Research Square

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IL-2 Increases Tams-Derived Exosomal Mir-375 to Ameliorate Hepatocellular Carcinoma Development and Progression

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Research Article

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Abstract

Background: Interleukin-2 (IL-2) is proved to play an irreplaceable role in anti-tumor regulation in numerous experimental and clinical trials. Tumor-associated macrophages (TAMs) is able to release exosomes to promote the development and progression of hepatocellular carcinoma (HCC) as part of microenvironment.

Methods: In this study, our intention is to explore the effects of the exosomes from TAMs with IL-2 treatment on HCC development.TAMs were collected and cultured from liver cancer tissues. The exosomes from the TAMs treated with IL-2 ($Exo^{IL2\text{-}TAM}$) or not (Exo^{TAM}) were identified and used to treat HCC cells. The HCC cells proliferation, apoptosis and metastasis were measured *in vivo* and *in vitro*. The changes of miR-375 in exosomes was explored to clarify whether it is reponsible to the anti-apoptotic effects of IL-2.

Results: Both decrease of cell proliferation and metastasis and increase of apoptosis were observed with $Exo^{IL2\text{-}TAM}$ treatment compared with Exo^{TAM} *in vivo* and *in vitro*. miR-375 was obviously abundant in $Exo^{IL2\text{-}TAM}$. Enriched miR-375 could be transmitted between TAMs and HCC cells via exosomes and was responbible for the increased apoptosis of HCC cells.

Conclusions: Taken together, IL-2 increases exosomal miR-375 from TAMs to attenuate hepatocellular carcinoma development. This study provides a new perspective to explain the mechanism how IL-2 inhibits hepatocellular carcinoma and implys the potential clinical value of exosomal miR-375 released by TAMs.

Background

Primary hepatocellular carcinoma (HCC) is the third most common malignant tumor in cancer mortality[1]. The treatment of HCC is mainly surgical resection and liver transplantation[2]. Because of its high degree of malignancy, the five-year survival rate is still less than 50% and the recurrence rate is high. Recurrence and metastasis have become the biggest obstacle to improve the therapeutic effect and survival rate of liver cancer[3]. The occurrence, development, invasion and metastasis of tumors are not only determined by the malignant tumor cells themselves, but also closely related to the tumor microenvironment[4–6].

It has been proved that there are a large number of tumor-associated macrophages (TAMs) in tumor microenvironment. Recent studies have confirmed that TAMs are mainly M2 type macrophages, which can promote tumor growth by affecting angiogenesis, immunosuppression, invasion and metastasis[7–9]. Exosomes are membrane vesicles with a diameter of 30–150 nm[10].Cells can secrete and widely exist in various body fluids. Exosomes carries RNA and protein components of parental cells and are involved in signal transduction and immune escape of tumor and the diagnosis and treatment of some diseases[11,12]. Compared with adjacent normal tissues, the expression levels of miRNAs changed in

many malignant tumors[1]. It has been reported that the exosomes derived from TAMs promoted HCC cell proliferation and metastasis[13–16].

IL-2 is a multifunctional cytokine in the immune system which effectively activates and enhances the phagocytosis and killing ability of macrophages[17,18]. IL-2 have been used in lots of clinical trials for the treatment of HCC[2]. However, it remains unkown whether IL-2 regulates the exosomes released from TAMs. Given the emerging role of IL-2 in cute HCC and regulating the function of macrophages, this study is aimed to explore the effects of exosomes from IL-2 treated TAMs on HCC development and the possible mechanisms.

Materials And Methods

Cell lines and reagents

Huh7, HepG2 and QJY-7703 cells cells were purchased from TongPai Biotechnology (Shanghai, China) and were maintained in Dulbecco's Modified Eagle Medium (DMEM, WISENT, CA, USA) containing 10% fetal bovine serum (FBS) (ExCell Bio, China). Recombinant human IL-2 was purchased fromSigma-Aldrich LLC.

Patients and Primary human TAMs isolation and culture from tissue specimens

Primary human HCC specimens were collected from the patients who suffered from hepatectomy at Yijishan Hospital of Wannan Medical College. Patient's consent was obtained and the procedures were approved by the Ethics Committee of Yijishan Hospital. Human fresh tumor samples were minced with scissors. The macrophages were isolated and cultured as previously described (refer to Tumor Microenvironment Study Protocols by Springer). TAMs were treated with (20ng/ml) for 24 hours before the supernatants were collected.

Isolation and labelling of exosomes

Exosomes were isolated from the cell culture media with Total Exosome Isolation Reagent (Thermo Scientific) according to the manufacturer's instructions. The purified exosomes were then labelled with the red fluorescent linker PKH26 (Umibio) according to the manufacturer's directions.

Exosomes and TAMs cells observed by transmission electron microscopy

The samples were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer at pH 7.3 for 3 hours at room temperature. After air drying, samples were mounted on specimen stubs and visualized using transmission electron microscope.

RNA isolation and qPCR

Total RNA was isolated from cells or mouse tissues using Trizol reagent (Invitrogen), following the manufacturer's instructions. The RNA was then analysed using real-time qPCR with SYBR Green PCR

Master mix (Roche Applied Science, Mannheim, Germany). The relative gene expression was normalized to U6.

agomiR and antagomiR transfection

The constructs of miR-375 from GenePharma (Shanghai) were utilized, and transfection was performed at a concentration of 200 × 10^{-3} M concentration using Lipofectamine 3000.

Western blot analysis

Exosomes or cells were lysed in RIPA containing protease inhibitors. A total of 20 μg of exosomes were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were then incubated with antibodies CD63 (1:1000; Abcam, Cambridge, MA, USA), Calnexin (1:1000; CST,USA), PCNA (1:1000; CST, USA), cyclin D1 (1:1000; CST, USA) E-cadherin (1:1000; CST, USA), N-cadherin (1:1000; CST, USA), tubulin (1: 5000, Abcam, Cambridge, UK),Bax (1:1000; CST,USA), Bcl-2 (1:1000; CST,USA), MMP-2 (1: 1000, Proteintech, Chicago, Illinois, USA), and MMP-9 (1: 1000, Proteintech, Chicago, Illinois, USA).

Quantification of apoptosis by flow cytometry

Apoptosis was determined using an Annexin V-FITC/PI apoptosis detection kit (eBioscience, USA). For both approaches cells were assessed via flow cytometry.

CCK8 assay

HepG2 cells were added to 96-well plates and cultured for 24, 48, 72, 96, 120 or 144 hours. Then, CCK-8 reagent was added to cells in serum-free medium for 2 hours, followed by measurements of absorbance at 450 nm.

EDU Assays

For EDU assays, HepG2 Cells were added to 24-well plates, and after incubation after 24 h with exosomes , EDU (Sigma-Aldrich) staining was conducted based on the protocols.

Migration assay

Cell invasion assays were conducted on 24-well Transwell cell culture chambers with 8-μm sized pores without precoated Matrigel (Corning, USA). HepG2 cells were suspended in 500 μl of medium and added to the upper inserts. After 24 hour of incubation, the cells remaining in the upper chamber were removed, and the cells on the lower surface of the chamber were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet.

Scratch test

A scratch assay was performed to assess cell migration *in vitro*. First, HepG2 cells were seeded in 6-well plates until a confluent monolayer was formed. Then, upon confluence, cells were scratched with a 10 μL sterile pipette tip. Pictures were then taken of the scratch at different time-points under the microscope. The cell migration rate was calculated as (width at 0 hours–width at 24 hours)/width at 0 hours.

Experimental animals

Xenograft mouse models

All animal experiments were performed in accordance with the approval of the Animal Ethics Committee of Youjiang Medical University for Nationalities. QJY-7703 cells in logarithmic growth phase were prepared into cell suspension. Axillary subcutaneous injection of cells (5×10^5) was performed BALB/c nude mice (4-6 weeks-old, n = 6 per group) accompanied with exosomes (40 ug/mL) injection via the tail vein every 2 days for 7 times. Tumor volumes were measured after 5 days every 10 days until mice were sacrificed 30 days later.

The liver and lung metastasis experiment

The 6–8 weeks old nude mice were divided into three randomized groups (n = 12 per group), and QJY-7703 (5×10^5) alone or with Exo^{TAM} (40 ug/mL) or with $Exo^{TAM\text{-}IL2}$ (40 ug/mL) in 100 ul were injected into the mice via tail vein every 2 days for 7 times. 30 days after cell injection, the mice were euthanized and were necropsied to assess metastatic burden. The tumor tissues, liver and lung tissues of mice were further examined by H&E and IHC staining.

TUNEL staining

Apoptotic cells in tumor tissues were detected by TUNEL assay according to the standard procedure. After fixed in 4% paraformaldehyde, the tissues were stained by 50 μL TUNEL reaction mixture (Roche) for 60 min at 37°C. The cell nucleus was stained with DAPI and observed through the Olympus microscope.

Bioinformatics analysis

The Cancer Genome Atlas (TCGA) databse and UALCAN database which is built on PERL-CGI with high quality graphics using javascript and CSS were used to explore the expression of miR-375 in HCC patients.

Statistical Analysis

Data are presented as mean ± SD. Comparisons between groups were performed using one-way ANOVA, and Tukey's procedure for multiple range tests was performed. All experiments for cell cultures were performed independently at least three times and in triplicate each time. Value of $P<0.05$ was considered to be significant.

Results

Phenotypic characteristics of TAMs

To obtain exosomes, we collected HCC tissues to Isolate and culture TAMs. The macrophages were identified by F4/80 as surface marker (Fig. 1A). After isolation of TAMs from HCC tissues, we first performed immunofluorescence to identify macrophage surface maker (F4/80) of TAMs (Fig. 1A). We treated TAMs with IL-2 (20ng/ml) for 24 hours, the morphology of macrophages changed, with increased volume and extended pseudopodia in IL-2 treated TAMs compared to Con TAMs under electron microscope (Fig. 1B).

The internalization of exosomes by HCC cells

The exosomes were isolated from macrophage supernatants with ultracentrifugation and were obersved by transmission electron microscope (TEM) which were hemisphere with one side depression with typical characteristics (Fig.2A). The diameter of exosomes ranging from 30 to 150 nm, as shown in Figure 2B. Western blot was used to further confirm that the isolated small spheres were exosomes by detecting CD63 and not Calnexin derived from TAMs (Fig. 2C). To determine the effects of exosomes on HCC cells, we examined whether exosomes could be taken up by HCCs. An immunofluorescence assay was performed by using exosomes labeled with PKH26, a red fluorescence dye. Red fluorescence was clearly observed in HCC cells around nuclei using the confocal microscope (Fig. 2D).

The effects of the exosmes on HCC development and progression*in vitro*

First, to evaluate the effects of exosomes on the HCC cells, Huh7, HepG2 and QJY-7703 cell were treated with PBS, Exo^{TAM} and $Exo^{IL2\text{-}TAM}$ and cell survival ability was assayed by MTT. The results showed that Exo^{TAM} promoted survival ability. However, $Exo^{IL2\text{-}TAM}$ reduced the cell survival ability compared to Exo^{TAM} (Fig. 2E).

Then, the effects of the exosmes on the proliferation and apoptosis of HpG2 cells were explored. The results revealed that when compared with the control (Con) group, the cell proliferation abilities were increased, whereas the apoptotic rate was decreased in the Exo^{TAM} group companied with related proteins. $Exo^{IL2\text{-}TAM}$ reversed these effects compared with Exo^{TAM} group(Figures 3A–3J).

Last, the effects of the exosmes on the migration and invasion of HpG2 cells were measured. Transwell assay and scratch test were used to explore whether IL-2 treatment affects TAMs derived exosomes to influence the migratory and invasive abilities of hepG2 cells. The cell migrative and invasive abilities were increased in Exo^{TAM} group compared to Con group, these abilites were reduced in $Exo^{IL2\text{-}TAM}$ group compared to Exo^{TAM} group (Figure 4).

Taken together, our findings indicated that the effects that exosomes fromTAMs promote proliferative, migratory and invasive behaviors and inhibited apopotosis were reversed by IL-2 treatment *in vitro*.

The effects of the exosmes on HCC development and progression *in vivo*

To test the above results *in vitro*, the *in vivo* xenograft model was used in nude mice. QJY-7703 cells were injected subcutaneously into the flanks of nude mice with exosomes injection via the tail vein. The tumors produced by co-injection of QJY-7703 and Exo^{TAM} were significantly larger and heavier than those produced by QJY-7703 cells alone. However, the tumors were smaller and lighter in $Exo^{IL2\text{-}TAM}$ group compared to Exo^{TAM} group (Figure 5A-C). The tumors were collected and the apoptosis of tumor tissues were detected by the TUNEL staining and western blot. The results showed that decreased positive expression of TUNEL and Bcl2 protein levels and increased Bax protein levels were found in Exo^{TAM} group. However, positive expression of TUNEL and Bcl2 protein levels increased and Bax protein levels decreased in $Exo^{IL2\text{-}TAM}$ group compared to Exo^{TAM} group(Figure 5D-H). Meanwhile, the protein levels of PCNA and cyclin D1 were reduced in $Exo^{IL2\text{-}TAM}$ group(Figure 5I-K).

To explore the effect of exosomes on tumor metastasis in vivo, we injected QJY-7703 cells alone, or with Exo^{TAM} , or with $Exo^{IL2\text{-}TAM}$ into nude mice via the tail vein. Quantitation of metastasis of the livers and lungs of mice in each group revealed that higher rates of hepatic and pulmonary metastases were found in Exo^{TAM} group compared with Con group, and the rates were lower in $Exo^{IL2\text{-}TAM}$ group(Fig. 6A-B). Furthermore, the IHC staining revealed that Vinmentin were significantly elevated in the Exo^{TAM} group, accompanied by increased protein levels related to EMT, but Vinmentin staining and protein levels related to EMT were reduced in $Exo^{IL2\text{-}TAM}$ group compared to Exo^{TAM} group(Fig. 6C-H).

These experiments indicated that *in vivo* results are consistent with *in vitro* results and IL-2 treatment ameliorates hepatocellular carcinoma development mediated by exosomes from TAMs.

The exosomal miR-375 was increased from IL-2 treated TAMs and promotes HCC cell apoptosis

The exosomal miRNAs was regarded as an important mechanism of Crosstalk between cancer cells and TAMs[19]. It was reportrd that miRNAs were reduced in TAMs compared with the macrophages from normal tissues and it is able to regulate the apoptosis of tumor cells[3].

It has been well documented that miRNAs, such as miR-120, miR-494, miR-143-3p, miR-375, miR-125a and miR-9-5p may inhibit cancer. As shown in Figure 7A, among six miRNAs miR-375 was obviously increased in exosomes derived from TAMs with IL-2 treatment. We analyzed miR-375 expression in HCC patients in the TCGA database and UALCAN database to explore the role of miR-375 in HCC. miR-375 was significantly elevated in HCC tissues compared with the control and the levels of miR-375 were significantly lower in patients with later stage than those with the early stage(Figure 7B-E).

The transport function of exosomes in HCC cells was analyzed with co-culture system. The TAMs transfected with FITC-miR-375 mimics were in apical chamber and the HCC cells were in the basolateral chamber. After 24-h co-culture, green fluorescence could be found in HCC cells (Figure 8A). To further confirm that exosomes released from TAMs is able to transfer miR-375 to HCC cells (Figure 8B for a

schematic design), we collected the conditioned media from TAMs to treat HCC cells. We found that miR-375 was increased in Group3. However, this increase was reversed by GW4869 in Group4 (Figure 7C).

Last, to study the function of exosomal miR-375, we inhibited miR-375 by antagomir and overexpressed miR-375 using the agomir in TAMs. HCC cells were treated with exosomes derived from the agomir and antagomir transfected TAMs treated with IL-2 or not respectively. The upregulation of miR-375 significantly reduced the rate of apoptosis. Consistently, decreased miR-375 caused increased the rate of apoptosis (Figure 8D). These results reflected that the inhibited apoptosis by exosomes from IL-2 treated TAMs is partly mediated by miR-375.

Discussion

The malignant behavior of tumor is not only determined by the characteristics of tumor cells, but also affected and regulated by various components in tumor microenvironment.TAMs, as the most abundant immune cells in tumor immune microenvironment, plays an important role in bridging the inflammatory mediators and tumors. Studies have shown that the degree of TAMs infiltration in HCC is negatively correlated with the prognosis of patients[20].

It was reported that most mature TAMs tend to M2 phenotype and function, including promoting angiogenesis, participating in tissue repair and reconstruction, regulating inflammatory mediators response and adaptive immunity. In a variety of malignant solid tumors, TAMs infiltration can promote tumor growth, angiogenesis, invasion and metastasis, and resist immune damage[21].

TAMs and HCC cells interact and interact to promote the progress of liver cancer: on the one hand, liver cancer cells secrete cytokines and chemokines, recruit macrophages to gather, domesticate and induce, and constantly adjust their own characteristics[22,23].On the other hand, mature TAMs participates in the progress of liver cancer through a variety of mechanisms, including: promoting angiogenesis to obtain sufficient nutrients for liver cancer cells in the state of rapid growth; participating in the degradation of basement membrane and matrix remodeling to prepare for local invasion and distant metastasis of tumor; negatively regulating the anti-hepatoma immune response to escape the immune system and provides a "safe haven" for tumor development [24].Therefore, the study of TAMs may help to find a molecular targeted drug that can effectively block the crosstalk between cancer cells and TAMs.

It is reported that exosomes mediate the intercellular information exchange which plays an important role in the occurrence and development of hepatocellular carcinoma[4–6]. Although the research on the relationship between cancers and exosomes from macrophages has become more and more popular in recent years, there is limited information on the exosomes derived from the cell lines[25–28]. Few studies have been carried out on the TAMs extracted directly from the tissues of patients[7–9]. Most of the studies on exosomes and their signaling pathways are only in the early stage, and many studies are limited to cell experiments and the results need to be further verified in animal models[27,29].We directly collected HCC tissues and isolated TAMs which retain some of their biological characteristics *in vivo*. More importantly, the effects of exosomes *in vivo* were explored.

Recent research has shown that IL-2 is a cytokine produced by activating T cells whose anti-tumor mechanism lies in stimulating and activating its effector cells, and then plays an anti-tumor role [29,30].Recent studies have shown that IL-2 can promote the synthesis and secretion of IFN - γ, and IFN - γ regulates macrophage polarization to M1 type.[31] LPS induces macrophage M1 polarization and the M1 macrophages release exosomes to potentiate the anticancer efficacy[10]. However, LPS is not an approach for human therapy.

miRNAs plays a key role in the occurrence and development of human malignant tumors[32,33]. In many malignant tumors, the expression levels of miRNA were changed to varying degrees compared with the surrounding normal tissues[11,12]. More and more *in vitro* experiments show that the changes of miRNAs can effectively affect the proliferation and metastasis of tumor cells[19,34].

It is reported that miR-375 inhibits human liver cancer development and progession [35–37]. In this study, we found that miR-375 is up-regulated in exosomes derived from TAMs treated with IL-2. Furthermore, to study the function of exosomal miR-375, we inhibited miR-375 by antagomir and overexpressed miR-375 using the agomir in HCC cells. The inhibition of miR-375 resulted in a decrease of apoptosis in HCC cells, while the overexpression by agomir resulted in an increase of apoptosis in HCC cells

Conclusions

Taken together, we have found that extracellular vesicles derived from TAMs promote the development and progression of hepatocellular carcinoma *in vivo* and *in vitro* and these effects of extracellular vesicles were reversed by IL-2 treatment. IL-2 increased the exosomal miR-375 which may be responsible for the increase of HCC cells apoptosis. These findings provides a new perspective to explain the mechanism that IL-2 inhibits HCC and provide basis for the clinical use of IL-2 .

Abbreviations

IL-2: interleukin-2

TAMs: tumor-associated macrophages

HCC: hepatocellular carcinoma

TUNEL: transferase-mediated deoxyuridine triphosphate-biotin nick end labeling

IFN-γ: interferon-γ

HCC: human hepatoellular carcinoma

Declarations

Ethics approval and consent to participate

All Clinical research were performed with the approval of the Ethics Committee of Yijishan Hospital, and informed consent was obtained from all patients. All the animal experiments were manipulated according to guidelines approved by the Animal Ethics Committee of Youjiang Medical University for Nationalities. .

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The data used to support the findings of this study are available from the corresponding authors upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

H.-q.C. designed research; C.H., T.C. and W.F. performed the cell experiments; L.-l.M., L.S. and L.-z.H. prepared the animal models; L.W. and W.- z.H. assisted in the study design and critically revised the raw manuscript. All the authors were involved in writing the manuscript. All authors read and approved the final manuscript.

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Figures

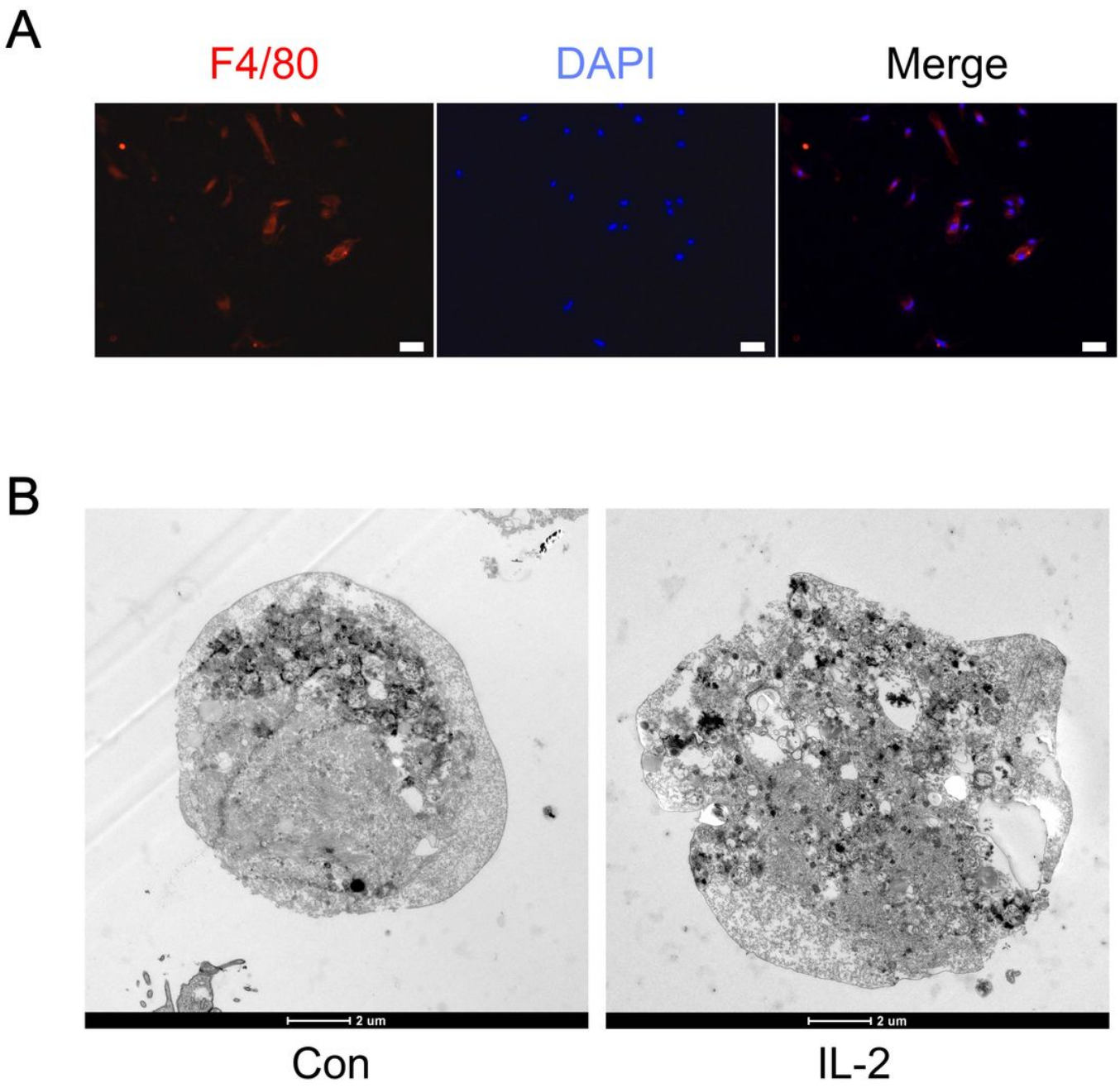

Figure 1

Identifications of TAMs. (A) Identification of TAMs surface marker (F4/80) by immunofluorescence. (B)The effect of IL-2 on the morphology of macrophages from different groups at 24 hours under a transmission electron microscope.

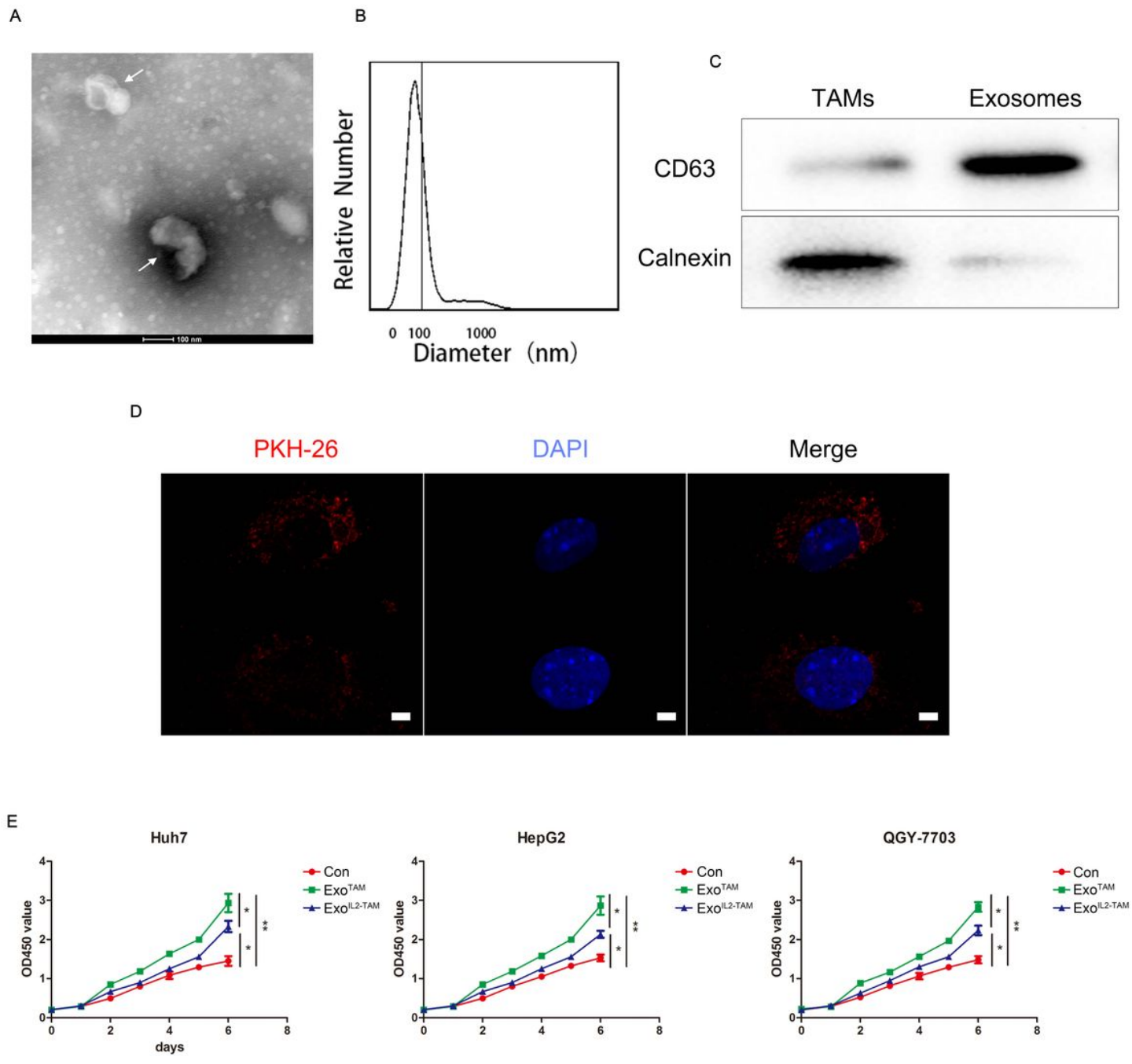

Figure 2

The exosomes internalization and their effects on the survival ability of HCC cells (A) Exosomes from TAMs observed under a transmission electron microscope; (B) NTA analysis of isolated exosomes; X-axis represents the diameter of the vesicle and the Y-axis represents the number of vesicles. (C) CD63 and Calnexin expression in TAMs and exosomes measured by western blot. (D) Internalization of PKH26-labeled exosomes (red) derived from TAMs by HpG2 cells.(E) Huh7, HepG2 and QJY-7703 cells were

treated with PBS, ExoTAM and ExoIL2-TAM for 0 to 6 days and cell survival ability was assayed by CCK8 assay. Error bars, SD. *P < 0.05; **P < 0.01.

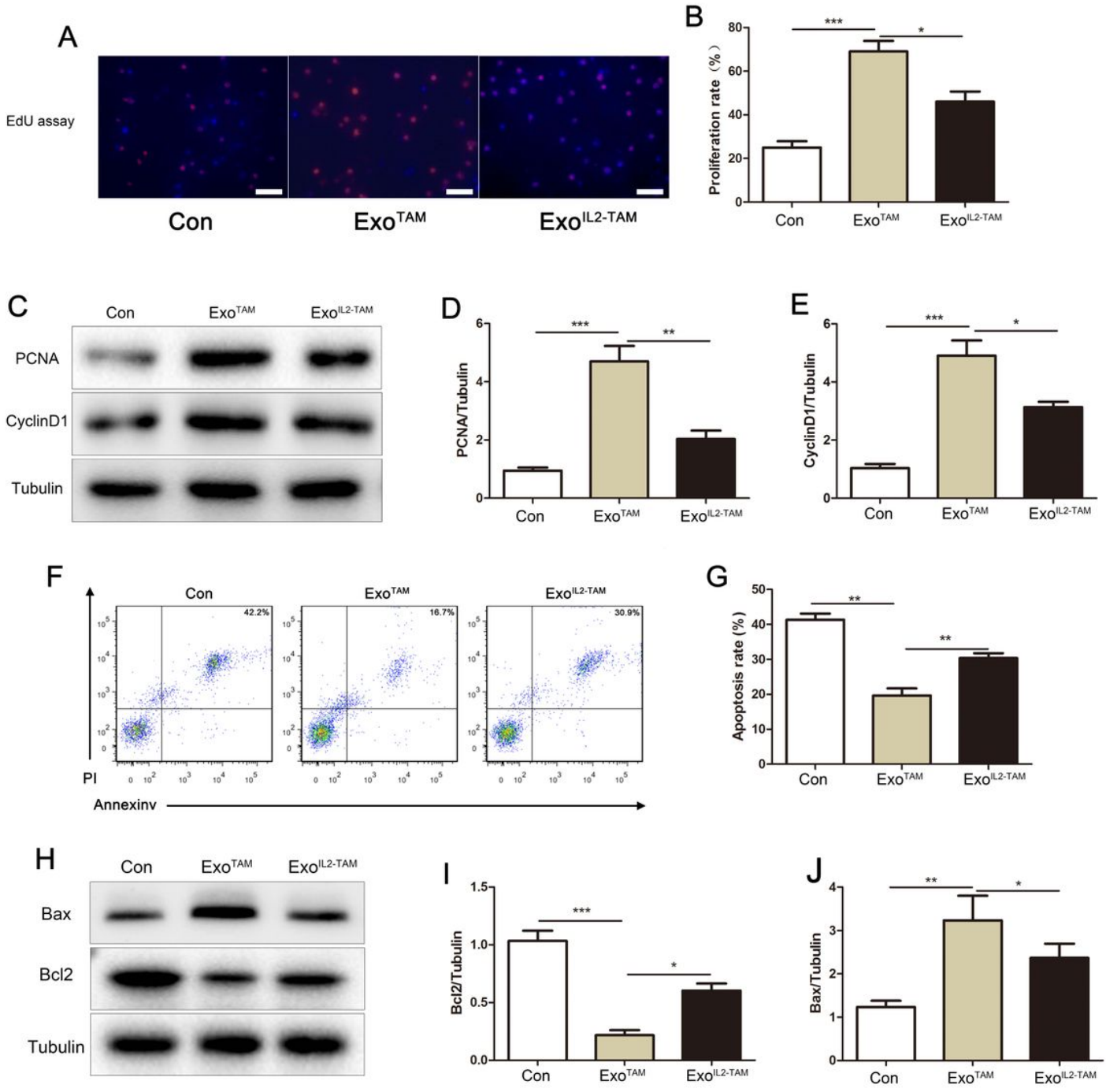

Figure 3

The effects of the exosmes on HCC cell proliferation and apoptosis. (A) Representative immunostaining images of EDU+cells in vitro ; (B) Percentages of EDU+ nuclei over total number of nuclei; (C-E) Western blot analysis of PCNA and cyclinD1 expression; (F-G) Flow cytometry for Annexin V-APC and propidium iodide (PI) staining in cells treated with exosomes for 24 h. The representative plots (F) and quantification

(G) are shown. (H-J) Western blot analysis of Bax and Bcl2 expression. (K) Representative electron microscopic images of mitochondria ultrastructure of HCC cells. Error bars, SD. *P < 0.05; **P < 0.01; ***P < 0.001.

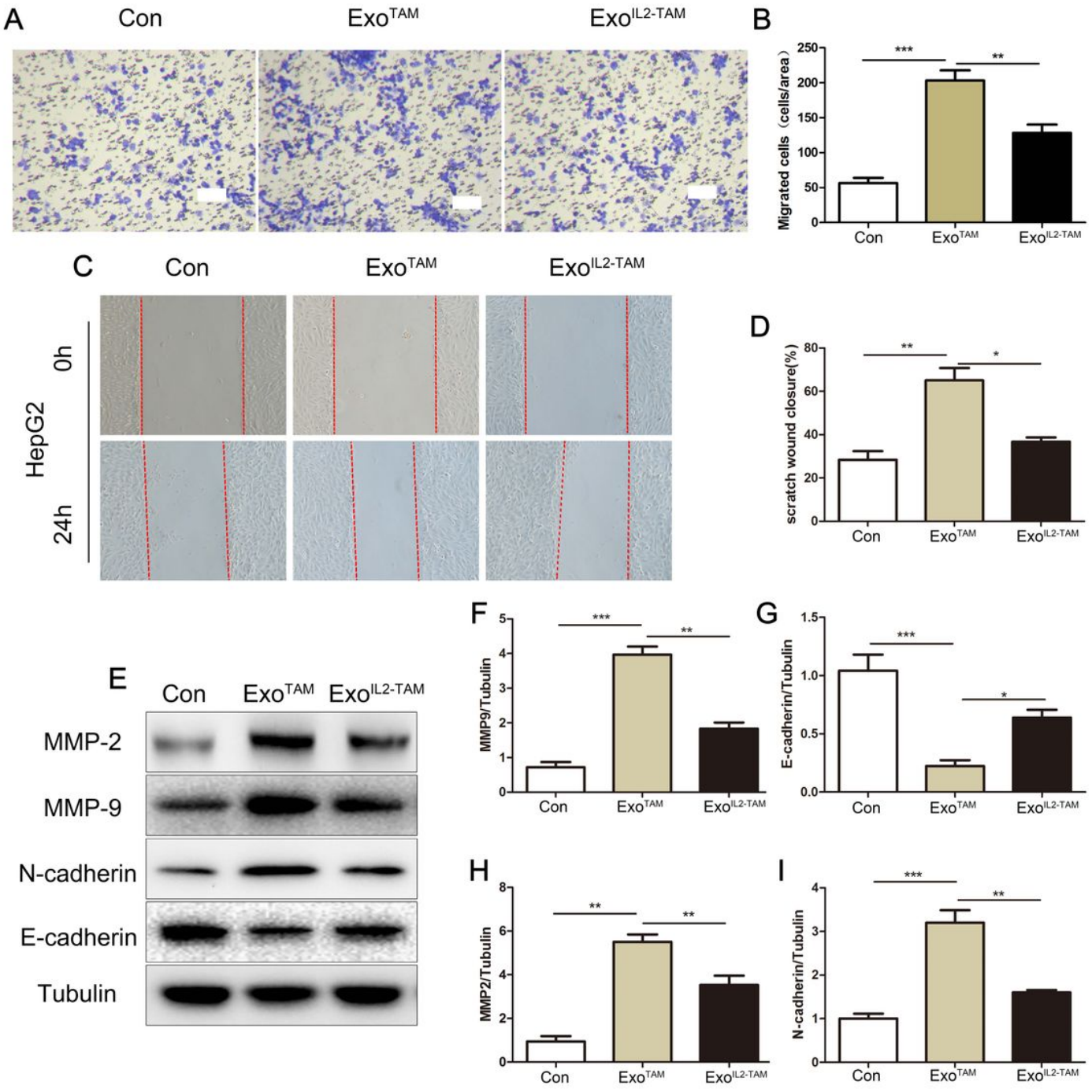

Figure 4

The effects of the exosmes on HCC cell invasion and migration in vitro (A) Representative photographs of migratory cells in transwell co-culture system.Scale bar = 100 μm; (B) Qualification of migratory cells in transwell coculture system; (C) Representative photographs of invasive cells in scratch test;

(D)Qualification of invasive cells in scratch test; (E-I) Western blot analysis of MMP2, MMP9, N-cadherin and E-cadherin expression. Error bars, SD. *P < 0.05; **P < 0.01; ***P < 0.001.

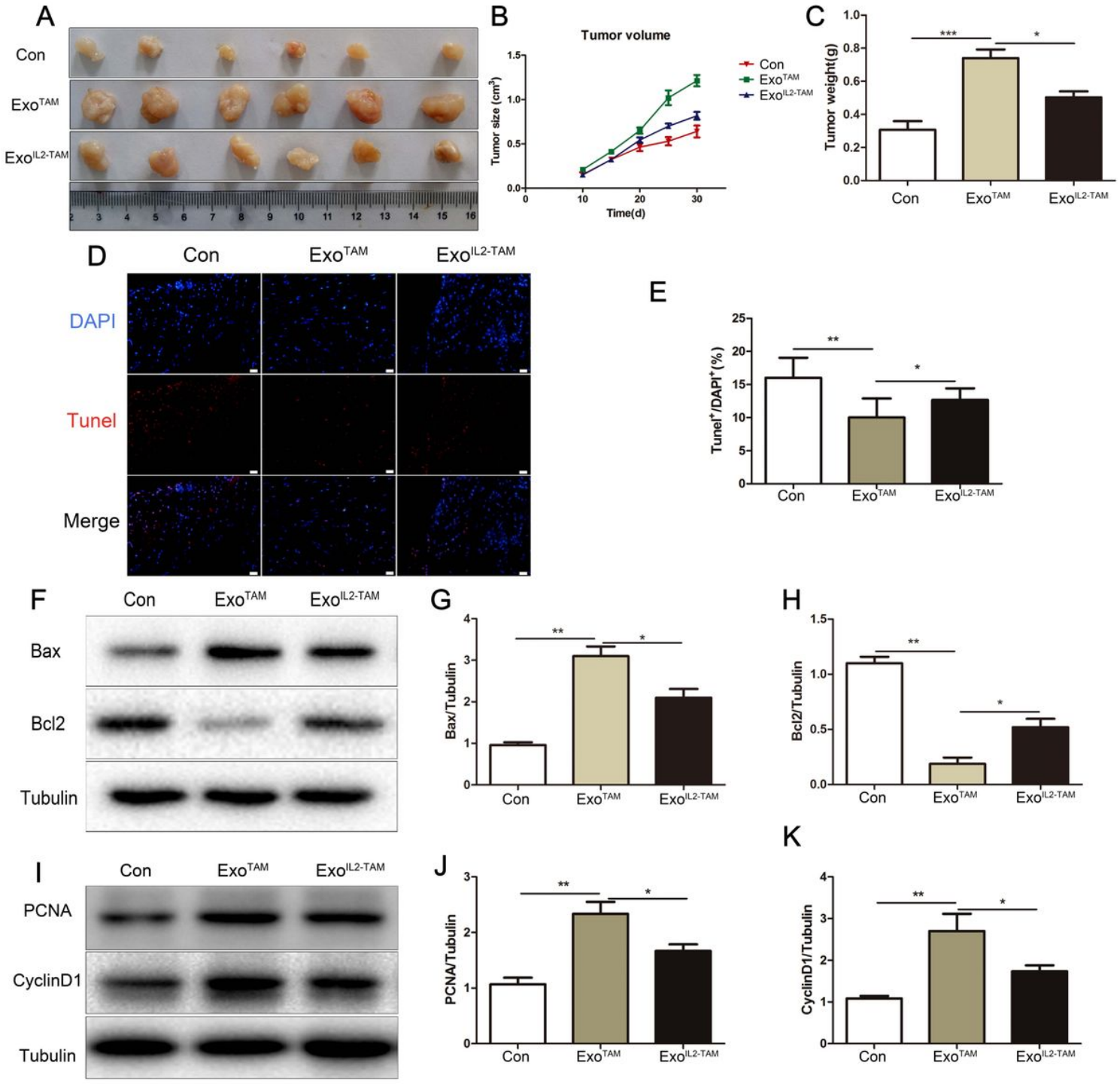

Figure 5

The effects of the exosmes on HCC progression in vivo (A-C) The morphological characteristics of tumor xenograft, tumor size and tumor weight. (n=6 per group). (D) Representative photographs of TUNEL-stained cancer sections from different groups. Apoptotic nuclei were identified by TUNEL staining (green), and total nuclei by DAPI (blue). Scale bar: 50 mm. (E) Percentages of TUNEL-positive nuclei over total

number of nuclei (n 4 to 5). (F-H) Western blot analysis of Bax and Bcl2 expression.(I-K) Western blot analysis of PCNA and cyclinD1 expression. Error bars, SD. *P < 0.05; **P < 0.01; ***P < 0.001.

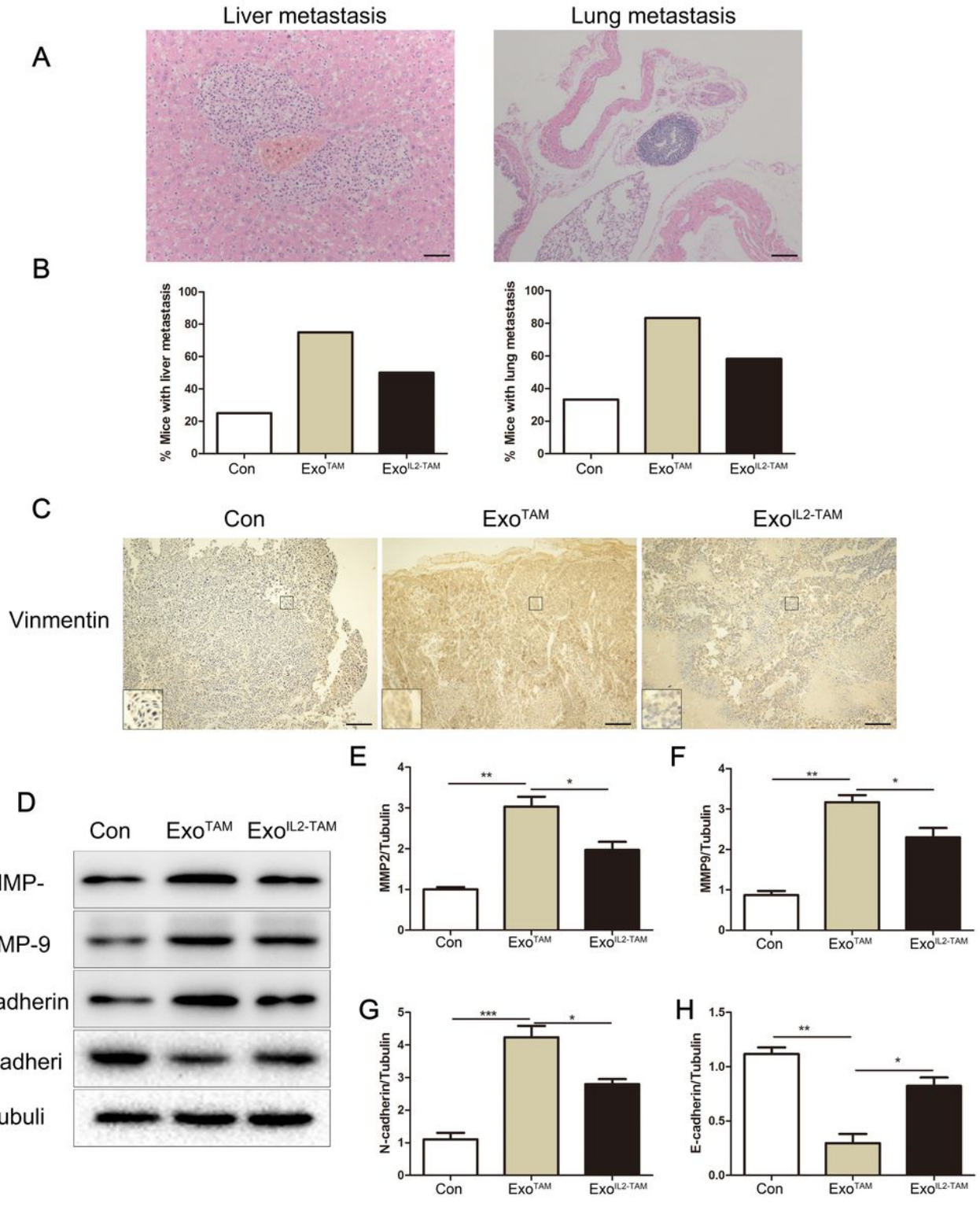

Figure 6

The effects of the exosmes on HCC metastasis in vivo. (A) Representative images of H&E–stained sections of metastatic nodules in liver and lung are shown. Scale bar, 200×. (B)Percentage of mice with metastasis is indicated from mice in Con, ExoTAM and ExoIL2-TAM groups (n = 12 per group). (C)

Representative images of IHC staining for Vinmentin protein of tumors. (D-H) Western blot analysis of MMP2, MMP9, N-cadherin and E-cadherin expression. Error bars, SD. *P < 0.05; **P < 0.01; ***P < 0.001.

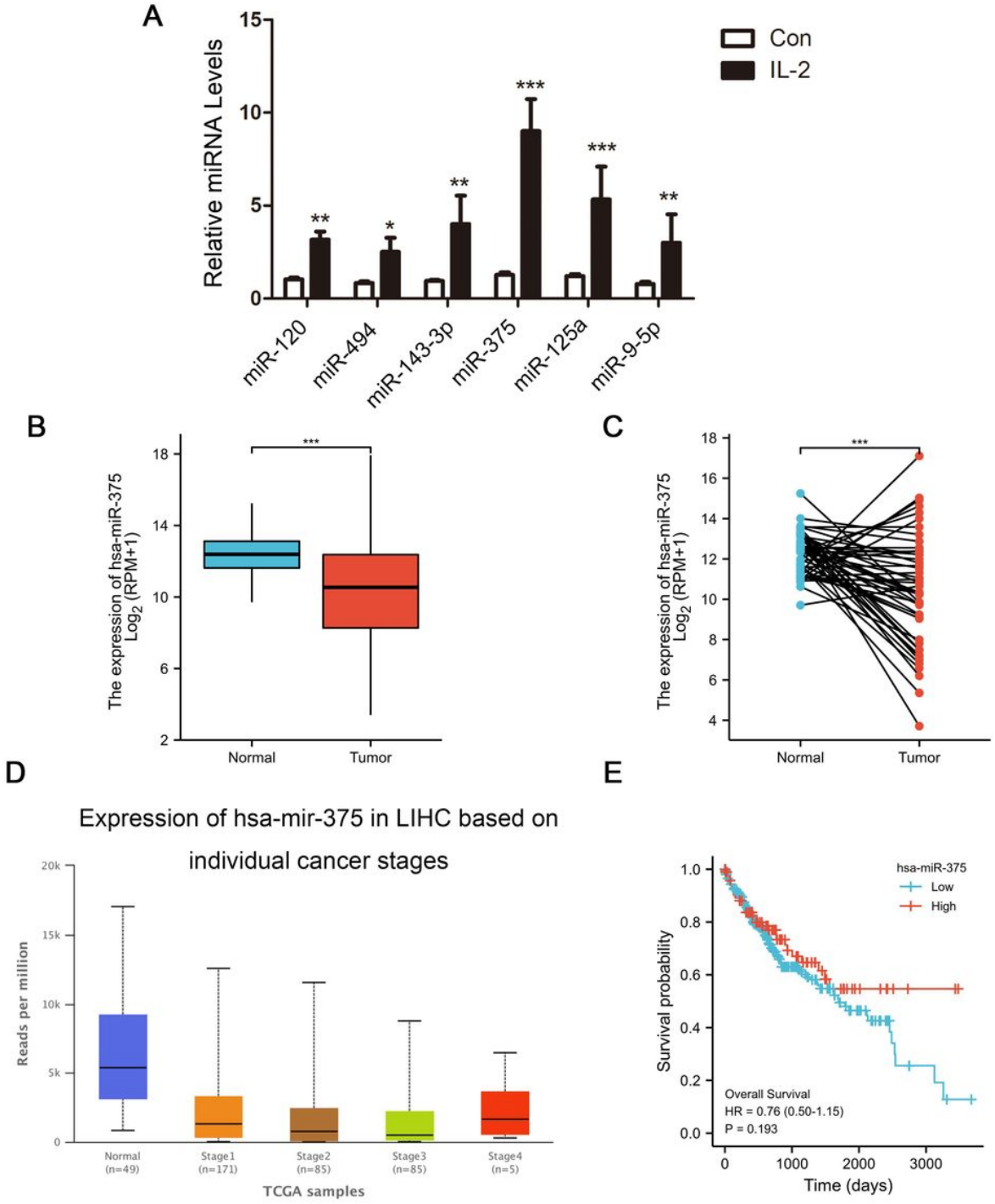

Figure 7

miR-375 expressions in exosomes from IL-2-induced TAMs and in HCC patients. (A) Six miRNAs expressions were detected by qRT-PCR in exosomes from TAMs . (B) No paired HCC tissues in TCGA database. (C) Paired HCC tissues in TCGA database. (D) HCC patients in different stages in UALCAN database. *P < 0.05; **P < 0.01; ***P < 0.001.

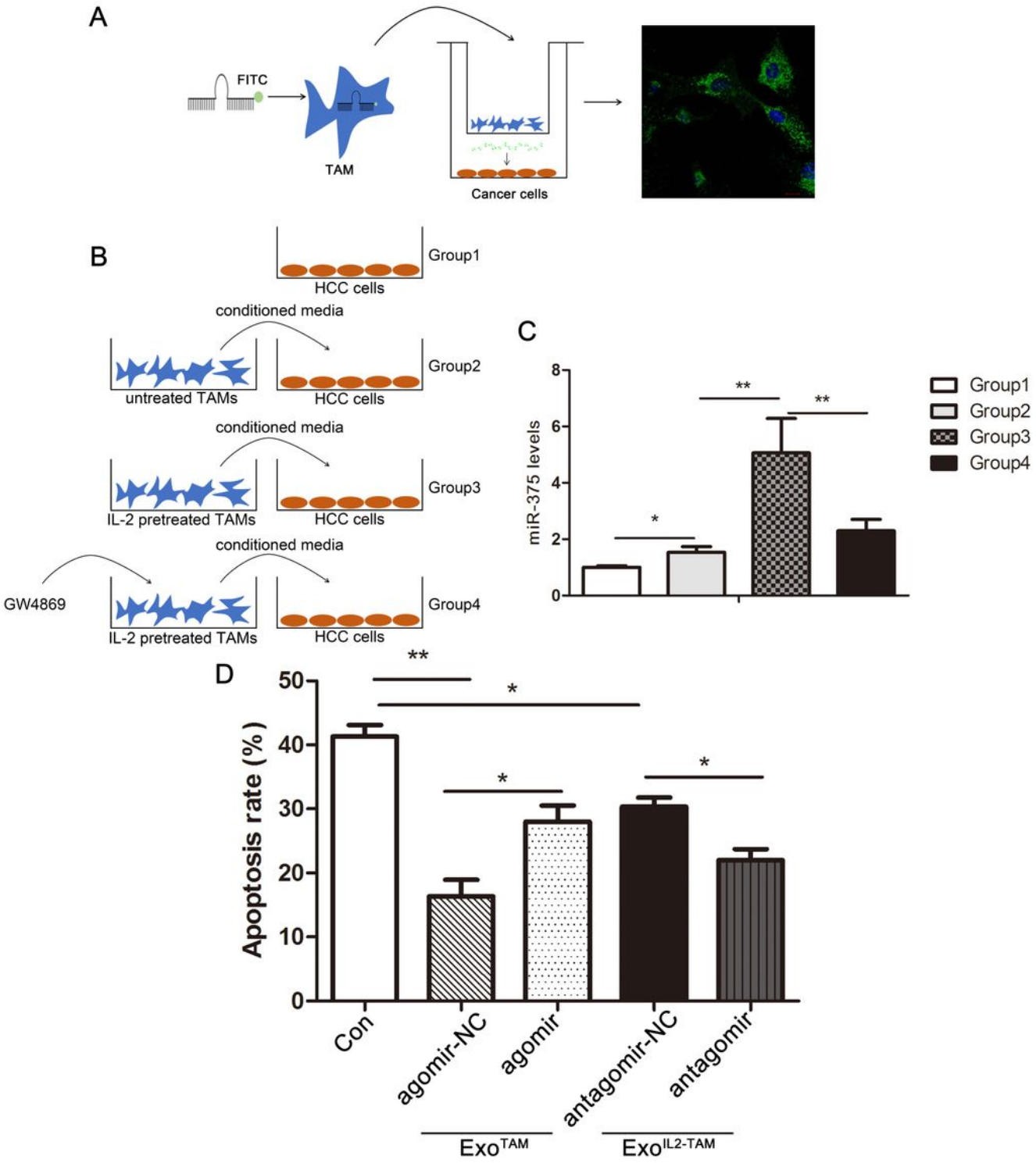

Figure 8

The effects of miR-375 in exosomes from IL-2-induced TAMs on apoptosis in HCC cells. (A) TAMs transfected with a FITC-labeled miR-375 mimic were co-cultured with HCCs in a transwell system. Representative images of the entry of miR-375 mimics into receptor HCC. Scale bar = 100 μm ; (B) Scheme of experiments for conditioned media from TAMs in the subsequent miR-375 levels analysis of HepG2 cells. (C) miR-375 levels in the different groups. (D) HepG2 cells were treated with exosomes

derived from the TAMs which were transfected with miR-375 agomir or antagomir respectively.The quantification of cell apoptosis was measured by flow cytometry for Annexin V-APC and PI. Error bars, SD. *P < 0.05; **P < 0.01; ***P < 0.001.