level of p62 in HCC tissues (n = 371) was much higher than that in normal liver tissues (n = 50) (Fig. 1A). The analysis of the survival curve of the TCGA dataset showed that the survival time of HCC patients with high expression of p62 (n = 91) was significantly shorter than that of patients with low expression of p62 (n = 274), suggesting that the level of p62 was a risk factor for poor prognosis of patients with HCC (Fig. 1B).

Moreover, the expression of p62 was upregulated with the decrease of tumor differentiation of HCC according to the TCGA dataset (Fig. 1C). Similar results were obtained in the clinical tissue samples collected specifically for this study. IHC analysis showed that the expression of p62 in HCC tissues was higher than that in adjacent non-tumor tissues (Fig. 1D,E). To screen suitable HCC cell lines for the follow-up experiments, the expression of p62 was explored in 7 HCC cell lines, including HepG2, SNU182, SNU387, Huh7, MHCC97H, Hep3B, and Li-7. p62 was upregulated in Huh7, MHCC97H, and Li-7, while it was silenced in HepG2, SNU182, SNU387, and Hep3B, according to the results of western blotting and RT-PCR (Fig. 1F,G).

3.2 p62 Promotes Cell Proliferation, Migration, and Invasion Abilities in HCC Cells

The high expression of p62 in clinical HCC tissues indicated that p62 may plays a potential tumor-promoting role in the occurrence and development of HCC. To further evaluate the role of p62 in HCC, stable p62-overexpressing HepG2 and p62-knockdown MHCC97H cell lines were established (Fig. 2A,C). The plasmids used for knockdown experiment have been verified by western blotting (**Supplementary Fig. 1**). The overexpression of p62 in HepG2 cells significantly promoted cell proliferation compared with control HepG2 cells as determined by CCK-8 assays, while knockdown of p62 inhibited cell proliferation of MHCC97H cells (Fig. 2B,D). Then, the abilities of migration and invasion in p62-overexpressing HepG2 cells and p62-knockdown MHCC97H cells were further evaluated. Wound healing assays showed that migration ability was significantly enhanced in p62-overexpressing HepG2 cells and weakened in p62-knockdown MHCC97H cells (Fig. 2E,F). In addition, transwell assays showed that the migration and invasion abilities were significantly promoted after overexpressing p62 in HepG2 cells, while knockdown of p62 inhibited the migration and invasion abilities of MHCC97H cells (Fig. 2G,H).

3.3 p62 Increases the Exosome Secretion but does not Affect the Size of Exosomes

As a multi-domain protein, p62 is widely involved in a variety of cellular life activities [5]. In our previous related studies, mass spectrometry was used to find the Vps4A-related protein [27]. Surprisingly, the mass spectrometry showed that p62 interacted with Vps4A (**Supplementary Table 3**). So, this study explored whether p62 affected the exosome pathway. Exosomes were isolated from culture medium of both control and p62-overexpressing HepG2 cells by precipitation reagent. The markers of exosomes including HSP90, Alix, Tsg101, and CD63 were examined by western blotting (Fig. 3A). Then, Nanoparticle Tracking Analysis (NTA) and Transmission Electron Microscopy (TEM) were used to identify the morphology and concentration of the exosomes (Fig. 3B,C). The results showed

that overexpression of p62 had no effect on the size but increased the number of the exosomes released into the culture medium, according to the concentration of NTA.

3.4 Exosomes Derived from p62-Overexpressing HepG2 Cells Promote the Proliferation, Migration, and Invasion of Recipient Cells

To investigate the bioactive effect of the exosomes derived from p62-overexpressing HepG2 cells, cell growth, wound healing, as well as transwell assays were performed. The recipient HepG2 cells were incubated with N-Rh-PE labeled exosomes for 24 h and observed under a confocal microscope to confirm that the recipient cells could absorb exosomes (Fig. 4A). HepG2 cells were incubated with different groups of exosomes, and it was found that exosomes from both control (CTexo) and p62-overexpressing HepG2 cells (p62exo) could promote the proliferation of the recipient cells. The promoting effect of exosomes in the p62-overexpressing group was stronger than that in the control group (Fig. 4B). Similar results were also obtained in wound healing and transwell assays. Exosomes derived from p62-overexpressing HepG2 cells could significantly promote the migration and invasion of the recipient cells (Fig. 4C,D). These results indicated that overexpression of p62 can promote the oncogenic behavior of HCC cells *in vitro* via exosomes.

3.5 p62 Interacts with β-Catenin and Down-Regulates the Expression of β-Catenin in HCC Cells

In previous studies, we found that Vps4A functions as a tumor suppressor to inhibit EMT by regulating the localization and exosome release of β-catenin in HCC [27]. We also found that p62 and Vps4A have interaction, according to mass spectrometry (**Supplementary Table 3**). Thus, the current study questioned whether p62 would also regulate the expression and localization of β-catenin. The interaction between p62 and β-catenin was confirmed by co-immunoprecipitation assay, and the results showed that p62 bound to endogenous β-catenin in Huh7 cells (Fig. 5A). After being stained with anti-p62 antibody and anti-β-catenin antibody, the Huh7 cells were visualized by confocal microscopy to confirm that p62 colocalizes with β-catenin (Fig. 5B). Western blotting showed that the expression of β-catenin was down-regulated after overexpressing p62 in HepG2 cells (Fig. 5C). Immunofluorescence showed that the fluorescence value and nuclear localization of β-catenin were decreased significantly after overexpressing p62, and the connection between the cells becomes less tight (Fig. 5D).

3.6 p62 Regulates the Expression and Localization of β-Catenin in the Cells and Exosomes

To confirm that p62 can regulate the localization of β-catenin, immunofluorescence tests of β-catenin were conducted in the control group and p62-overexpressing group respectively. Surprisingly, p62-overexpression re-
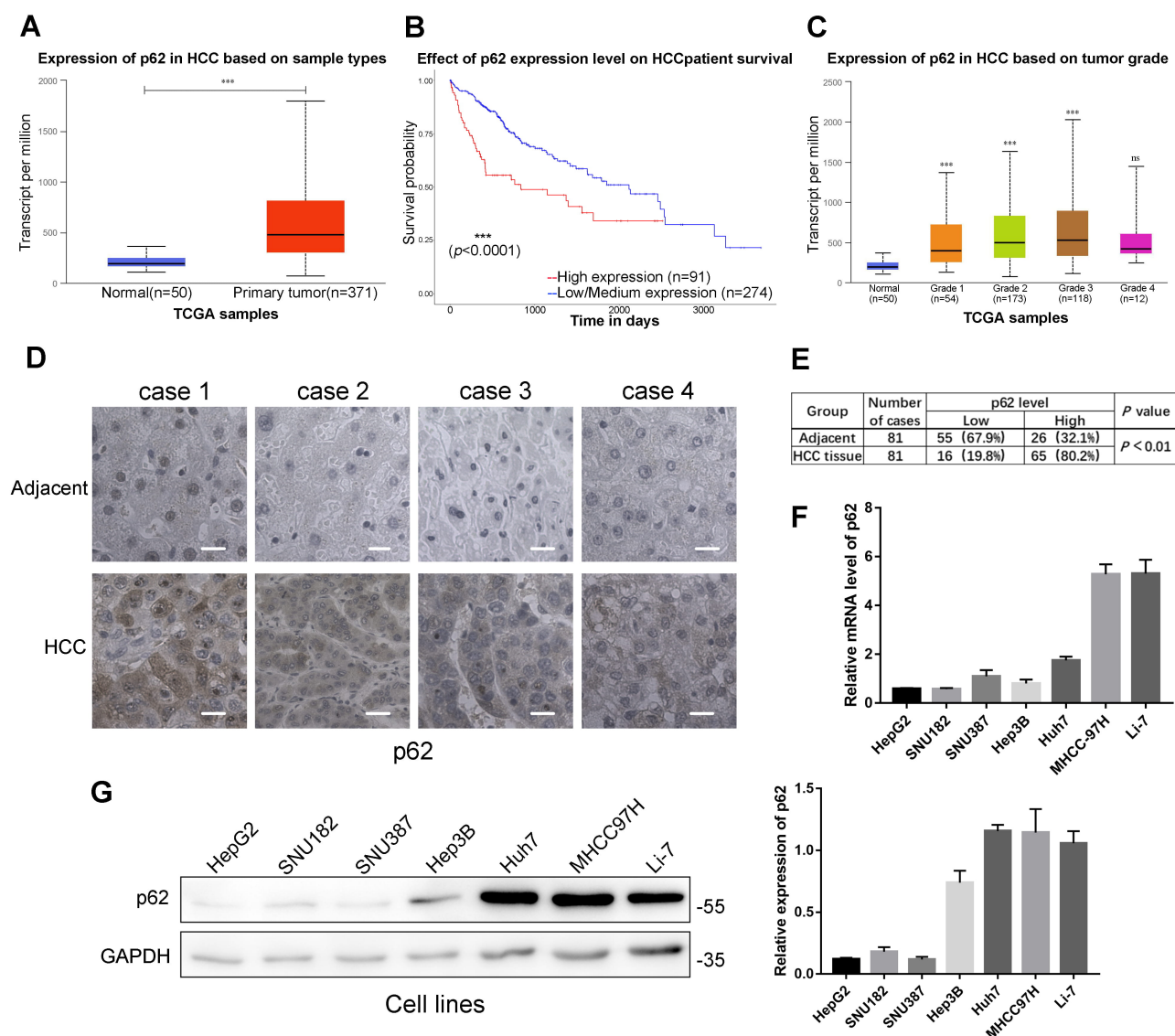
Fig. 1. p62 is highly expressed in HCC tissues and indicates poor prognosis in human HCC. (A) The expression of p62 in HCC based on sample types from TCGA dataset ($p < 0.001$). (B) The effect of p62 expression level on HCC patient survival ($p < 0.0001$). (C) The expression of p62 in HCC based on tumor grade from TCGA dataset (Grade 1, Well differentiated, n = 54, $p < 0.001$; Grade 2, Moderately differentiated, n = 173, $p < 0.001$; Grade 3, Poorly differentiated, n = 118, $p < 0.001$; Grade 4, Undifferentiated, n = 12, $p = 0.051$). (D) Representative images of IHC staining of p62 in 81 HCC tissues and adjacent non-tumor tissues. The high (down) and low (up) expression levels of p62 were evaluated semi-quantitatively by the staining intensity. Scale bars, 20 μm (high score: 7–12; low score: 0–6). (E) Chi-square analysis of the p62 levels in 81 HCC tissues and adjacent non-tumor tissues ($p < 0.01$). (F) Relative mRNA level of p62 in 7 human HCC cell lines. (G) The expression of p62 in 7 human HCC cell lines based on western blotting.

duced both nuclear and plasma membrane localization of β-catenin (Fig. 6A). Previous related studies, as well as other researchers, have proven that β-catenin can be packaged into exosomes [27,28]. The content of β-catenin in the exosomes of the p62-overexpressing group was higher than both that of the wild type group (WT) and the control group, suggesting that p62 can promote the entry of β-catenin into exosomes and release out of cells (Fig. 6B). Several key proteins of Wnt/β-catenin pathway including phosphorylated GSK3β^{Ser9}, active GSK3β^{Tyr216}, and to-

tal GSK3β were detected, and it appeared that the expression of total GSK3β was significantly upregulated after overexpressing p62 in HepG2 cells, in which the expression of active GSK3β^{Tyr216} increased mainly (Fig. 6C). A highly selective GSK3 inhibitor LY2090314 was then used to inhibit the activity of GSK3β. The results showed that 20 nM LY2090314 could significantly downregulate both total GSK3β and active GSK3β^{Tyr216}, while the expression of phosphorylated GSK3β^{Ser9} did not change significantly, and the expression of β-catenin was subsequently upregu-
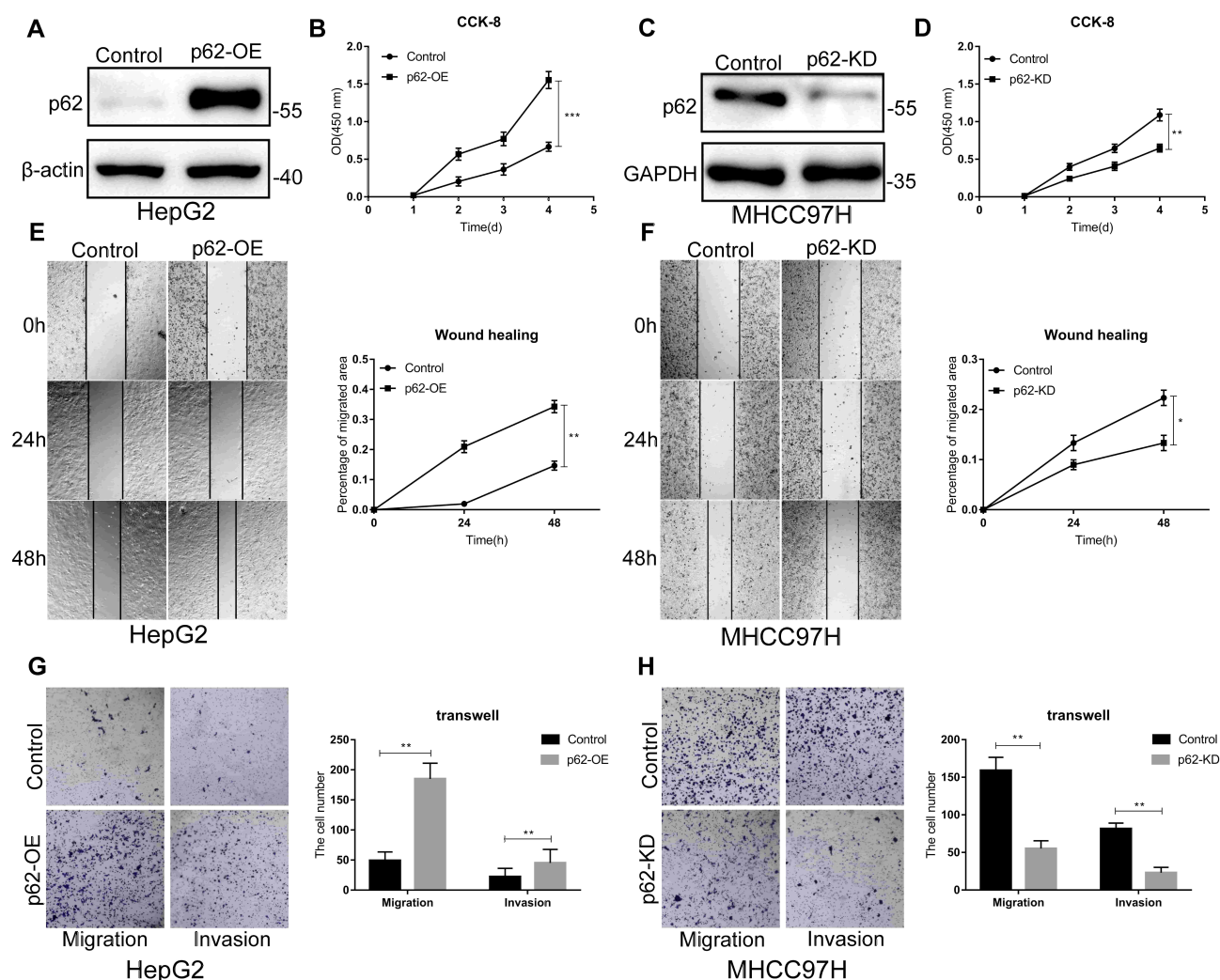
Fig. 2. p62 promotes cell proliferation, migration, and invasion abilities in HCC cells. (A) HepG2 was stably transduced with a p62-overexpression lentiviral vector (p62-OE), and the control group was transduced with the corresponding lentiviral carrying an empty vector (Control). (B) Overexpression of p62 significantly promoted cell proliferation in HepG2, according to CCK-8 assays, compared to that of the control groups (***$p < 0.001$). (C) MHCC97H was stably transduced with a p62-knockdown lentiviral vector (p62-KD), and the control group was transduced with the corresponding lentiviral carrying an empty vector (Control). (D) Knockdown of p62 significantly inhibited cell proliferation in MHCC97H, according to CCK-8 assays, compared to that of the control groups (**$p < 0.01$). (E) Overexpression of p62 promoted migration ability in HepG2 cells according to wound healing studies (**$p < 0.01$). (F) Knockdown of p62 inhibited migration ability in MHCC97H cells according to wound healing studies (*$p < 0.05$). (G and H) Transwell assays were used to evaluate the migration and invasion of the indicated cells. Bars represent the standard error of the mean $\pm$ SD from three independent experiments (**$p < 0.01$).

lated after using LY2090314 in p62-overexpressing HepG2 cells (Fig. 6D). To further confirm that β-catenin can be excreted through exosomes, 10 μM GW4869 was used to inhibit the formation of external vesicles. Although using GW4869 alone could not significantly increase the expression of β-catenin in p62-overexpressing HepG2 cells, the combination of GW4869 and LY2090314 could significantly increase the protein level of β-catenin in p62-overexpressing HepG2 cells (Fig. 6D).

3.7 p62 Promotes Tumor Growth of HCC and Down-Regulates the Expression of β-Catenin In Vivo

The experiments of subcutaneous exnograft tumor formation in nude mice were carried out to explore the function of p62 in HCC *in vivo*. The tumor volumes of nude mice were significantly increased in the mice injected with p62-overexpressing HepG2 cells compared with those injected with control HepG2 cells (Fig. 7A,B), concomitant with significantly greater tumor weights at the end of the animal experiments (Fig. 7C). Western blotting and IHC assays were used to detect the expression level of rele-
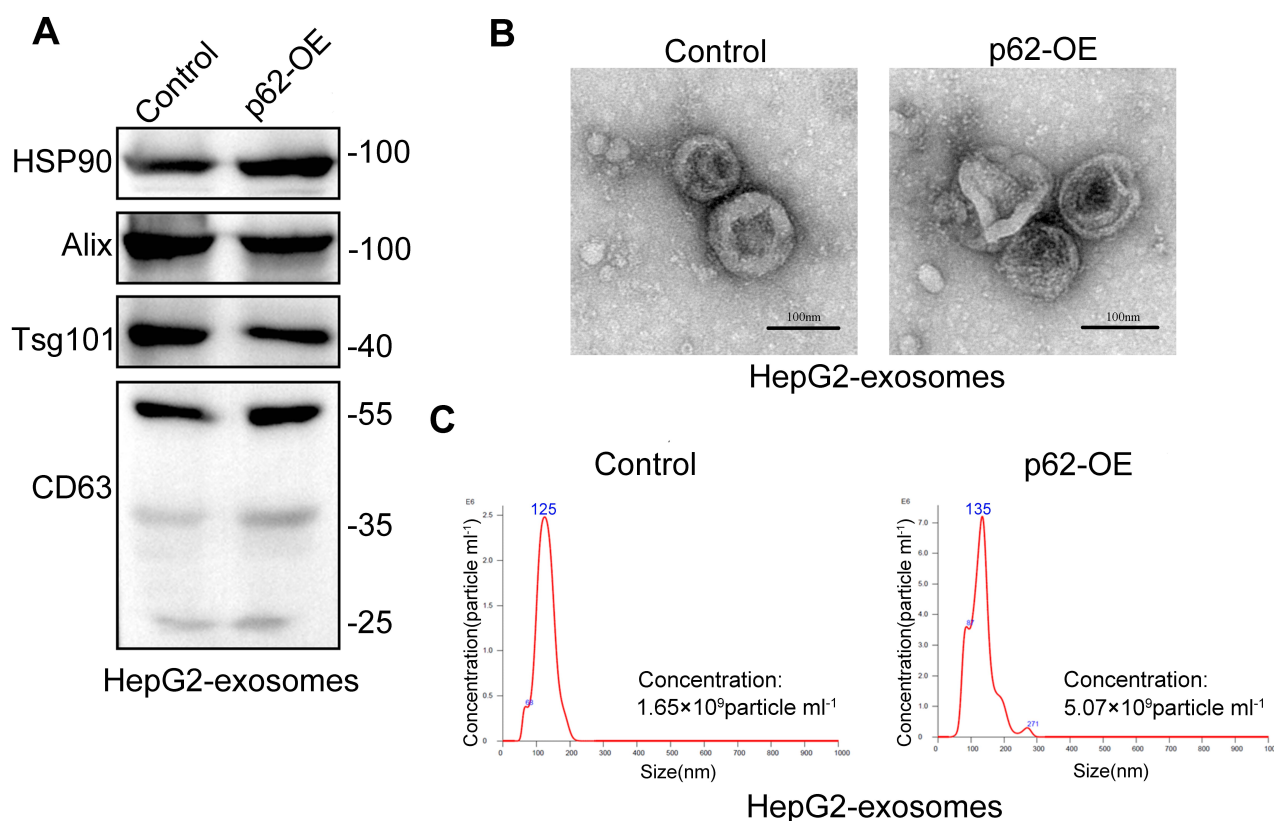
Fig. 3. p62 increases exosome secretion but does not affect the size of exosomes. (A) Immunoblotting showed the typical exosomal markers (HSP90, Alix, Tsg101, and CD63) in isolated exosomes. (B) Exosomes isolated from the culture medium of HepG2 control cells and p62-overexpressing cells by precipitation reagent were identified by Transmission Electron Microscopy. (C) Nanoparticle Tracking Analysis showed that the majority of isolated exosomes were about 130 nm, and the exosome concentration in the p62-overexpressing HepG2 group (5.07×10^9/mL) was higher than that in the control group (1.65×10^9/mL).

vant proteins in exnograft tumor tissues. The results of western blotting and IHC showed that the expression level of p62 was higher in exnograft tumors injected with p62-overexpressing HepG2 cells than those injected with control HepG2 cells while the expression level of β-catenin was lower in exnograft tumors injected with p62-overexpressing HepG2 cells than those injected with control HepG2 cells (Fig. 7D,E). These results indicated that p62 promotes tumor growth of HCC and down-regulates the expression of β-catenin *in vivo*.

4. Discussion

As a key protein of autophagy, p62 is indispensable in the growth and development of organisms [4]. Moreover, because p62 has multiple domains, it can participate in many signal pathways, affecting not only cell selective autophagy, but also metabolic reprogramming, antioxidation and so on [5]. In recent years, SQSTM1/p62 has been reported in many kinds of tumors as an oncogene, and its importance has been given increasing attention [6–10]. However, the mechanism of p62 in promoting cancer invasion and metastasis remains unclear.

Based on the TCGA dataset and clinical samples obtained from the center, the data confirmed that p62 was highly expressed in HCC tissues and highly metastatic HCC cell lines. Further experiments showed that by overexpressing p62 in HepG2 cells, the abilities of cell growth, mobility, and invasiveness were promoted, while the opposite results we got in p62-knockdown MHCC97H cells. This effect of regulating cancer biological behaviors was supported by previous reports [29,30]. In a previous related study on Vps4A, a key protein of endosomal sorting complex required for transport (ESCRT), an interaction was observed between Vps4A and p62 in mass spectrometry (**Supplementary Table 3**). Thus, the current study boldly speculated that p62 may affect the exosome pathway to regulate the secretion of cytoplasmic materials [27]. The overexpression of p62 can increase the concentration but not change the size of exosomes in HepG2 cells according to the results of NTA and TEM. The recipient HepG2 cells incubated with exosomes derived from both control and p62-overexpressing HepG2 cells showed enhanced cell proliferation, migration, and invasion abilities. Previous related studies also supported these results [26]. In the cur-
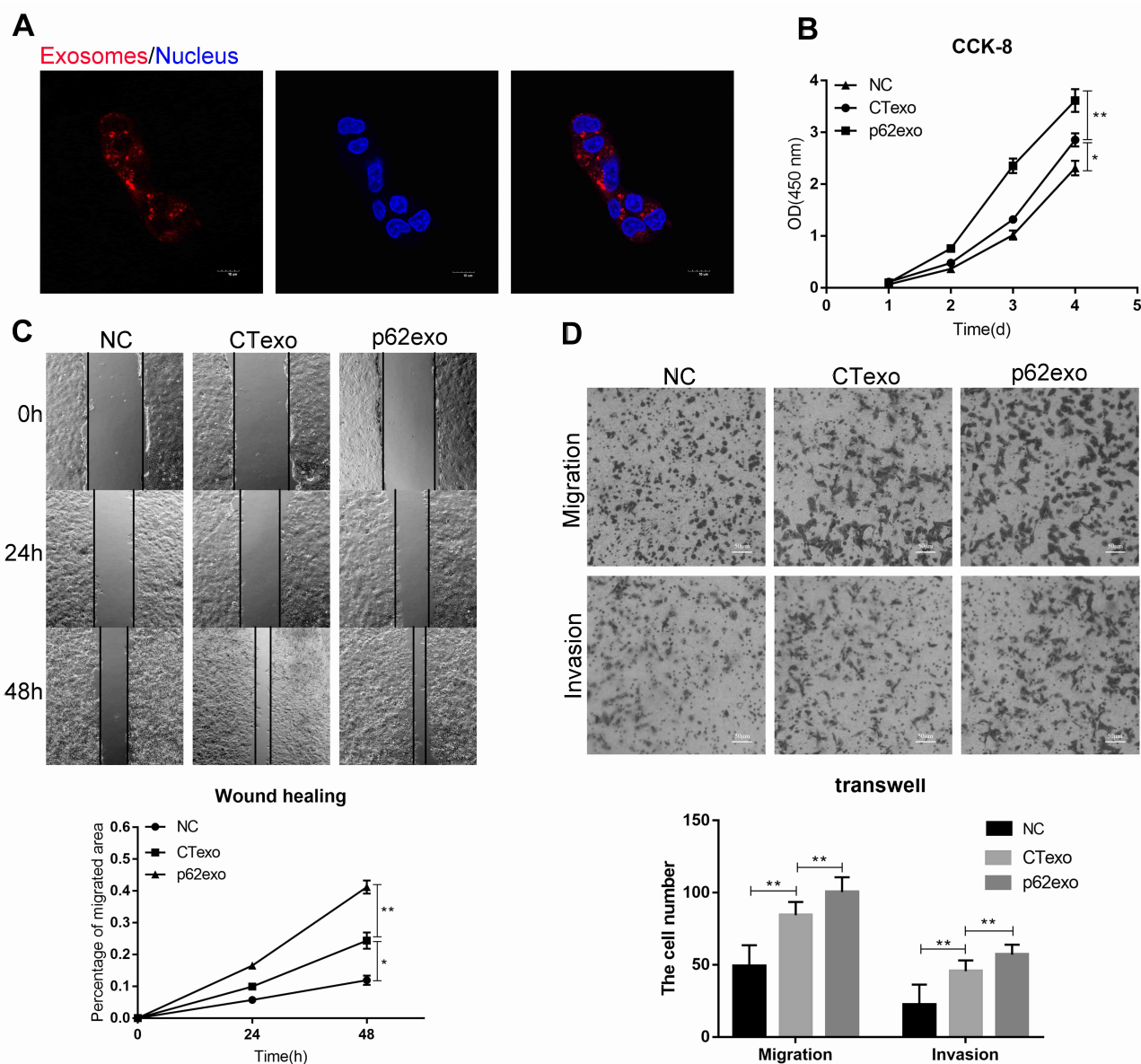
Fig. 4. Exosomes derived from p62-overexpressing HepG2 cells promote the proliferation, migration, and invasion of recipient cells. (A) The recipient HepG2 cells were incubated with N-Rh-PE labeled exosomes for 24 h and observed under a confocal microscope (Red, N-Rh-PE, Exosomes; Blue, DAPI, Nucleus). (B) Cell proliferation (CCK-8) assays were performed using HepG2 cells cocultured with exosomes derived from HepG2 control cells and p62-overexpressing cells (*$p < 0.05$, **$p < 0.01$). (C,D) Wound healing assays and transwell assays were used to evaluate the migration and invasion of the indicated cells cocultured with exosomes derived from HepG2 control cells and p62-overexpressing cells. Bars represent the standard error of the mean $\pm$ SD from three independent experiments (Represents Student's t-test, *$p < 0.05$, **$p < 0.01$).

rent study, the exosomes derived from p62-overexpressing HepG2 cells had a stronger promoting effect than in the control group, suggesting that p62 could strengthen the oncogenic behavior of HCC cells via exosomes. However, the mechanism of how p62 finely regulates the exosomes pathway needs further research.

In previous related studies, β-catenin was found to regulate the process of cancer invasion and metastasis depend on its localization [27]. We confirmed the interaction and colocalization between p62 and β-catenin, according to the results of co-immunoprecipitation and immunofluorescence assays. These results have not been mentioned in previous studies on the cascade of autophagy and the Wnt pathway [31,32]. Further experiments showed that the expression of β-catenin was down-regulated after overexpressing p62 in HepG2 cells. Moreover, the overexpression of p62 changed the intercellular morphology from tight to loose. The decrease in stickiness between tumor
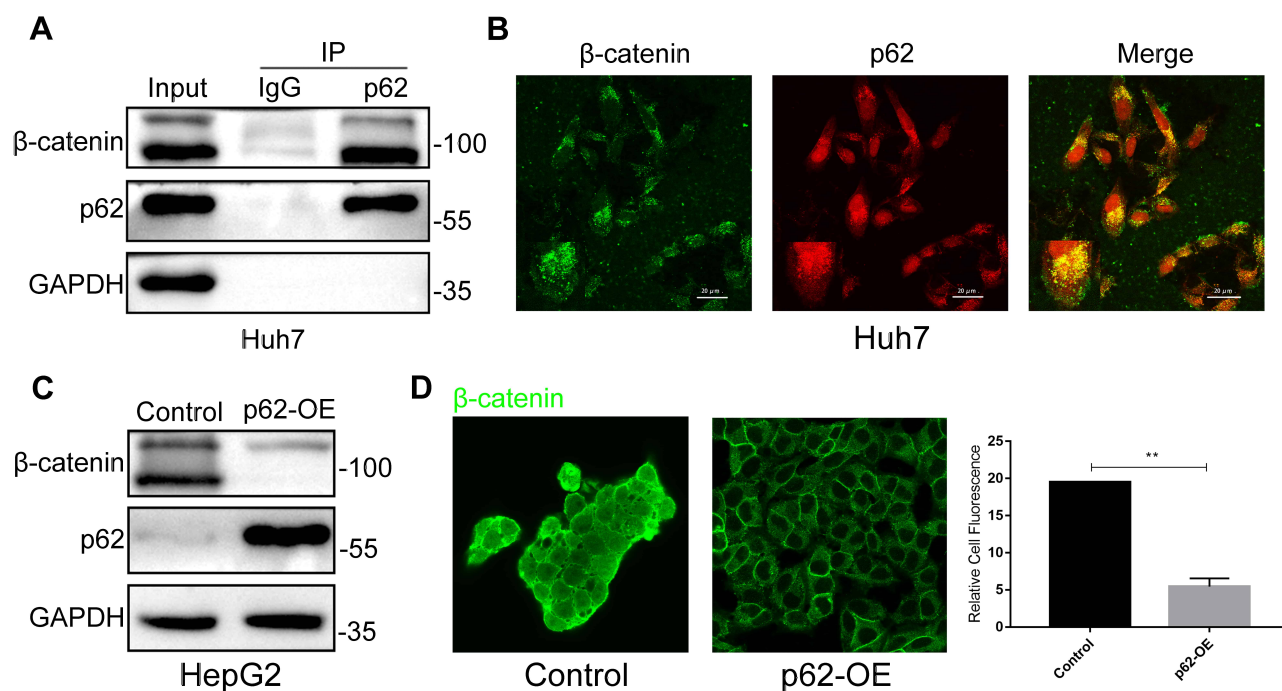
Fig. 5. p62 interacts with β-catenin and down-regulates the expression of β-catenin in HCC cells. (A) p62 interacts with endogenous β-catenin. Immunoprecipitation assays using a monoclonal anti-p62 antibody were performed in Huh7 cells, and immunoblotting analyses were carried out using the indicated antibodies. (B) p62 colocalizes with β-catenin. Huh7 cells stained with anti-β-catenin antibody and anti-p62 antibody were analyzed by confocal microscopy. (C) Western blotting showed that the expression of β-catenin was down-regulated after overexpressing p62 in HepG2 cells. (D) Immunofluorescence assays were used to observe the expression and localization of β-catenin. Under the same conditions, the fluorescence quantification of β-catenin of HepG2 control cells and p62-overexpressing cells were observed by confocal microscopy. Bars represent the standard error of the mean $\pm$ SD from three independent experiments (**$p < 0.01$).

cells contributes to invasion and metastasis [25,33]. Subsequently, immunofluorescence assays were used to confirm that p62 can regulate the intracellular localization of β-catenin. Overexpression of p62 reduced the protein level of β-catenin in both the nucleus and membrane.

The expression of exosomal β-catenin were upregulated after overexpressing p62 in HepG2 cells according to the results of western blotting. It has been shown that p62 mediates the exosome pathway to increase the secretion of β-catenin. Although many factors can affect the degradation of β-catenin, the protein GSK3β is considered as an important regulator in this process [34,35]. The overexpression of p62 upregulates the total GSK3β and active GSK3β^{Tyr216}, resulting in increased degradation of β-catenin, while there is no significant change in the expression of phosphorylated GSK3β^{Ser9}. To confirm this effect, LY2090314, a specific inhibitor of GSK3, was used to inhibit the activity of GSK3β [36]. The results showed that reducing the active form of GSK3β (GSK3β^{Tyr216}) could indeed antagonize the effect of p62 on β-catenin. Moreover, the combined application of LY2090314 and external vesicle formation inhibitor GW4869 significantly increased the content of β-catenin in HepG2 cells, which fur-

ther confirmed the conclusion that β-catenin could be secreted via exosomes [28]. As a multifunctional protein, p62 still has many uncertainties in its molecular mechanism. Some scholars have pointed out that p62 can increase the degradation of GSK3β in glioblastoma cells [37], which seems to be contrary to our experimental results. Some scholars have found that GSK3β can enhance the radiosensitivity by inhibiting autophagy in non-small cell lung cancer [38]. The relationship between p62 and GSK3β may not be a simple linear relationship, and more in-depth mechanisms need to be investigated in further research.

We also established the HCC xenograft tumors in nude mice by using the p62-overexpressing HepG2 cells and the control HepG2 cells. The tumor growth curve and the histogram of tumor weight showed that overexpression of p62 in HepG2 aggravates xenograft tumor growth. Meanwhile, the expression level of p62 and β-catenin in xenograft tumors were detected by western blotting and IHC, the results showed that p62 down-regulates the expression of β-catenin *in vivo*, which further confirm the findings in our previous *in vitro* experiments.

At the same time, the level of EMT-related markers including Vimentin, Twist1, and Snail were increased af-
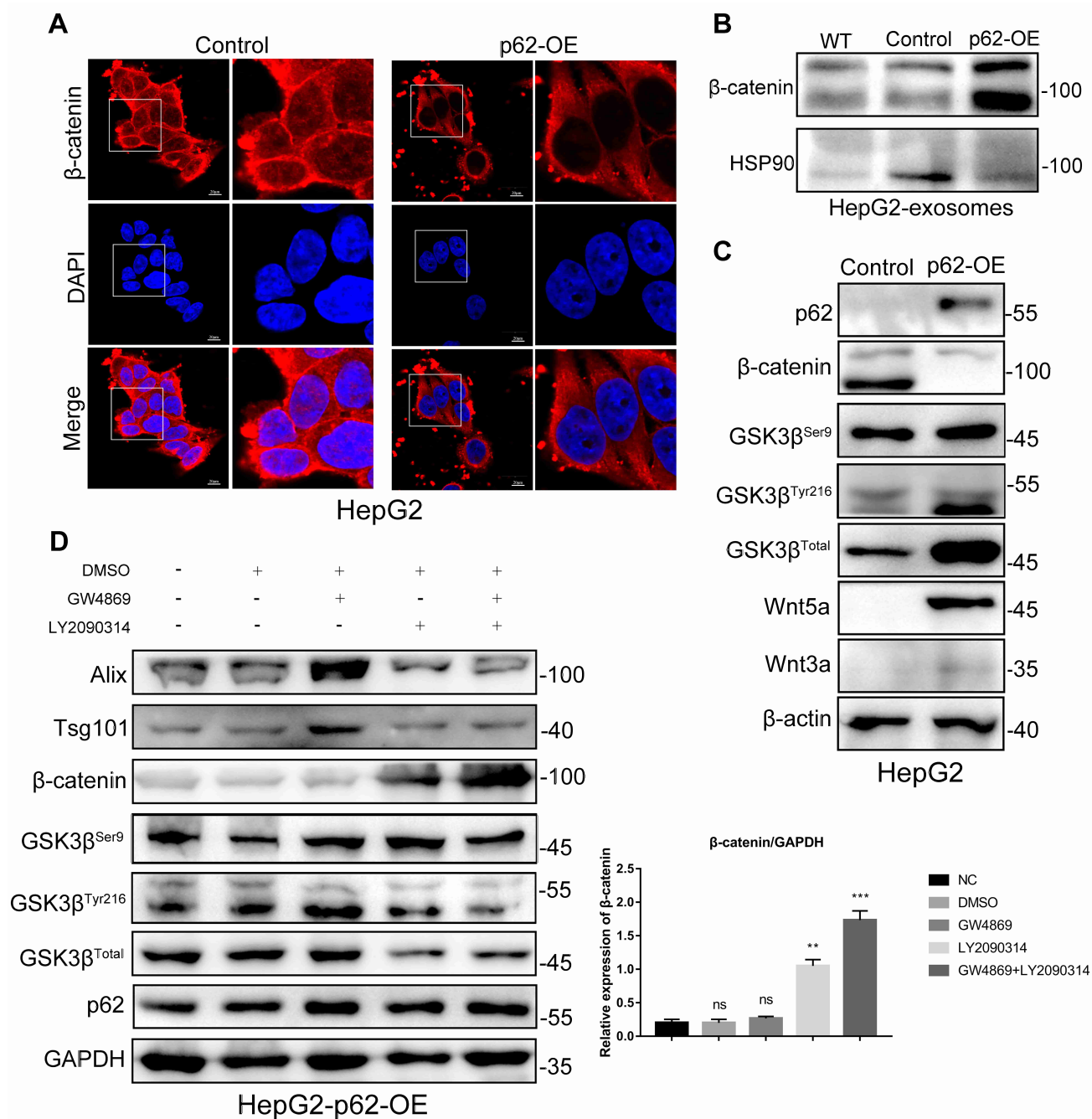
Fig. 6. p62 regulates the expression and localization of β-catenin in the cells and exosomes. (A) Control HepG2 group (Control) and p62-overexpressing group (p62-OE) were immunostained with anti-β-catenin antibody and analyzed by confocal microscopy. (B) The exosomes derived from the cell supernatant of the HepG2 wild type group (WT), the control group (Control) and the p62-overexpressing group (p62-OE), respectively. Western blotting showed that overexpression of p62 increased the expression of β-catenin in exosomes. (C) Detection of related proteins in the Wnt/β-catenin pathway by western blotting. (D) GW4869 and LY2090314 were used to inhibit the secretion of exosomes and activity of GSK3β, respectively, and western blotting was used to detect the changes of β-catenin in p62-overexpressing HepG2 cells. Bars represent the standard error of the mean $\pm$ SD from three independent experiments (ns = not significant, **$p < 0.01$, ***$p < 0.001$).

ter overexpressing p62, while the expression of E-cadherin was down-regulated (**Supplementary Fig. 2**). As we know that, β-catenin can regulate EMT by changing its localiza-

tion [25,39]. When the nuclear localization of β-catenin is increased, it can promote EMT of cancer cells. On the contrary, when the membrane accumulation of β-catenin is in-
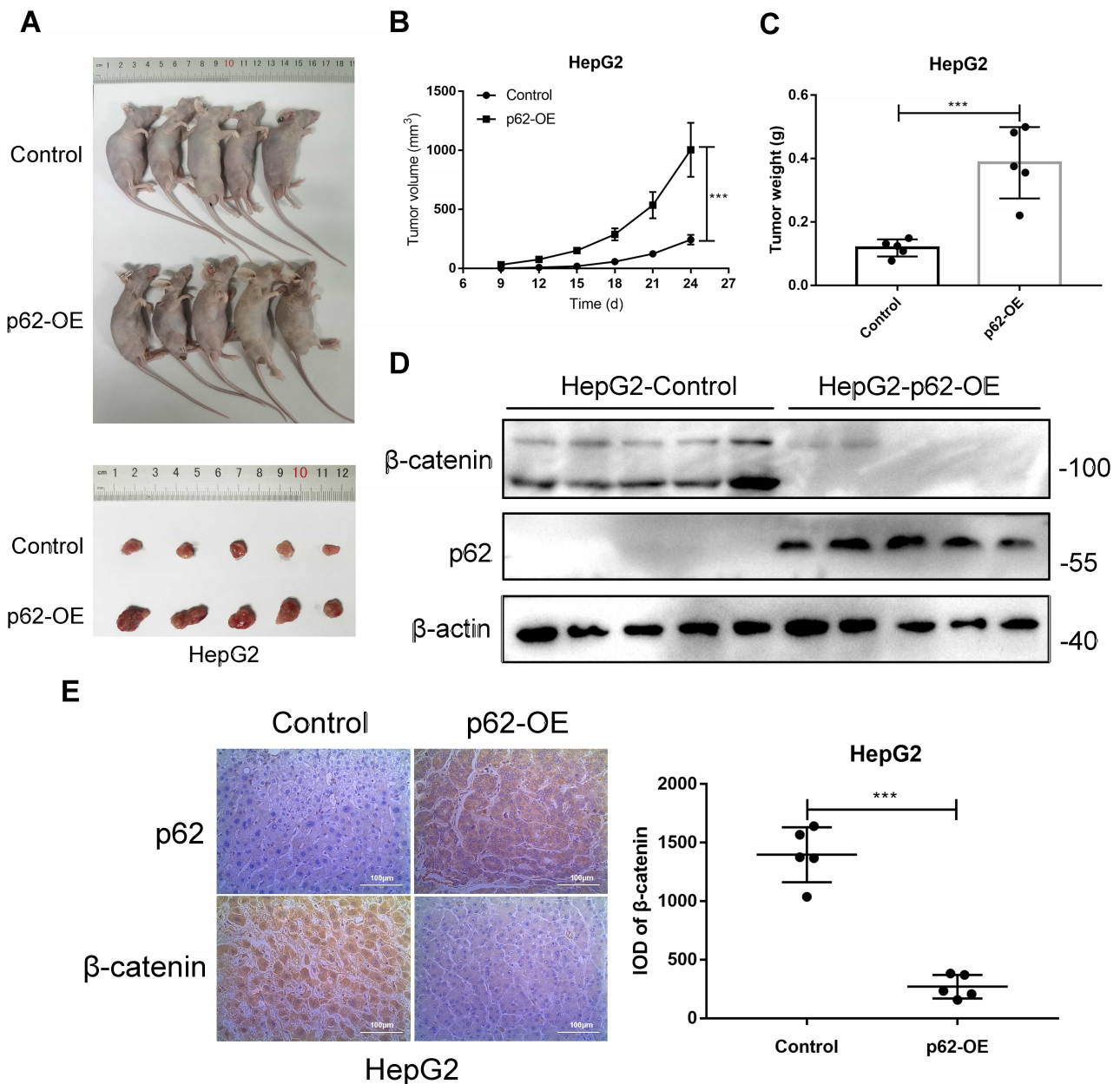
Fig. 7. p62 promotes tumor growth of HCC and down-regulates the expression of β-catenin *in vivo*. (A) Images of HepG2 xenograft tumors showed that the overexpression of p62 promotes the tumor growth in nude mice (n = 5) compared to that of the control group (n = 5). (B) The xenograft tumor growth curve of p62-overexpressing HepG2 and control HepG2 (***$p < 0.001$). (C) The histogram of tumor weight showed that the overexpression of p62 in HepG2 cells formed larger xenografts compared with those of the control group (***$p < 0.001$). (D) Expression of β-catenin and p62 in HepG2 xenograft tumors were detected by western blotting. (E) IHC demomstrated that the expression level of β-catenin in p62-overexpressing HepG2 xenograft tumors (n = 5) was lower than that in control HepG2 xenograft tumors (n = 5). Scale bar = 100 μm. (***$p < 0.001$).

creased, it can inhibit the abilities of invasion and metastasis by promoting the adhesion between cancers cells [23,24]. Surprisingly, after overexpressing p62 in HepG2 cells, although the level of EMT was enhanced, β-catenin did not accumulate in the nucleus but down-regulated, whether in the nucleus or in the cytoplasm. The results suggested that p62 can promote the level of EMT in HCC but not by

activating the Wnt/β-catenin pathway, and more in-depth mechanisms need further research.

5. Conclusions

Taken together, this study demonstrated that p62 functions as an oncogene to promote cell proliferation, migration, and invasion abilities in HCC cell lines by regulating

the cell structure and secretion of exosomes. p62 downregulate the expression of β-catenin to weaken the intercellular junction. Moreover, the results of *in vivo* experiments also corroborate our findings. In addition, detection of the intact composition of exosomes will also need to be investigated in further research. These results reveal an important role of p62 in the regulation of β-catenin, which may provide new aspects for the diagnosis and treatment of HCC.

Abbreviations

HCC, hepatocellular carcinoma; EMT, epithelial-mesenchymal transition; N-Rh-PE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl); TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis; ESCRT, endosomal sorting complex required for transport.

Author contributions

WY, JM and PL designed the research study. WY, JW, ZZ and WZ performed the research. WY, LL, AL, and JX analyzed the data. All authors contributed to the manuscript writing and revision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). All enrolled patients signed informed consent, and the study was approved by the ethics committee of the Sun Yat-sen Memorial Hospital, Sun Yat-sen University [approval number: 2017(107)].

Acknowledgment

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/27/3/10.31083/j.fbl2703089.

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA-A Cancer Journal for Clinicians. 2018; 68: 394–424.

[2] Villanueva A. Hepatocellular Carcinoma. New England Journal of Medicine. 2019; 380: 1450–1462.

[3] Kulik L, El-Serag HB. Epidemiology and Management of Hepatocellular Carcinoma. Gastroenterology. 2019; 156: 477–491.e1.

[4] Moscat J, Diaz-Meco MT. p62 at the crossroads of autophagy, apoptosis, and cancer. Cell. 2009; 137: 1001–1004.

[5] Moscat J, Karin M, Diaz-Meco MT. p62 in Cancer: Signaling Adaptor beyond Autophagy. Cell. 2016; 167: 606–609.

[6] Zhang J, Yang S, Xu B, Wang T, Zheng Y, Liu F, et al. p62 functions as an oncogene in colorectal cancer through inhibiting apoptosis and promoting cell proliferation by interacting with the vitamin D receptor. Cell Proliferation. 2019; 52: e12585.

[7] Li SS, Xu L, Zhou W, Yao S, Wang C, Xia J, et al. p62/SQSTM1 interacts with vimentin to enhance breast cancer metastasis. Carcinogenesis. 2017; 38: 1092–1103.

[8] Jiang X, Huang Y, Liang X, Jiang F, He Y, Li T, et al. Metastatic prostate cancer-associated P62 inhibits autophagy flux and promotes epithelial to mesenchymal transition by sustaining the level of HDAC6. The Prostate. 2018; 78: 426–434.

[9] Li T, Jiang D, Wu K. p62 promotes bladder cancer cell growth by activating KEAP1/NRF2-dependent antioxidative response. Cancer Science. 2020; 111: 1156–1164.

[10] Pölönen P, Jawahar Deen A, Leinonen HM, Jyrkkänen HK, Kuosmanen S, Mononen M, et al. Nrf2 and SQSTM1/p62 jointly contribute to mesenchymal transition and invasion in glioblastoma. Oncogene. 2019; 38: 7473–7490.

[11] Umemura A, He F, Taniguchi K, Nakagawa H, Yamachika S, Font-Burgada J, et al. p62, Upregulated during Preneoplasia, Induces Hepatocellular Carcinogenesis by Maintaining Survival of Stressed HCC-Initiating Cells. Cancer Cell. 2016; 29: 935–948.

[12] Denk H, Stumptner C, Abuja PM, Zatloukal K. Sequestosome 1/p62-related pathways as therapeutic targets in hepatocellular carcinoma. Expert Opinion on Therapeutic Targets. 2019; 23: 393–406.

[13] Shao H, Im H, Castro CM, Breakefield X, Weissleder R, Lee H. New Technologies for Analysis of Extracellular Vesicles. Chemical Reviews. 2018; 118: 1917–1950.

[14] Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nature Reviews Immunology. 2002; 2: 569–579.

[15] Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annual Review of Cell and Developmental Biology. 2014; 30: 255–289.

[16] Yáñez-Mó M, Siljander PR, Andreu Z, Zavec AB, Borràs FE, Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. Journal of Extracellular Vesicles. 2015; 4: 27066.

[17] Becker A, Thakur BK, Weiss JM, Kim HS, Peinado H, Lyden D. Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. Cancer Cell. 2016; 30: 836–848.

[18] Syn N, Wang L, Sethi G, Thiery J, Goh B. Exosome-Mediated Metastasis: From Epithelial-Mesenchymal Transition to Escape from Immunosurveillance. Trends in Pharmacological Sciences. 2016; 37: 606–617.

[19] Lee SC, Tan HT, Chung MC. Prognostic biomarkers for prediction of recurrence of hepatocellular carcinoma: current status and future prospects. World Journal of Gastroenterology. 2014; 20: 3112–3124.

[20] Stamos JL, Weis WI. The beta-catenin destruction complex. Cold Spring Harbor Perspectives in Biology. 2013; 5: a007898.

[21] Wu H, Lu XX, Wang JR, Yang TY, Li XM, He XS, et al. TRAF6 inhibits colorectal cancer metastasis through regulating selective autophagic CTNNB1/β-catenin degradation and is targeted for GSK3B/GSK3β-mediated phosphorylation and degradation. Autophagy. 2019; 15: 1506–1522.

[22] Bernaudo S, Salem M, Qi X, Zhou W, Zhang C, Yang W, *et al*. Cyclin G2 inhibits epithelial-to-mesenchymal transition by disrupting Wnt/β-catenin signaling. Oncogene. 2016; 35: 4816–4827.

[23] Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. Nature Reviews Molecular Cell Biology. 2014; 15: 178–196.

[24] Giannelli G, Koudelkova P, Dituri F, Mikulits W. Role of epithelial to mesenchymal transition in hepatocellular carcinoma. Journal of Hepatology. 2016; 65: 798–808.

[25] Basu S, Cheriyamundath S, Ben-Ze'ev A. Cell-cell adhesion: linking Wnt/beta-catenin signaling with partial EMT and stemness traits in tumorigenesis. F1000Research. 2018; 7: 1488.

[26] Wei JX, Lv LH, Wan YL, Cao Y, Li GL, Lin HM, *et al*. Vps4a functions as a tumor suppressor by regulating the secretion and uptake of exosomal microRNAs in human hepatoma cells. Hepatology. 2015; 61: 1284–1294.

[27] Han Q, Lv L, Wei J, Lei X, Lin H, Li G, *et al*. Vps4a mediates the localization and exosome release of β-catenin to inhibit epithelial-mesenchymal transition in hepatocellular carcinoma. Cancer Letters. 2019; 457: 47–59.

[28] Chairoungdua A, Smith DL, Pochard P, Hull M, Caplan MJ. Exosome release of β-catenin: a novel mechanism that antagonizes Wnt signaling. Journal of Cell Biology. 2010; 190: 1079–1091.

[29] Saito T, Ichimura Y, Taguchi K, Suzuki T, Mizushima T, Takagi K, *et al*. P62/Sqstm1 promotes malignancy of HCV-positive hepatocellular carcinoma through Nrf2-dependent metabolic reprogramming. Nature Communications. 2016; 7: 12030.

[30] Taniguchi K, Yamachika S, He F, Karin M. p62/SQSTM1-Dr. Jekyll and Mr. Hyde that prevents oxidative stress but promotes liver cancer. FEBS Letters. 2016; 590: 2375–2397.

[31] Gao C, Cao W, Bao L, Zuo W, Xie G, Cai T, *et al*. Autophagy negatively regulates Wnt signalling by promoting Dishevelled degradation. Nature Cell Biology. 2010; 12: 781–790.

[32] Petherick KJ, Williams AC, Lane JD, Ordóñez-Morán P, Huelsken J, Collard TJ, *et al*. Autolysosomal β-catenin degradation regulates Wnt-autophagy-p62 crosstalk. EMBO Journal. 2013; 32: 1903–1916.

[33] Heuberger J, Birchmeier W. Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. Cold Spring Harbor Perspectives in Biology. 2010; 2: a002915.

[34] Sempou E, Biasini E, Pinzón-Olejua A, Harris DA, Málaga-Trillo E. Activation of zebrafish Src family kinases by the prion protein is an amyloid-β-sensitive signal that prevents the endocytosis and degradation of E-cadherin/β-catenin complexes *in vivo*. Molecular Neurodegeneration. 2016; 11: 18.

[35] Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. Nature Reviews Cancer. 2013; 13: 11–26.

[36] Kunnimalaiyaan S, Schwartz VK, Jackson IA, Clark Gamblin T, Kunnimalaiyaan M. Antiproliferative and apoptotic effect of LY2090314, a GSK-3 inhibitor, in neuroblastoma *in vitro*. BMC Cancer. 2018; 18: 560.

[37] Li H, Li J, Zhang G, Da Q, Chen L, Yu S, *et al*. HMGB1-Induced p62 Overexpression Promotes Snail-Mediated Epithelial-Mesenchymal Transition in Glioblastoma Cells via the Degradation of GSK-3β. Theranostics. 2019; 9: 1909–1922.

[38] Ren J, Liu T, Han Y, Wang Q, Chen Y, Li G, *et al*. GSK-3β inhibits autophagy and enhances radiosensitivity in non-small cell lung cancer. Diagnostic Pathology. 2018; 13: 33.

[39] McCrea PD, Maher MT, Gottardi CJ. Nuclear signaling from cadherin adhesion complexes. Current Topics in Developmental Biology. 2015; 53: 129–196.