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miR-210 promotes hepatocellular carcinoma progression by modulating macrophage autophagy through PI3K/AKT/mTOR signaling

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Abstract

Background: Tumor-associated macrophages (TAMs) play an important role in tumor development. Increasing research suggests that miR-210 may promote the progression of tumor virulence, but whether its pro-carcinogenic effect in primary hepatocellular carcinoma (HCC) is via an action on M2 macrophages has not been examined.

Methods: Differentiation of THP-1 monocytes into M2-polarized macrophages was induced with phorbol myristate acetate (PMA) and IL-4, IL-13. M2 macrophages were transfected with miR-210 mimics or miR-210 inhibitors. Flow cytometry was used to identify macrophage-related markers and apoptosis levels. The autophagy level of M2 macrophages, expression of PI3K/AKT/mTOR signaling pathway-related mRNAs and protein were detected by qRT-PCR and Western blot. HepG2 and MHCC-97H HCC cell lines were cultured with M2 macrophages conditioned medium to explore the effects of M2 macrophage-derived miR-210 on the proliferation, migration, invasion and apoptosis of HCC cells.

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Authors' contributions

RCX and WCH conceived and designed the project. BSM, ZYD, ZJ, YYY, WJD and FMM carried out most of the experiments. BSM wrote the manuscript, BSM and LBZ performed the statistical analysis, all authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval and consent to participate

The study is a general cell experiment and no ethical application is required.

Consent for publication

Not applicable.

Results: qRT-PCR showed increased expression of miR-210 in M2 macrophages. Autophagy-related gene and protein expression was enhanced in M2 macrophages transfected with miR-210 mimics, while apoptosis-related proteins were decreased. MDC staining and transmission electron microscopy observed the accumulation of MDC-labeled vesicles and autophagosomes in M2 macrophages in the miR-210 mimic group. The expression of PI3K/AKT/mTOR signaling pathway in M2 macrophages was reduced in miR-210 mimic group. HCC cells co-cultured with M2 macrophages transfected with miR-210 mimics exhibited enhanced proliferation and invasive ability as compared to the control group, while apoptosis levels were reduced. Moreover, promoting or inhibiting autophagy could enhance or abolish the above observed biological effects, respectively.

Conclusions: miR-210 can promote autophagy of M2 macrophages via PI3K/AKT/mTOR signaling pathway. M2 macrophage-derived miR-210 promotes the malignant progression of HCC via autophagy, suggesting that macrophage autophagy may serve as a new therapeutic target for HCC, and targeting miR-210 may reset the effect of M2 macrophages on HCC.

Keywords

miR-210; Macrophage; Autophagy; Primary hepatocellular carcinoma

1. Introduction

To date, primary liver cancer has become one of the leading causes of cancer deaths worldwide, with more than 900,000 new cases each year, of which 830,000 deaths, and the number of incidences and deaths is still increasing year by year [1]. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, accounting for about 75%–85% [1]. Metastasis and drug resistance are the key reasons for poor prognosis of the primary HCC [2]. The role of autophagy in HCC is closely related to its development stage, and how to balance the cell death and cell survival mechanisms caused by autophagy is the focus of treatment for HCC [3].

Tumor-associated macrophages (TAMs) are the main components of immune cells in the tumor microenvironment (TME). TAMs are usually divided into M1 (classically activated) phenotype with pro-inflammatory response and M2 (selectively activated) phenotype with anti-inflammatory response [4]. In macrophages, autophagy is also a key regulatory process that not only controls the stability of the intracellular environment but also participates in the regulation of a wide range of specific immune functions [5]. Autophagic activation of TAMs in HCC has been found to promote M2 phenotypic polarization and function [6]. In this context, the relationship between macrophage autophagy and HCC is of great interest.

A number of studies have explored the role of TAMs in tumor progression and immune escape [7]. However, the role of TAMs-derived miRNAs in tumorigenesis is little studied.

Several studies have revealed a correlation between miR-210 and poor prognosis in malignant tumors such as liver, lung, colorectal and pancreatic cancers [8–11]. However, the role and function of miR-210 in the interaction between HCC cells and TAMs remain unknown.

PI3K/AKT/mTOR signaling pathway functions as a classic dysregulated pathway involved in hepatocarcinogenesis and plays a significant role in various biological processes such as proliferation, metastasis, chemo- and radiotherapy resistance, energy metabolism, and autophagy in HCC [12–14]. Knowledge on the involvement of miR-210 and PI3K/AKT/mTOR pathway would provide new insight into TAMs mediated HCC progression and identify new therapeutic target.

In present study, we aimed to explore whether the pro-cancer effects of miR-210 on HCC were associated with autophagy of macrophages. Our results showed that macrophage-derived miR-210 could regulate autophagy of macrophages through PI3K/AKT/mTOR signaling pathway, thus promoting the malignant biological behavior of HCC.

2. Methods

2.1. Cell lines and cell culture

The human HCC cell lines HepG2 and MHCC-97H and the human monocytic leukemia cell line THP-1 were purchased from the Sebacon Biotechnology Co (Shanghai, China). HepG2 and MHCC-97H were cultured in Dulbecco's Modified Eagle Medium (Sebacon, Shanghai, China). THP-1 cells were cultured in RPMI 1640 Medium (Sebacon, Shanghai, China). The media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.05 mM β-mercaptoethanol, and 1% double antibiotics (Sebacon, Shanghai, China). THP-1 monocytes were treated with 150 nM phorbol 12-myristate 13-acetate (PMA) (P8139, Sigma-Aldrich, St Louis, MO, USA) for 24 h to be differentiated into macrophages.

2.2. Macrophage polarization

The THP-1 cells were differentiated into M0 macrophages by incubation in 320 nmol/L PMA for 18 h (h). To generate M2-polarized macrophages, THP-1 cells were treated with 320 nmol/L PMA for 12 h and then cultured with 100 nmol/L PMA plus 20 ng/ml IL-4 and 20 ng/ml IL-13 for a further 48 h.

2.3. Flow cytometry

Flow cytometry (Beckman, NAVIOS) was used to identify M1 and M2 macrophages and to detect apoptosis levels. Flow cytometry antibodies included: FITC anti-human CD68 (321103, BioLegend), PE anti-human CD11b (301305, BioLegend), FITC anti-human CD206 (Sc-20060FITC, Santa cruz).

Annexin V-FITC/PI apoptosis kit (UNICOBIO) was used for the detection of apoptosis in macrophages or HCC cells. The protocol in the JC-1 kit (Beyoncé) was followed. Finally, the cells went for flow cytometry analysis and the data were analyzed by Kaluza Analysis software.

2.4. miRNA transfection

MiR-210 mimics, miR-210 inhibitors, negative control mimics (NC mimics) and negative control inhibitors (NC inhibitors) were synthesized by GenePharma Co.(Shanghai, China).

Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the supplier's protocol. 48 h after transfection was considered as the harvest time.

2.5. Quantification of cytokines by enzyme-linked immunosorbent assay (ELISA)

IL-10 and TGF-β cytokine concentrations in M2 macrophages of different subgroups were measured using human TGF-β1 ELISA Kit and human IL-10 ELISA Kit according to the manufacturer's instructions.

2.6. Immunofluorescence (IF)

IF staining was used to detect macrophage autophagy. After aspirating the blocking solution, a sufficient amount of primary antibody (MDC, 1:200, dilution rabbit, Bioss, bs-1761R) was added and the samples were incubated at 37 °C for 60min. After removing the primary antibody, the samples were washed slowly 3 times. A sufficient amount of immunofluorescent secondary antibody (goat anti-rabbit 1:400) was added and the samples were incubated for 30 min at 37 °C, in the dark. The secondary antibody was removed, and the slices were washed 3 times in PBST before being removed and sealed with an anti-fluorescence quenching blocker (containing DAPI, ebiogo, B024). Finally, fluorescent sections were scanned with a digital section scanner (Pannoramic MIDP).

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Detection of mRNA expression levels of genes related to macrophages was made by qRT-PCR. cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser reverse transcription kit (TaKaRa). The qRT-PCR was performed using Novostart SYBR qPCR SuperMix Plus (novoprotein). The primer sequences used for qRT-PCR are shown in Table 1 β-Actin was used as an internal control and relative expression was calculated according to the $2^{-\Delta\Delta Ct}$ method.

2.8. Western blot

Western blotting was used to detect protein abundance. Protein samples were separated by SDS-PAGE gels and transferred to PVDF membranes (Millipore) pre-soaked in methanol. After the transfer of the membrane was completed, the immunoblots were incubated in a blocking solution (5% skim milk powder) for 2 h at room temperature. The primary antibodies were diluted with diluent to the appropriate ratios as per manufactures' manual. The samples were incubated overnight at 4 °C with gently shaking. On the following day, blots were incubated with 1:20,000 dilutions of horseradish peroxidase (HRP)-labeled secondary antibody for 2 h at room temperature. The samples were then washed 3 times with washing solution (PBST) for 10 min each. Finally, staining with the ECL luminescence kit (Thermo) was performed to detect the proteins. The analysis of the film strips was performed with Image J software and the intensity of the proteins normalized to the corresponding β-actin bands. The antibodies used and their dilution ratios were as follows:anti-Arg-1 (1:200, Santa Cruz), anti-IL-8 (1:500, Bioworld), anti-IL-10 (1:1000, Bioss), anti-TGF-β1 (1:500, Bioss), anti-VEGF (1:500, Abcam), anti-LC3B (1:2000, Cell Signaling), anti-Beclin-1(1:1000, abcam), anti-P62 (1:5000, Proteintech), anti-PI3K p85α (1:2000, Bioss), anti-p-PI3K p85 (1:1000, abcam), anti-AKT (1:1000, Cell Signaling), anti-

p-AKT(1:2000, Cell Signaling), anti-mTOR (1:1000, CST), anti-p-mTOR (1:1000, Cell Signaling), anti-β-actin (1:2000, Zsbio).

2.9. CCK8 assays

Cell Counting Kit-8 (BIOSS) was used to detect HCC cell viability. HCC cells in exponential growth phase were digested with trypsin, subsequently collected by centrifugation and made into a cell suspension, and the cell concentration was adjusted to 5–10 × 10^4/ml. 100 μl of cell suspension was added to each well of a 96-well plate, and the edge wells were filled with sterile PBS. After 0, 24, 48, 72 h of incubation, 10 μl CCK8 was added to each well and the incubation was continued for 1–4 h. The absorbance value of each well was measured at OD450nm on a Microplate Reader (Bio-Rad, Hercules, CA, USA).

2.10. Invasion assay

Transwell assay was performed to detect the invasiveness of HCC cells. After addition of serum-free medium for resuspension and counting, the cell concentration was adjusted to 2 × 10^5/ml, and 100 μl of cell suspension was added to matrix gel-coated Transwell cell culture insert, and the culture was continued for 24 h. The inserts were removed from the transwell chambers, fixed with 4% paraformaldehyde at room temperature for 30 min and then stained with 0.5% crystal violet for 15–30 min. The inserts were rinsed several times with water, and the cells on the bottom membrane surface of the upper chamber were carefully wiped off with a wet cotton swab, randomly photographed and counted under the microscope, and the results were counted. The experiment was repeated 3 times.

2.11. Statistical analysis

Statistical analysis was performed using SPSS v22.0 software. Data were expressed as mean ± standard error. The statistical significance of the differences between multiple groups was tested using ANOVA (one-way analysis of variance), and comparison between any two groups was done using Student's *t*-test. Statistical charts were carried out using GraphPad Prism v8.0 software. *P*-values <0.05 were considered statistically significant for the differences.

3. Results

3.1. Establishment of human M2-polarized macrophages

Human THP-1 monocytes were successfully differentiated into macrophages (M0 macrophages) after being induced by PMA, as shown in Fig.1A. To obtain M2-polarized macrophages, IL-4 and IL-13 were then used to stimulate M0 macrophages. Analysis by flow cytometry revealed a significant increase in the expression of CD11b and CD68 (macrophage surface markers) in M0 macrophages differentiated from THP-1, while CD206 (M2 macrophage surface markers) expression increased in M2 polarized macrophages (Fig. 1B). Subsequently, the mRNA expression of TGF-β1and Arg-1 (M2 macrophage markers) and miR-210–5p were detected by qRT-PCR (see section 3.2). As expected, the mRNA expression levels of TGF-β1 and Arg-1 were greater in M2 macrophages, confirmation M2 phenotype (Fig. 1C). In addition to these, Arg-1 protein levels were significantly elevated in

M2 macrophages (Fig. 1D). Taken together, we successfully polarized THP monocytes into M2-polarized macrophages.

3.2. miR-210 promotes autophagy and inhibits apoptosis in M2 macrophages

To investigate the effect of miR-210 on autophagy and apoptosis in M2 macrophages, miR-210 mimics, miR-210 inhibitors, negative control mimics (NC mimics) and negative control inhibitors (NC inhibitors) were transfected into M2 macrophages, separately. qRT-PCR showed that miR-210 levels in M2 macrophages were significantly increased after the transfection (Fig. 2A). The mRNA levels of autophagy-related genes ATG5 and Beclin-1 were elevated in M2 macrophages transfected with miR-210 mimics, whereas the mRNA levels of autophagy-related genes were decreased in the group transfected with miR-210 inhibitors. These data suggest that miR-210 stimulates the expression of the key autophagic genes in M2 macrophages. Consistently, Western blot assays showed increased protein expression of Beclin-1 and LC3-II/LC3-I and decreased P62 protein accumulation in M2 macrophages transfected with miR-210 mimics, whereas the opposite result was observed in the group transfected with miR-210 inhibitors (Fig. 2B). The morphological characteristics of autophagic cells were detected by MDC staining, and the accumulation of MDC-labeled vesicles in M2 macrophages of the miR-210 mimic group was observed (Fig. 2C). Moreover, the formation of double-membrane autophagosomes in M2 macrophages transfected with miR-210 mimics was also observed by transmission electron microscopy, which was not detected in the miR-210 inhibitor group (Fig. 2D). All these results proved that miR-210 promoted autophagy in M2 macrophages.

Apoptosis and mitochondrial membrane potential changes in M2 macrophages with different miR-210 expression levels were examined by flow cytometry. These results revealed that upregulated miR-210 inhibited the apoptosis of M2 macrophages (Fig. 2E–F).

There was increased expression of IL-8, IL-10, TGF-β1 and VEGF in M2 macrophages in the upregulated miR-210 group compared to that in other groups (Fig. 2G). The contents of TGF-β1 and IL-10 in the supernatant of M2 macrophages were determined by ELISA. The results were shown in Fig. 2H, and upregulation of miR-210 raised the concentrations of TGF-β1 and IL-10. We therefore propose that miR-210 enhances the immunosuppressive ability of M2 macrophages.

In summary, our data suggest that miR-210 promotes autophagy and suppresses apoptosis of M2 macrophages. These opposing effects thus form a synergy in the immunosuppressison and hence cancer survival.

3.3. miR-210 targets PI3K/AKT/mTOR signaling pathway and inhibits its expression in M2 macrophages

Considering PI3K/AKT/mTOR as the ‘common signal’ of autophagy and apoptosis [15], the expression levels of PI3K/AKT/mTOR signaling pathway were examined in M2 macrophages with different miR-210 expression levels. In M2 macrophages transfected with miR-210 mimics, qRT-PCR results showed reduced expression of PI3K, AKT and mTOR, and Western blot analysis further showed reduced expression of marker proteins of PI3K/AKT/mTOR signaling pathway, which was completely reversed in M2 macrophages

transfected with miR-210 inhibitors (Fig. 3A–B). The above data support the notion that miR-210 targets PI3K/AKT/mTOR signaling pathway and inhibits its expression in M2 macrophages.

3.4. miR-210 preconditioned M2 macrophages promote proliferation, invasion and inhibit apoptosis of HCC cells

To determine the effect of M2 macrophages with altered miR-210 levels on HCC cell proliferation, invasion, and apoptosis, M2 macrophages transfected with miR-210 mimics and miR-210 inhibitors were co-cultured with HepG2 or MHCC-97H cells, respectively (Fig. 4A). The results of CCK8 assay and cell invasion assay showed that the cell viability and invasion ability of HepG2 cells and MHCC-97H cells were enhanced after co-culture with M2 macrophages transfected with miR-210 mimics, while the cell viability and invasion ability of HCC cells co-cultured with M2 macrophages transfected with miR-210 inhibitors were both decreased compared to those of the negative control groups (Fig. 4B–C). Flow cytometric detection of apoptosis and mitochondrial damage in HCC cells revealed a decrease in the percentage of apoptotic and mitochondrial damaged cells in HepG2 and MHCC-97H cells co-cultured with M2 macrophages transfected with miR-210 mimics compared to that in negative controls and blank controls. However, HCC cells co-cultured with M2 macrophages transfected with miR-210 inhibitors cells showed an increase in the percentage of apoptotic and mitochondrial-damaged cells (Fig. 4D–E).

These results demonstrate that M2 macrophages under miR-210 interventions inhibite HCC cells apoptosis and promote HCC cells survival and invasion ability.

3.5. miR-210-mediated autophagy of M2 macrophages contributes to its promotion of tumor invasion, proliferation and inhibition of apoptosis

To investigate whether autophagy regulated by macrophage-derived miR-210 contributes to the progression of HCC. We intervened the autophagy by adding the autophagy agonist rapamycin at a concentration of 200 nM or the autophagy inhibitor CQ at 50 μM to the co-culture system of M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors with HCC cells. Western blot and qRT-PCR showed the corresponding changes in autophagy-related proteins and mRNA (Fig. 5A–B). In addition to this, transmission electron microscopy also confirmed the altered autophagy (Fig. 5C). The ability of miR-210 in M2 macrophages to promote the proliferation and invasion of HCC cells HepG2 and MHCC-97H as well as to inhibit their apoptosis was further enhanced by the addition of rapamycin (Fig. 5D–G). In contrast, in the CQ groups, the promotion of proliferation and invasion and also the inhibition of apoptosis of HCC cells by miR-210 of M2 macrophage-derived miR-210 was reversed (Fig. 5H–K). Taken together, these consistent results demonstrate that M2 macrophage-derived miR-210-mediated autophagy contributes to its promotion of tumor proliferation, invasion and inhibition of apoptosis.

4. Discussion

Several studies have revealed a correlation between TAMs and HCC malignancy, prognosis and treatment resistance [16,17]. An in-vitro study found that TAMs reduced the pro-

apoptotic effect of sorafenib by inducing autophagy in HCC cells [18]. A dual role of autophagy in cancer has been widely demonstrated. miR-210 has been reported as an independent prognostic factor for HCC, positively correlated with its pathological grade, TNM stage, and angiogenesis [19]. In lung cancer, miR-210 overexpression regulates proliferation and autophagy in carcinoma cells through targeted inhibition of autophagy-associated protein 7 (ATG7) [20]. This is also similar to studies reporting the pro-oncogenic role of miR-210 in other cancers, such as miR-210–5p promoting oncogenic autophagy in osteosarcoma [21]. Our present study suggests that miR-210 expression is upregulated in M2 macrophages. miR-210 has been reported to be upregulated at variable fold changes in twelve cancer types, which also include HCC [22]. In a study of infectious diseases, miR-210 was found to be a non-genetic immunomodulator, and macrophages upregulated miR-210 in response to infection [23]. The novelty of our present study is prominent upregulation of miR-210 in M2 macrophages, rather than its change in HCC cells. This opens the possibility that the tumorigenic microenvironment promotes cancer malignancy via miR-210 upregulation in M2 macrophages. The selective over-expression of miR-210 in M2 macrophages is intriguing. It is possible that the abundance of Toll-like receptor (TLR) family members in macrophages confers selectivity for their miR-210 upregulation [24].

More importantly, the present study demonstrated that that miR-210 promoted autophagy and inhibited apoptosis in M2 macrophages (Fig. 2). Zhang et al. [25] screened some key genes involved in the development of osteosarcoma, including miRNAs, which may be related to the metastasis of osteosarcoma. They found miR-210 overexpression elevated Beclin-1 expression and decreased p62 expression in osteosarcoma cells. miR-210 may therefore function by promoting autophagy to influence the development of osteosarcoma. Shao et al. [26] showed that the administration of miR-29a significantly upregulated LC3II expression in macrophages, that miR-29a elevation enhanced macrophage autophagy. This is similar to our findings that miR-210 promotes autophagy in M2 macrophages. Notably, the expression of anti-inflammatory factors IL-8, IL-10, TGF-β1, etc. was increased in the miR-210 overexpression group, and the upregulation of immunosuppressive factors' expression has been shown to promote tissue repair and tumor progression, which is consistent with our findings [27]. To our knowledge, this is the first report that miR-210 promotes autophagy and inhibits apoptosis in M2 macrophages.

PI3K/AKT/mTOR serves as a common signaling pathway for HCC and autophagy, and the aberrant activation of this signaling pathway in different diseases is closely associated with miR-210 overexpression [28]. Our data show that miR-210 can target and inhibit PI3K/AKT/mTOR expression in M2 macrophages (Fig. 3). Similarly, miRNA-29a mediates macrophages autophagy to inhibit atherosclerotic plaque formation through downregulating the PI3K/AKT/mTOR pathway [26]. It is known that PI3K/AKT/mTOR is negatively correlated with autophagy. An inhibitory effect of miR-210 on PI3K/AKT/mTOR as shown in our study would favor autophagy. This serves an important mechanism for autophagy-promoting effect of miR-210 on M2 macrophages [29].

The next question to answer is whether an action of miR-210 on M2 macrophages as demonstrated in our experiments could actually affect the progression of HCC. We sought to investigate in this context whether miR-210 affects the HCC cell phenotype after

M2 macrophages treatment. In the experiments where M2 macrophages transfected with miR-210 were co-cultured with HepG2 or MHCC-97H cells, miR-210 was found to promote the invasion and proliferation of M2 macrophage-interacted HCC cells, yet inhibit their apoptosis (Fig. 4). miR-210 is upregulated in HCC and enhances the hypoxia-induced migration and invasion of HCC cells by targeting VMP1 [30]or by suppressing TIMP2 expression via HIF-1α/miR-210/HIF-3α regulatory feedback circuit [31]. miR-210 also promotes proliferation and reduces apoptosis of HCC cells [32]. M2 macrophages have been shown to promote tumor cell growth, metastasis and invasion [33]. Our findings demonstrate that miR-210 can exert an action on M2 macrophages which in turn promote HCC malignancy. Therefore, miR-210 and M2 macrophages may have synergistic pro-cancer effects. Notably, when we added miR-210 inhibitors to the co-culture system, the pro-cancer effects of M2 macrophages were attenuated (Fig. 4), establishing a causal relationship. We reason that the biological link between miR-210 and M2 macrophages in HCC affects the progression of HCC. Next, the autophagy agonist rapamycin or inhibitor CQ was added to the co-culture system. Results showed that the pro-HCC effect of M2 macrophages was further enhanced after promoting autophagy, while inhibition of autophagy suppressed miR-210 overexpression or M2-induced pro-cancer effects. This may be related to the fact that autophagy activation reduces the stability of NF-κB p65 and hence proinflammatory response and exacerbates poor M2 antigen presentation, facilitating tumorigenesis among other functions [10]. In macrophages, autophagy is involved in regulating specific immune functions such as pathogen clearance, antigen presentation, and extracellular proliferation [34]. Disruption of autophagy in the TME has been shown to engage antitumor immunity [35]. Furthermore, it has been reported that LC3-associated phagocytosis (LAP) deficiency induces pro-inflammatory gene expression, triggers STING-mediated type I interferon responses in TAMs, and promotes T cell-mediated antitumor immunity [36]. This suggests that atypical autophagy in TAMs may contribute to immune suppression in the TME. In ovarian cancer, genetic deficiency of autophagy element FAK family interacting protein of 200 kDa (FIP200) in macrophages resulted in TIM4+ TAM loss via ROS-mediated apoptosis, and elevated T cell immunity and tumour suppression in vivo, inhibiting peritoneal ovarian cancer progression [37]. Thus, autophagy activation in tumour-associated residential macrophages supports TAMs survival and enforces immunosuppression in the TME. In HCC, our data consistently demonstrated a malignancy-promoting role of autophagy in TME. This study uncovers the ability of miR-210 as an autophagic agonist of M2 macrophages to promote HCC progression via PI3K/AKT/mTOR pathways. The finding that M2 macrophages with miR-210 promoted autophagy excrete more anti-inflammatory cytokines TGF-β1 and IL-10 suggests that autophagic M2 macrophages can exert tumour-promoting action on HCCs via these cytokines. The specific mechanism underlying this effect remains to be explored.

The shortcomings of this study are that we used only one type of macrophage (THP-1) and the molecular mechanism of miR-210 promoting autophagy in M2 macrophages affecting HCC cell phenotype was not explored in greater depth. As the priority of this first study was to establish a definitive role of miR-210 in autophagy in M2 macrophages and such an action can promote HCC invasion and malignancy, M2 macrophages and HCC cell lines were used and the interactions studied in co-culture. Further studies in vivo experiments

with interventions of miR-210 memetics and inhibitors will be required to assess its translational value. Finally, the role of miR-210 in macrophage-HCC interactions remains to be clarified by more experiments.

5. Conclusions

In summary, the key findings of this study support the role of miR-210 in promoting autophagy of M2 macrophages, likely through PI3K/AKT/mTOR signaling pathway in HCC progression and malignancy. It adds new knowledge on the role of miR-210 in macrophages research and TAMs-targeted tumor immunotherapy. Specifically, this study provides new insights into the pathogenesis of HCC, and a theoretical basis for exploration of the key molecules and pathway involved for better diagnosis and treatment of HCC.

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Availability of data and materials

The data are available from the corresponding author upon reasonable request.

List of abbreviations

HCC	Hepatocellular carcinoma
TAMs	Tumor-associated macrophages
TME	tumor microenvironment
FBS	fetal bovine serum
PMA	phorbol 12-myristate 13-acetate
PBS	phosphate buffered saline
NC mimics	negative control mimics
NC inhibitors	negative control inhibitors
ELISA	enzyme-linked immunosorbent assay
IF	Immunofluorescence
PBST	PBS-Tween
ANOVA	one-way analysis of variance
ATG7	autophagy-associated protein 7

TLR	Toll-like receptor
TNBC	triple-negative breast

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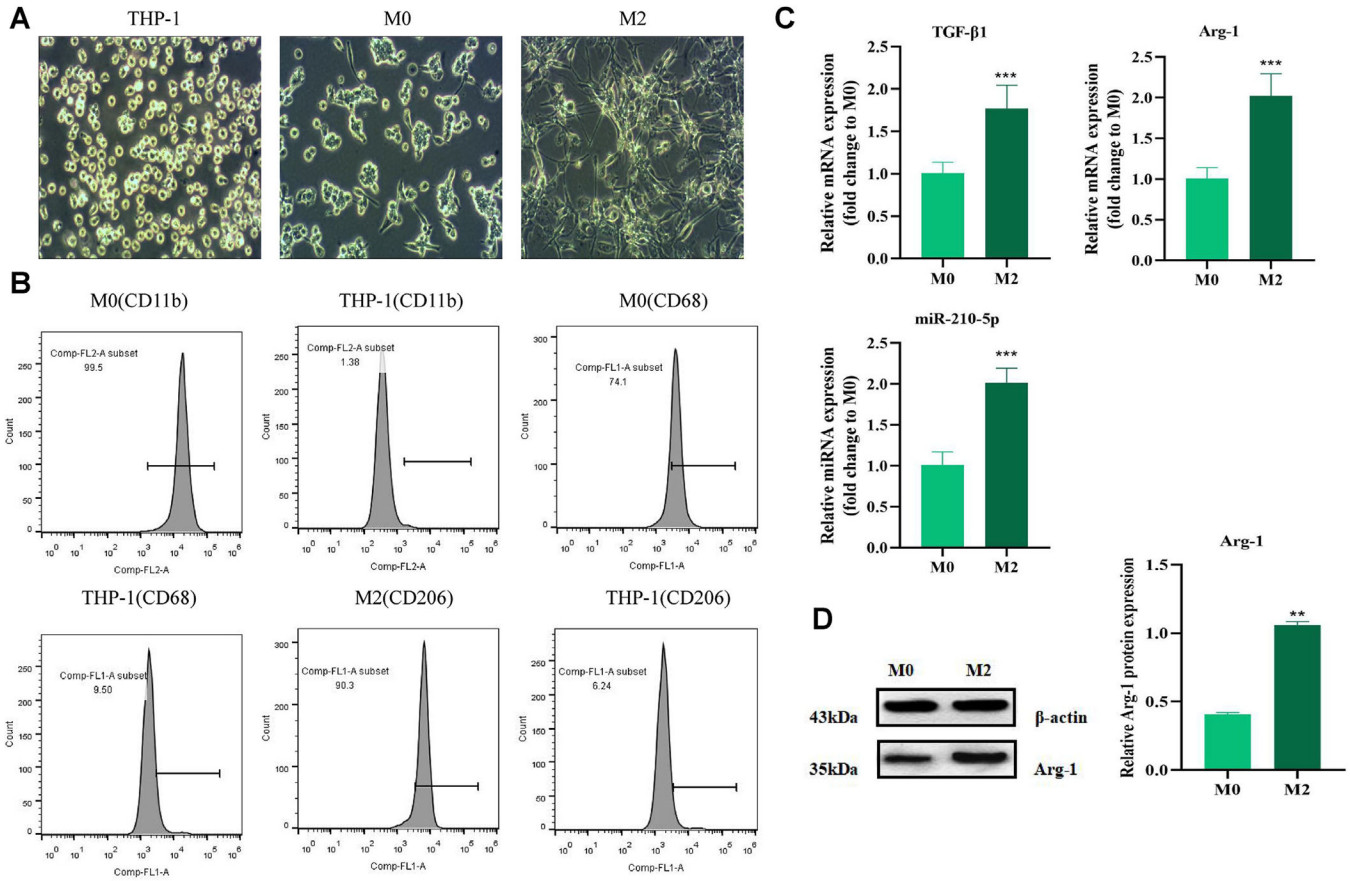


Fig. 1.

Differentiation of human THP-1 monocytes to M2 macrophages. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (A) Morphology of THP-1 monocytes, M0 macrophages (non-polarized THP-1 cells), and M2 polarized macrophages. (B) Flow cytometry showed cell surface expression of CD11b, CD68 and CD206 in the THP-1, M0, M2 populations. (C) The levels of expression of M2 macrophage markers mRNA and miR-210 were examined by qRT-PCR. All results showed relative fold changes relative to M0 (non-polarized THP-1) cells. (D) Detection of Arg-1 protein expression in M2 macrophages by Western blot.

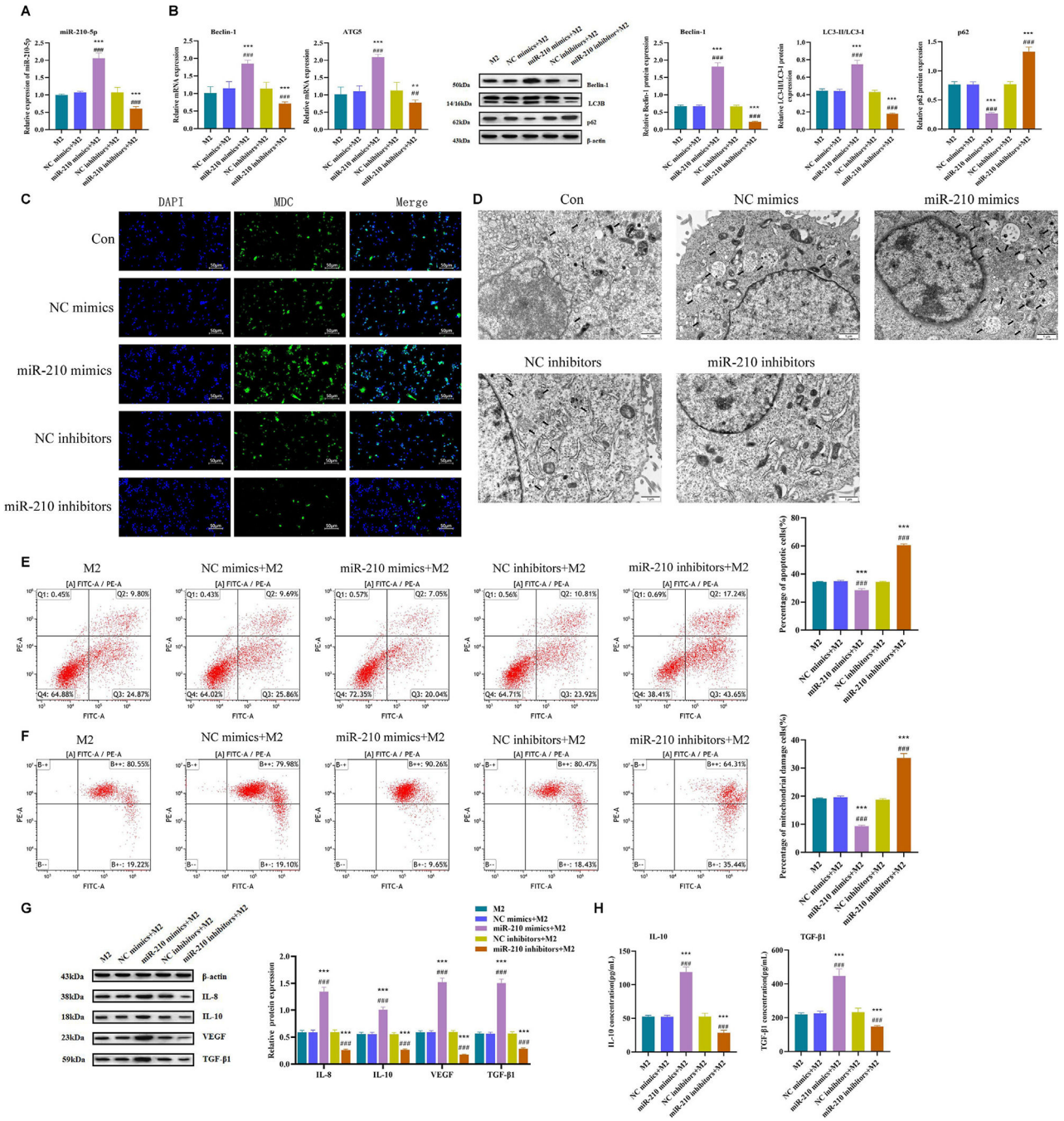


Fig. 2.

miR-210 promotes autophagy and inhibits apoptosis in M2 macrophages. M2 macrophages were transfected with miR-210 mimics and miR-210 inhibitors, respectively. "*" indicates compared with NC mimics group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$."#" indicates compared with NC inhibitors group, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$. $P > 0.05$ for unlabeled groups, the difference is not significant. (A)Expression of miR-210–5p in M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors. (B)Expression of autophagy-associated proteins in M2 macrophages transfected with miR-210 mimics

or miR-210 inhibitors. (C)Representative images of MDC fluorescent staining showing M2 macrophages undergoing autophagy. (D)TEM observation of autophagosomes in M2 macrophages. Scale bar = 1 μm. (E)Flow cytometry analyzed the apoptosis levels of M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors. (F)The JC-1 kit detected changes in mitochondrial membrane potential in M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors. (G)Western blot detected the expression of IL-8,IL-10,VEGF and TGF-β1 proteins in M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors. (H)The concentrations of IL-10 and TGF-β1 in the culture supernatants of M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors were measured by ELISA.

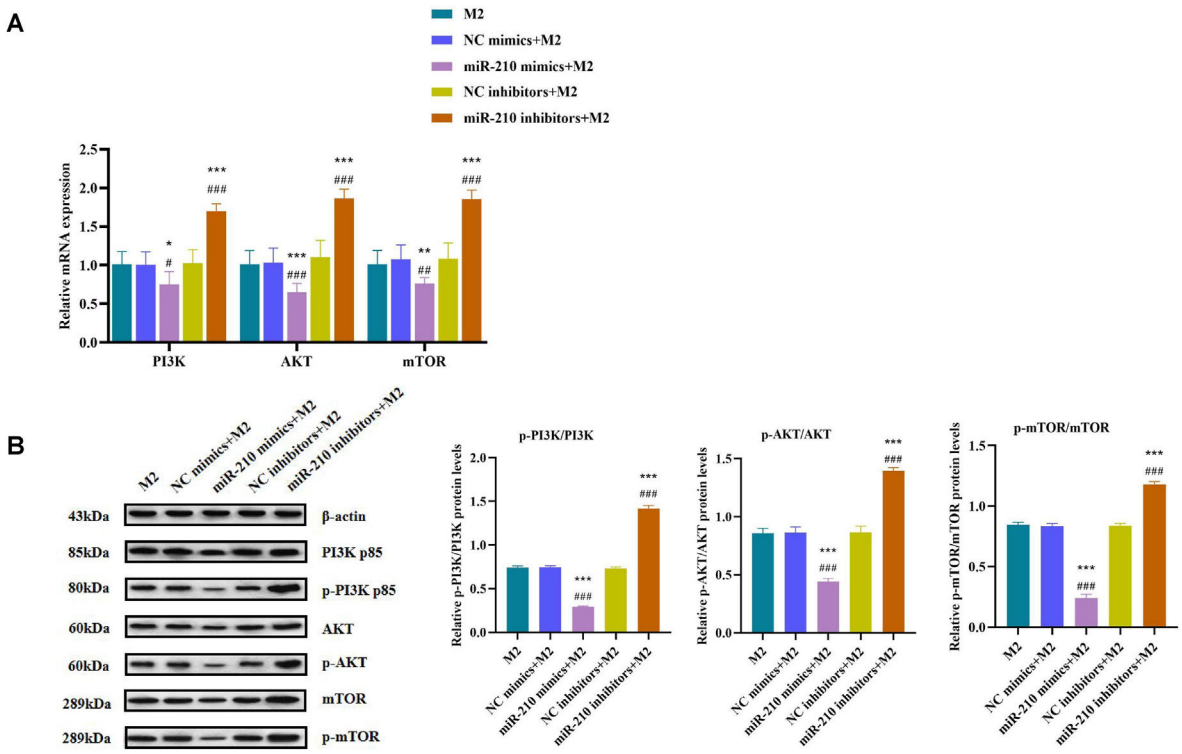


Fig. 3.

miR-210 targetes PI3K/AKT/mTOR signaling pathway in M2 macrophages. M2 macrophages were transfected with miR-210 mimics and miR-210 inhibitors, respectively. "*" indicates compared with NC mimics group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$."#" indicates compared with NC inhibitors group, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$. $P > 0.05$ for unlabeled groups, the difference is not significant. (A)Detection of PI3K, AKT and mTOR mRNA expression in M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors by qRT-PCR. (B)Western blot to determine the expression of PI3K, AKT and mTOR and phosphorylated proteins in M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors.

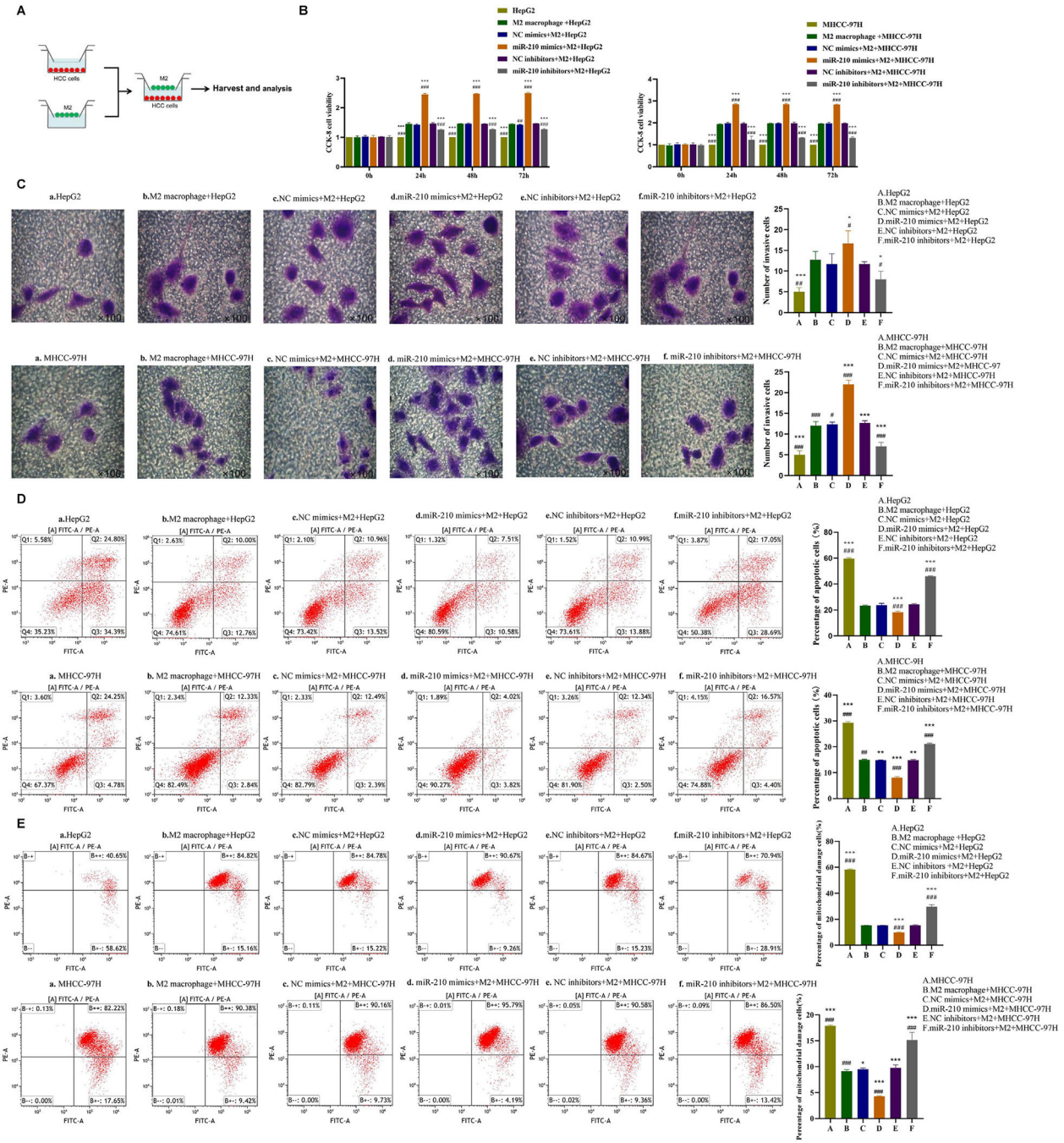
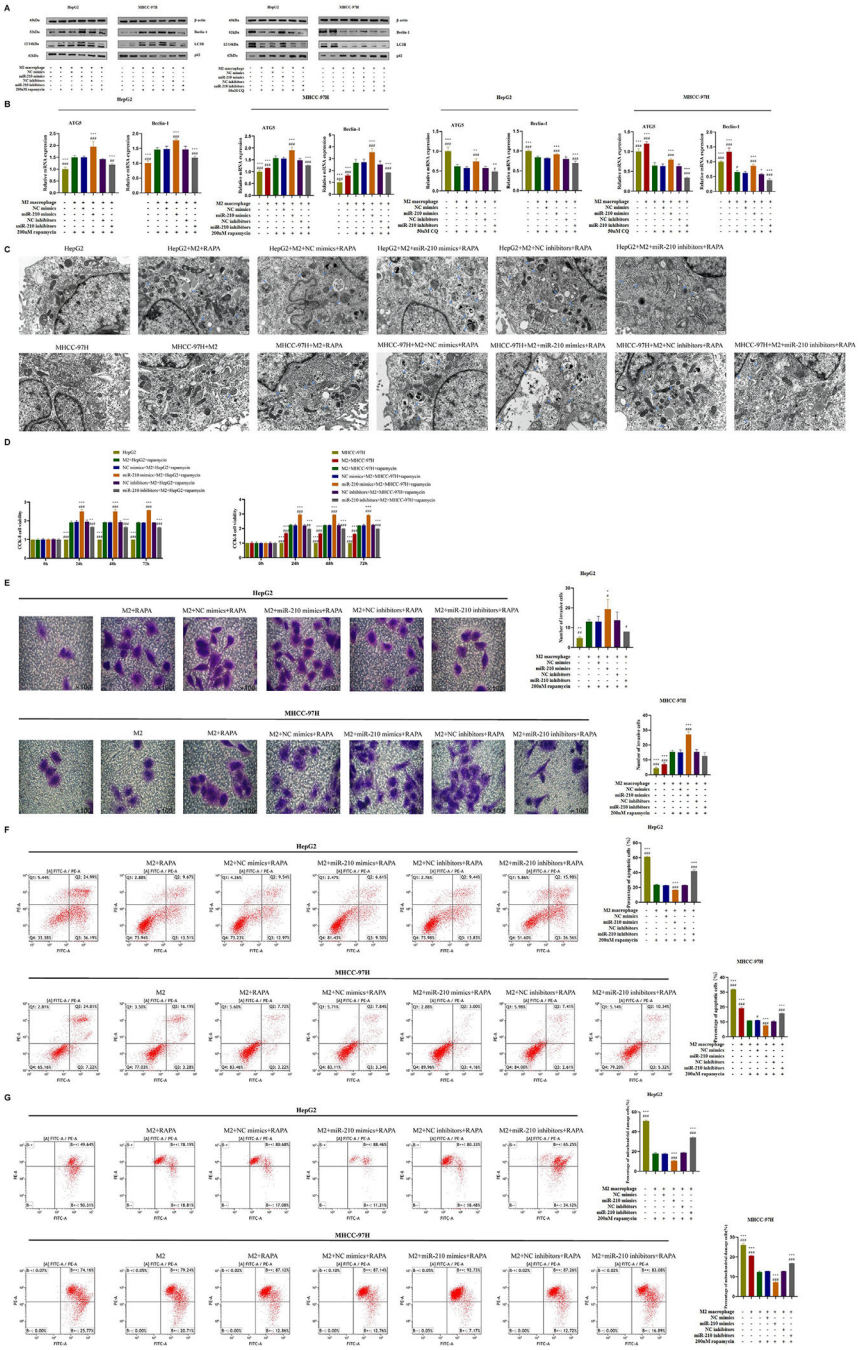


Fig. 4.

miR-210 preconditioned M2 macrophages promote proliferation, invasion and inhibit apoptosis of HCC cells. "*" indicates compared with M2 macrophage + HepG2/MHCC-97H group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. "#" indicates compared with NC inhibitors group, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$. $P > 0.05$ for unlabeled groups, the difference is not significant. (A)Co-culture model of M2 macrophages and HCC cells. (B)The proliferation of HCC cells co-cultured with M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors and the control groups were detected by CCK-8 assay. (C)The

invasive ability of HCC cells co-cultured with M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors and control cells was examined by transwell invasion assay. (D)Apoptosis of HCC cells co-cultured with M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors and control cells was detected by flow cytometry. (E)JC-1 staining and flow cytometry were used to detect mitochondrial damage in hepatoma cells co-cultured with M2 macrophages transfected with miR-210 mimics or miR-210 inhibitors and control cells.



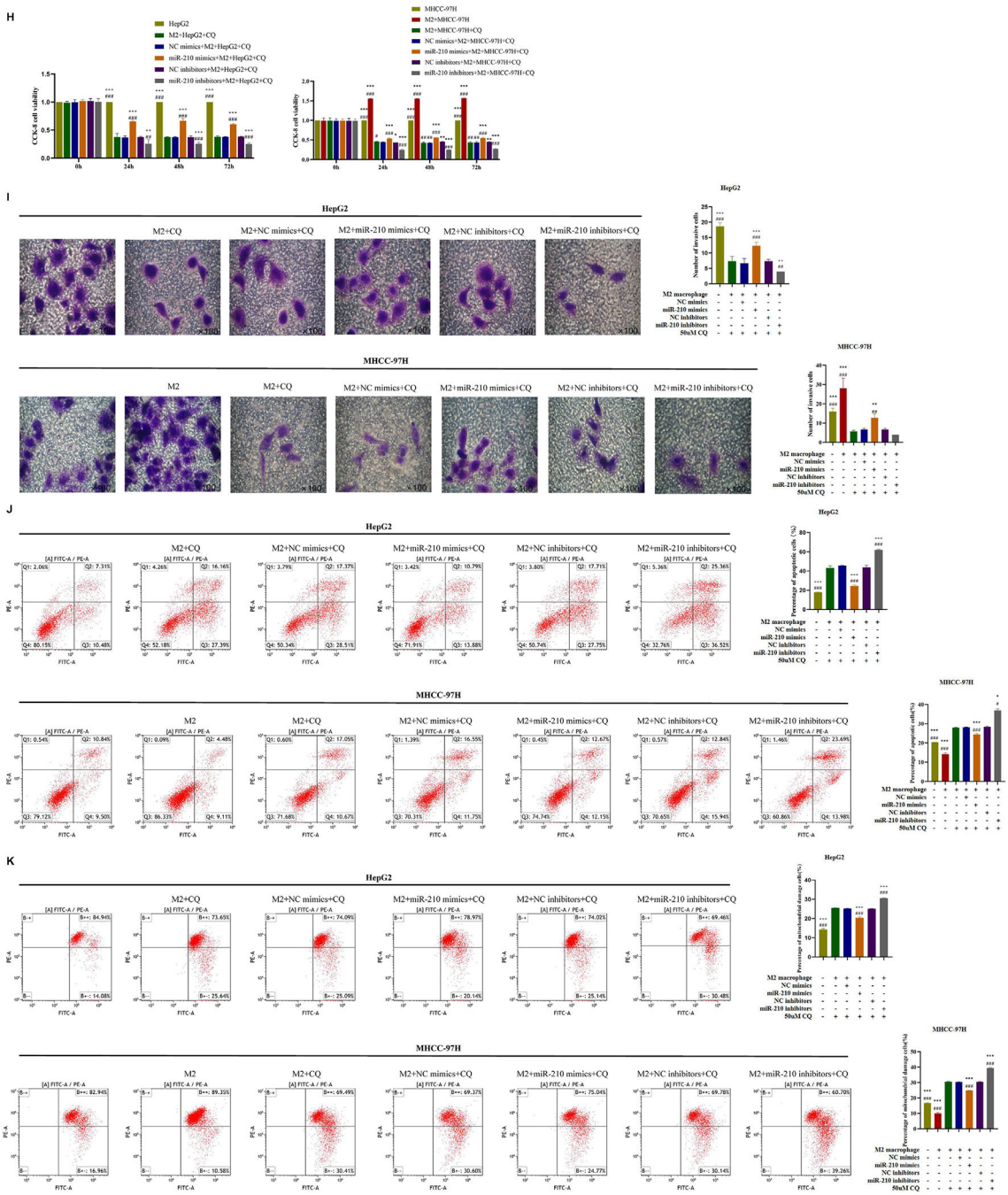


Fig. 5.

miR-210-mediated autophagy of M2 macrophages contributes to its promotion of tumor invasion, proliferation and inhibition of apoptosis. “*” indicates compared with M2 macrophage + HepG2/MHCC-97H + 200nM rapamycin/50μM CQ group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. “#” indicates compared with NC inhibitors + M2+HepG2/MHCC-97H + 200nM rapamycin/50μM CQ group, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$. $P > 0.05$ for unlabeled groups, the difference is not significant. (A)Expression of autophagy-associated proteins Beclin-1,LC3B and p62 in HCC cells of each group after adding 200 nM rapamycin

or 50uM CQ. (B)Detection of Beclin-1 and ATG5 mRNA expression in HCC cells of each group after adding 200 nM rapamycin or 50uM CQ by qRT-PCR. (C)TEM observation of autophagosomes in HCC cells. Scale bar = 500 nm. (D)Detection of proliferation of HCC cells in each group after adding 200 nM rapamycin by CCK-8 method. (E)Invasion of HCC cells in each group after adding rapamycin. (F)Flow cytometry detection of apoptosis in each group of HCC cells after adding rapamycin. (G)JC-1 staining was used to detect the mitochondrial damage in each group of HCC cells after the addition of rapamycin. (H) Detection of proliferation of HCC cells in each group after adding 50uM CQ by CCK-8 method. (I)Invasion of HCC cells in each group after adding CQ. (J)Flow cytometry detection of apoptosis in each group of HCC cells after adding CQ. (K)JC-1 staining was used to detect the mitochondrial damage in each group of HCC cells after the addition of CQ.

Table 1

Primer sequences for quantitative real-time PCR.

Gene	Amplicon Size (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
β-actin	96	CCCTGGAGAAGAGCTACGAG	GGAAGGAAGGCTGGAAGAGT
miR-210-5p	60	AGCCCCTGCCCACCGC	AGTGCAGGGTCCGAGGTATT
TGF-β1	133	GAGCCCTGGACACCAACTAT	CAGAAGTTGGCATGGTAGCCA
Arg-1	160	ACACTCCACTGACAACCACA	TCCACGTCTCTCAAGCCAAT
Bcl-2	103	GCGGCCTCTGTTTGATTTCT	TCACTTGTGGCCCAGATAGG
ATG5	131	CTGGGCTGGTCTTACTTTGC	GGCCAAAGGTTTCAGCTTCA
Beclin-1	163	GGCTCCCGAGGGATGG	GGGCTGTGGTAAGTAATGGA
PI-3K	138	TGTGGAGCTCGCTAAAGTCA	CACTCCTGCCCTAAATGGGA
AKT	167	CTTTCGGCAAGGTGATCCTG	GTACTTCAGGGCTGTGAGGA
mTOR	178	GGCCAATGACCCAACATCTC	CATGATGCGATGCTCGATGT