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MicroRNA200a Enhances Antitumor Effects in Combination with Doxorubicin in Hepatocellular Carcinoma

Xiao Cui [a,b], Dachen Zhou [a], Qiang Du [b], Peiqi Wan [c], Kun Dong [d], Hui Hou [a,*], David A. Geller [b,*]

[a] Department of Surgery, The Second Hospital of Anhui Medical University, Hefei, Anhui, China
[b] Department of Surgery, University of Pittsburgh, Pittsburgh, USA
[c] Department of Infectious Diseases, The First Affiliated Hospital of Guangxi Medical University, Nanning, China
[d] Department of Pediatric Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning, China

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ABSTRACT

Hepatocellular carcinoma (HCC) is often treated with doxorubicin. MicroRNAs have been shown to have important regulatory roles in cancer and serve as a target in chemoresistance. In this study, we investigated the effects of specific microRNA-200a (miR-200a) on HCC tumor cell growth and effect of doxorubicin-mediated cytotoxicity. Our results show miR-200a is downregulated in human HCC and HCC tumor cell lines. Increasing miR-200a expression inhibited HCC growth and synergized with the antitumor effects of doxorubicin. Inhibiting endogenous miR-200a promoted tumor growth and chemotherapeutic resistance. Increasing miR-200a expression inhibited tumor metabolism (ATP production, mitochondrial respiration, glycolysis), while inhibition of endogenous miR-200a reversed these effects. MiR-200a expression also increased autophagy and synergized with doxorubicin-mediated cytotoxicity. This study identifies a novel role of miR-200a in potentiating doxorubicin-mediated therapeutic effects in HCC.

Introduction

Hepatocellular carcinoma (HCC) is an important cause of death in patients with cirrhosis, ranking sixth in most common malignancies and third in cause of cancer deaths. Therapeutic resistance is a challenge in HCC treatment and often limits the effectiveness of antitumor drugs [1]. Elucidating the molecular signaling mechanisms in HCC may facilitate treatment strategies to improve the poor prognosis.

MicroRNAs have been shown to have important roles in cancer prognosis and serve as a target in chemoresistance [2]. MicroRNA-200a (miR-200a) is a member of miR-200 family and is known to exert effects on tumor progression, metastases, and anoikis in various tumor types including HCC [3,4]. Specific miR-200a target proteins include ZEB1/ZEB2, SIRT1, YAP1 modulating TGFβ, PI3K/AKT, and Hippo signal pathway [5].

While miR-200a has been shown to act as a potential biomarker for tumor diagnosis, the role of miR-200a in HCC treatment response is unknown. Our previous study showed that miR-200a slowed HCC progression by targeting CXCL1 to modify the host immune response [6]. MicroRNAs have promising roles in reprogramming tumor metabolism and autophagy, which are important in understanding chemotherapeutic resistance in tumors [7,8]. In this study, we investigated the effects of miR-200a in combination with doxorubicin, which is commonly used to treat HCC. We showed that miR-200a enhanced the antitumor effects of doxorubicin in HCC by directly regulating tumor metabolism and autophagy.

Materials and Methods

Human HCC samples were obtained from 30 patients who had undergone liver resection at the University of Pittsburgh between 2010 and 2017. All HCC patients were confirmed by pathological diagnosis. No neoadjuvant treatment for HCC was performed in these patients. The study protocol was approved by the university institutional review board committee. Written informed consent was obtained from all patients.

Cell Lines

The human cell lines Huh 7 and HepG2 were obtained from American Type Culture Collection (ATCC, Manassas, VA) maintained in Dulbecco's modified Eagle's media (GE Healthcare Bio-Sciences, Pittsburgh, PA) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD),

* Address all Correspondence to: David A. Geller, UPMC Liver Cancer Center, Montefiore 7 South, 3459 Fifth Avenue, Pittsburgh, PA 15213, or Hui Hou, Department of Surgery, The Second Hospital of Anhui Medical University, 678 Furong Road, Shushan District, Hefei, Anhui, China, 230601.
E-mail addresses: Hui0402@hotmail.com, (H. Hou), gellerda@upmc.edu. (D.A. Geller).

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100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA). Human normal liver hepatocytes were obtained from the NIH LTCDS program at the University of Pittsburgh. Cells were maintained in modified William's medium E media with 5% bovine calf serum. Cells were cultured at 37°C in incubator with 5% CO_2.

Real-Time Polymerase Chain Reaction

RNA isolated from cell lines and human HCC and adjacent nontumor tissue was obtained by treating with Trizol solution (Invitrogen, Carlsbad, CA). To test the expression of miR-200a, TaqMan miRNA cDNA synthesis kit and TaqMan universal master mix II, no UNG (Thermofisher, Pittsburgh, PA) were employed for cDNA synthesis and real-time polymerase chain reaction. The expression of miR-200a was calculated as $2^{-\triangle\triangle CT}$. U6 expression was used as the normalized control. All tests were performed three times.

Immunoblotting

Total protein was isolated from cell lines by using cell lysis buffer (Cell Signaling Technology, Denver, MA) according to manufacturer protocol. The Western blot assay was performed as standard procedure [9]. LC I, LC II, GAPDH, P62, β-catenin, and β-actin anti-human primary antibodies were used (Cell Signaling Technology, Danvers, MA). Pyruvate kinase M2 isoform (PKM2) and transcription factor A (TFAM) anti-human primary antibodies were purchased from Abcam, Cambridge, UK, and then incubated with secondary antibodies labeled with infrared dyes (Li-COR Bioscience, Lincoln, NE).

Immunohistochemistry Staining

Five-micrometer–thick paraffined sections were cut from paraffin- embedded specimens. Staining protocol used a standard process; Ki-67 antibody (Abcam, Cambridge, UK) and caspase 3 antibody (CST, Danvers, MA) were used. Images were obtained by Nikon E-800 microscope.

Transfection and Stable Expression Clone Selection

Packaged lentiviral pGC-GFP-miR-200a mimic and pGCSIL-GFP-miR-200a inhibitor were amplified (Genechem, Shanghai, China). Huh7 and HepG2 cells were transfected with lentivirus according to protocol. Stable expression clone was selected by adding puromycin (InvivoGen, San Diego, CA) and using flow cytometry (BD, Franklin Lake, NJ).

Cell Proliferation Assay

CCK8 Kit (Dojindo Molecular Technologies, Rockville, MD) was used to detect the proliferation of the cells according to the protocol instruction to test cell viability. Half-maximal inhibitory concentration (IC50) was tested by adding doxorubicin (Sigma Aldrich, St. Louis, MO) at 0, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 10, 100, and 1000 μM. The absorbance data were measured by BioTek microplate reader (BioTek, Winooski, VT). Rapamycin and 3-methyladenine (3-MA) were added as described (Sigma Aldrich).

Cell Cycle Assay

Cells were washed with ice-cold PBS and fixed with 70% ethanol when the confluence reached 80%. Cells were resuspended with propidium iodide solution (Thermo Fisher, Waltham, MA), separated, and assessed using flow cytometry (BD, Franklin Lake, NJ).

Cell Apoptosis Assay

Cells were washed with ice-cold PBS solution, resuspended, and incubated with Annexin V-APC (Ebioscience, San Diego, CA). The apoptosis rate was measured by cytometry (BD, Franklin Lake, NJ).

HCC Xenograft Animal Model Study

Murine experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and the Second Hospital of Anhui Medical University. Transfected Huh7 cells (1×10^7/mice) were injected into the right flank subcutaneously with 200 μl serum-free DMEM in 8-week-old male BALB/c (nu/nu) mice (Jackson laboratory, Bar Harbor, ME). Doxorubicin (4 mg/kg) (Sigma-Aldrich, St. Louis, MO) was injected through tail vein once every 2 days starting 6 days after tumor cell injection. Tumor size and mice weight (volume = length × width × width/2) were measured every 5 days until the 25th day after tumor cell injection. Mice were sacrificed and tumor mass was harvested for immunohistochemistry.

Autophagic Flux Analysis

mRFP-GFP-LC3 plasmid (HanBio, Shanghai, China) was used in transfected Huh 7 cells according to the manufacturer's instructions. Twenty-four hours later, cells were fixed with 4% paraformaldehyde and detected by a confocal microscope (Nikon A1, Nikon, Japan). Yellow (autophagosomes) and red puncta (autolysosomes) were quantified as autophagic flux. Three-methyladenine and rapamycin (Sigma-Aldrich, St. Louis, MO) were used to activate or inhibit autophagy. The experiments were repeated in triplicate.

Cell Metabolism Assay

The XF cell mito-stress test kit (Agilent Technologies, Wilmington, DE) was employed to evaluate cellular metabolism by using XF-24 analyzer (Agilent Technologies, Wilmington, DE) according to the manufacturer's introduction. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in triplicate.

Lactate Measurement

Secreted lactate from culture medium was measured using Glycolysis cell-based assay kit (Cayman, Ann Arbor, MI); excitation and emission wavelength was set at 530/590 nm in microplate reader in duplicate.

Statistical Methods

Data analysis in cells was performed with GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA). The difference among groups was compared using unpaired Student's *t* test or one-way analysis of variance. Clinical data analysis was done using SPSS 19.0 (SPSS, Inc., Chicago, IL). Statistical significance was set at $P < .05$.

Results

MiR-200a Expression Is Decreased in HCC

In 30 patients undergoing hepatic resection for HCC, miR-200a expression was significantly decreased in the HCC tumors compared to paired nontumor background liver (Figure 1*A*). Likewise, miR-200a expression was significantly decreased in human hepatoma cell lines Huh7 and HepG2 compared to normal primary human hepatocytes (hHC) (Figure 1*B*).

Tumors with low miR-200a expression tended to be predominantly fibrotic or cirrhotic (17/19 patients) (Table 1). While our sample size of 30 HCC patients showed statistically decreased miR-200a expression in the HCC tumors compared to paired nontumor background liver, we acknowledge that this was only 30 patients. Therefore, we also explored the possible correlation between miR-200a and clinicopathological characteristics in 377 HCC patients from The Cancer Genome Atlas database using analysis tool Oncomir [10]. The analysis shows that miR-200a is downregulated in HCC tissues compared with paired tissues, confirming the finding in a larger sample size (data not shown).

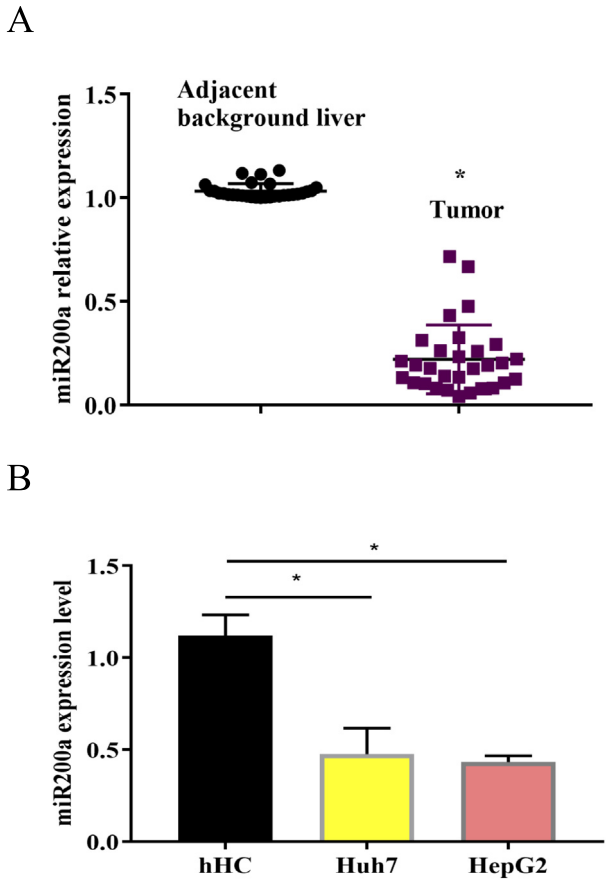


Figure 1. (A) MiR-200a expression levels were measured in human HCC tumors and adjacent background liver. (B) MiR-200a expression levels were measured in human HCC tumor cell lines Huh7 or HepG2, or normal human hepatocytes (hHC). * indicates $P < .05$.

MiR-200a Inhibits HCC Proliferation

To evaluate the function of miR-200a in HCC growth, we used lentivirus to stably transfect miR-200a mimic or miR-200a inhibitor into Huh7 and HepG2 cells, respectively (Fig. 2, *A* and *B*). Lv-miR-200a mimic resulted in >800-fold (Huh7) and >50-fold (HepG2) induction of miR-200a expression compared to vector control, while miR-200a inhibitor (Lv-anti-miR-200a) decreased miR-200a by over 50% in both cell lines.

MiR-200a expression inhibited HCC cell viability (proliferation), while miR-200a inhibition promoted cell growth (Figure 2, *C* and *D*). In cell cycle assay, miR-200a increased the percentage of cells in G1/G0 phase and decreased percentage in G2/M compared to controls (Figure 2*E* and Supplementary Fig. S1, *A-C*).

MiR-200a Impairs HCC Proliferation and Augments Doxorubicin-Induced Apoptosis in HCC Cells

We performed proliferation assay to investigate the effects that miR-200a on HCC proliferation and apoptosis in combination with doxorubicin. Increasing miR-200a expression significantly reduced HCC proliferation (Figure 3*A*) and IC50 of doxorubicin (Figure 3*B*) compared to control in Huh7 cells. Similar results were seen in HepG2 cells (S1*D*). With doxorubicin treatment, endogenous miR-200a induced S stage arrest (Figure 3, *C* and *E*; Supplementary Figs. S1*E* and S2*A*). MiR-200a expression alone did not induce apoptosis but did enhance apoptosis triggered by doxorubicin treatment, while miR-200a inhibition decreased doxorubicin-mediated apoptosis (Figure 3, *D* and *F*). Inhibition of endogenous miR-200a reduced apoptosis induced by doxorubicin. (Figure 3, *D* and *E*; Supplementary Fig. S2, *B* and *C*).

MiR-200a Synergizes with Doxorubicin to Inhibit HCC Tumor Growth In Vivo

We investigated the effect of miR-200a in mice xenografts in combination with doxorubicin. HCC gross tumor size (Figure 4*A*), tumor volume (Figure 4, *B*, *C*, and *D*), and tumor weight (Figure 4*E*) were significantly decreased by miR-200a expression. MiR-200a expression also synergized with doxorubicin to further inhibit tumor growth, while MiR-200a inhibition had minimal change from control. In the doxorubicin treatment group, the mice weight decreased (Figure 4*F*), and MiR-200a expression decreased Ki-67, increased caspase-3 protein, and potentiated effects of doxorubicin shown by immunohistochemistry staining (Figure 4, *G*, *H*, and *I*). We have performed new experiments and show that microRNA200a also enhances antitumor effects in combination with sorafenib in HCC cells. Lentiviral delivery of Mir-200a enhanced the growth inhibitory effect of sorafenib in human hepatoma Huh7 cells, while inhibition of miR-200a diminished the effects of sorafenib (data not shown).

MiR-200a Inhibits Oxidative Phosphorylation (OXPHOS) and Glycolysis Metabolism in HCC

MiR-200a negatively regulates β-catenin and TFAM by directly binding the 3′UTR sequence [11,12], thereby regulating glycolysis and OXPHOS in tumors. β-Catenin promotes glycolysis by activating PKM2 and AKT-mTOR signaling pathway [13,14], while deletion of TFAM suppresses glucose consumption and metabolism in tumors cells [15,16]. Here, we investigated the effects of miR-200a on mitochondrial metabolism by measuring OCR and glycolysis ECAR. Expression of miR-200a altered OCR in Huh 7 cells in a time-dependent manner (Figure 5*A*). Expression of miR-200a in Huh 7 cells decreased ATP production (Figure 5*B*), maximal respiration (OCR) (Figure 5*C*), glycolysis ECAR (Figure 5, *D* and *E*), and lactate concentration (Figure 5*F*). Doxorubicin decreased HCC tumor cell ATP production and

Table 1
Patient Tumor Differentiation and MiR-200a Expression

Patients' Clinical Characteristics	MiR-200a High Expression (*n*)	MiR-200a Low Expression (*n*)	*P* Value
Etiology			.964
HCV	2	4	
HBV	1	3	
Nonalcoholic steatohepatitis and alcohol liver disease	4	6	
Uncertain etiology	4	6	
Tumor size (cm)	5.7 ± 4.3	7.2 ± 4.4	.450
Differentiation			.042*
Well	7	4	
Moderately	3	12	
Poorly	1	3	
Liver background			.032*
Normal	5	2	
Fibrosis or cirrhosis	6	17	

* *means P* value < 0.05.

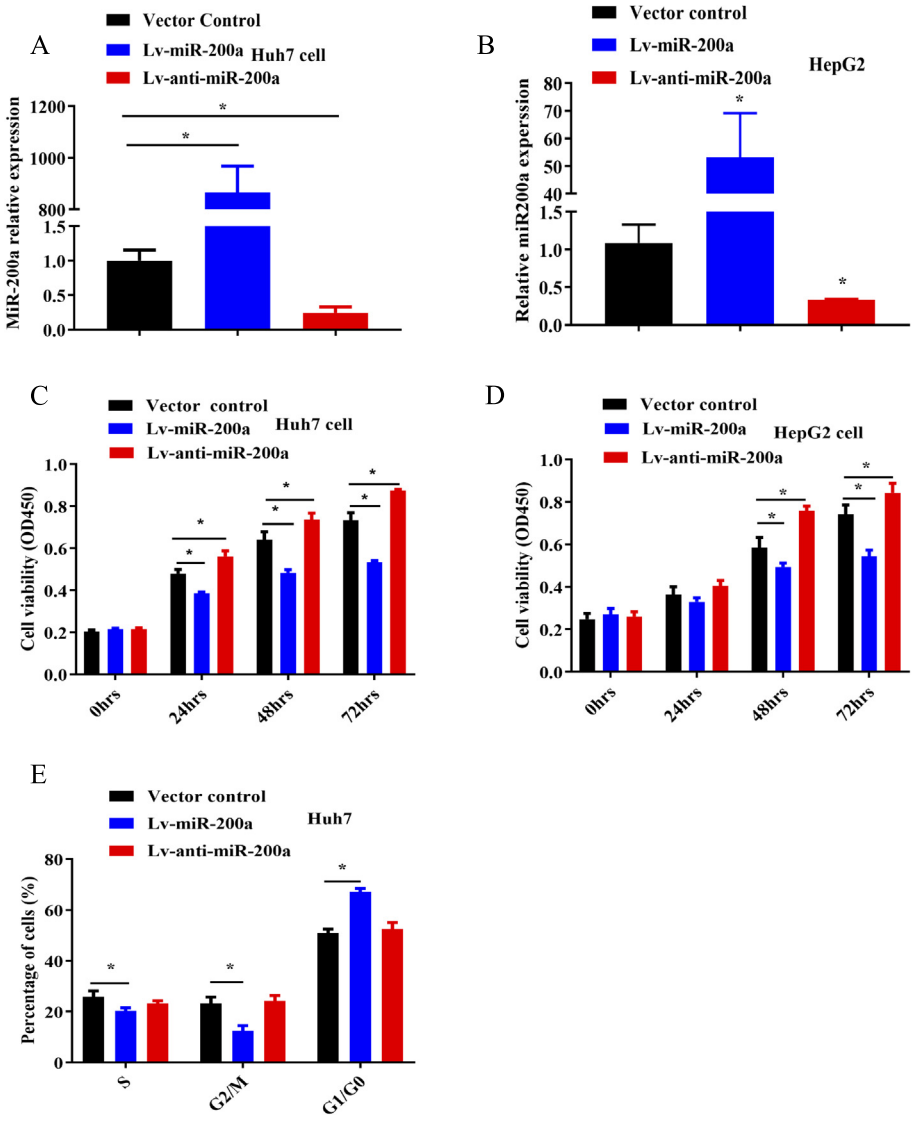


Figure 2. MiR-200a expression induced changes in HCC cell proliferation and cell cycle. (A, B) The effects of stable miR-200a expression in Huh7 and HepG2 cell lines. (C, D) The effects of miR-200a on the proliferation capacity on HCC cells. Upregulating miR-200a inhibited cell proliferation, while downregulating endogenous miR-200a promoted proliferation. (E) MiR-200a induced G1 cycle arrest in Huh7 cells ($P < .05$).

maximal respiration, and this was potentiated by miR-200a (Figure 5, *B* and *C*). Expression of miR-200a decreased β-catenin, PKM2, and TFAM and increased p53 protein expression, while inhibition of miR-200a had the opposite effects (Figure 5*G*).

MiR-200a Promotes Autophagy in HCC Cells

Autophagy is a lysosomal degradation process in cell under stress which exerts multiple roles in cancer proliferation [17]. Reprogramming metabolism in tumor cells favors autophagy induction to promote cell death and decrease chemotherapeutic resistance [18]. MiR-200a expression increased autophagy with increased ratio of LC3II/LC3I and decreased p62 proteins (Figure 6*A*). Likewise, miR-200a expression increased autolysosomes (red fluorescence) and autophagosomes compared to control (Figure 6, *B* and *C*), while inhibition of miR-200a decreased autophagy.

Increasing Autophagy Synchronized the Antitumor Effects of miR-200a in Doxorubicin Treatment

Increasing miR-200a promoted autophagy in combination with doxorubicin (Figure 7, *A* and *B*). Autophagy level decreased when endogenous miR-200a expression was inhibited (Figure 7, *A* and *B*). These results indicate that miR-200a expression enhanced autophagy and synergistically increased the doxorubicin-mediated cytotoxicity in HCC. To further confirm these effects, rapamycin (autophagy inducer) or 3-MA (autophagy inhibitor) was added. The synergistic effects of miR-200a expression on doxorubicin-induced autophagy were abolished by 3-MA, while rapamycin reversed the Lv-anti-miR-200a–mediated inhibition of autophagy (Figure 7, *A* and *B*). Cell viability was increased by inhibition of miR-200a in doxorubicin-treated cells at 48-72 hours, and this was reversed by rapamycin (Figure 7*C*). Cell viability was decreased by miR-200a expression and reversed by autophagy inhibition with 3-MA (Figure 7*D*).

Discussion

In HCC, resistance to chemotherapeutic drugs limits the effectiveness of antitumor treatment.

Host factors and tumor heterogeneity are important factors for HCC survival [19]. MicroRNAs regulate tumor growth and chemotherapeutic reactions by targeting tumor cells and associated noncancer cells [20,21]. In this study, the major findings are: 1) miR-200a is down-regulated in human HCC and HCC tumor cell lines; 2) increasing miR-200a expression

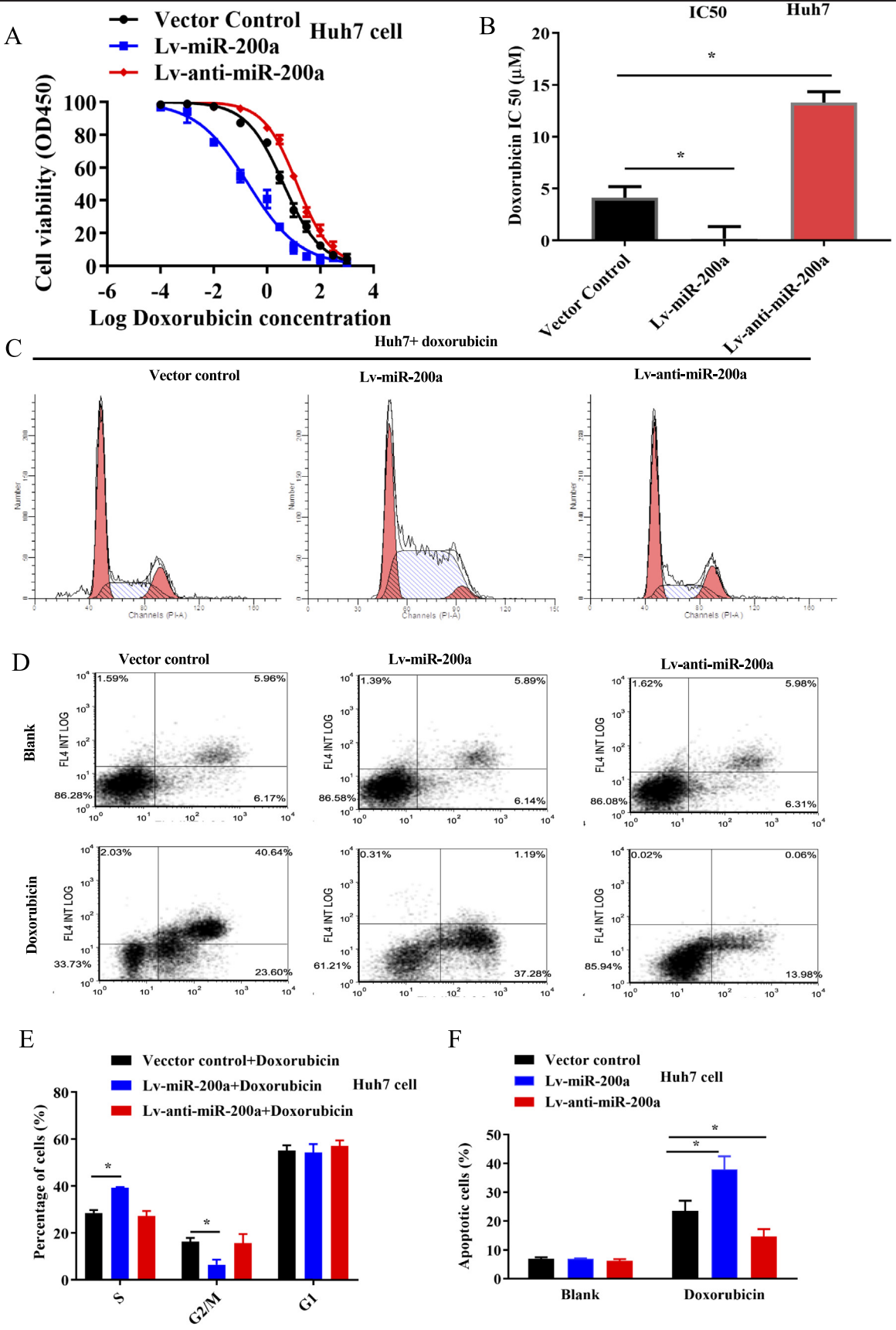


Figure 3. MiR-200a enhanced the antitumor effects of doxorubicin. (A, B) miR-200a reduced the IC50 of doxorubicin in Huh7 cells. (C, E) miR-200a combined with doxorubicin induced S stage cycle arrest in HCC cell. (D, F) miR-200a strengthened apoptosis effect of doxorubicin ($P < .05$).

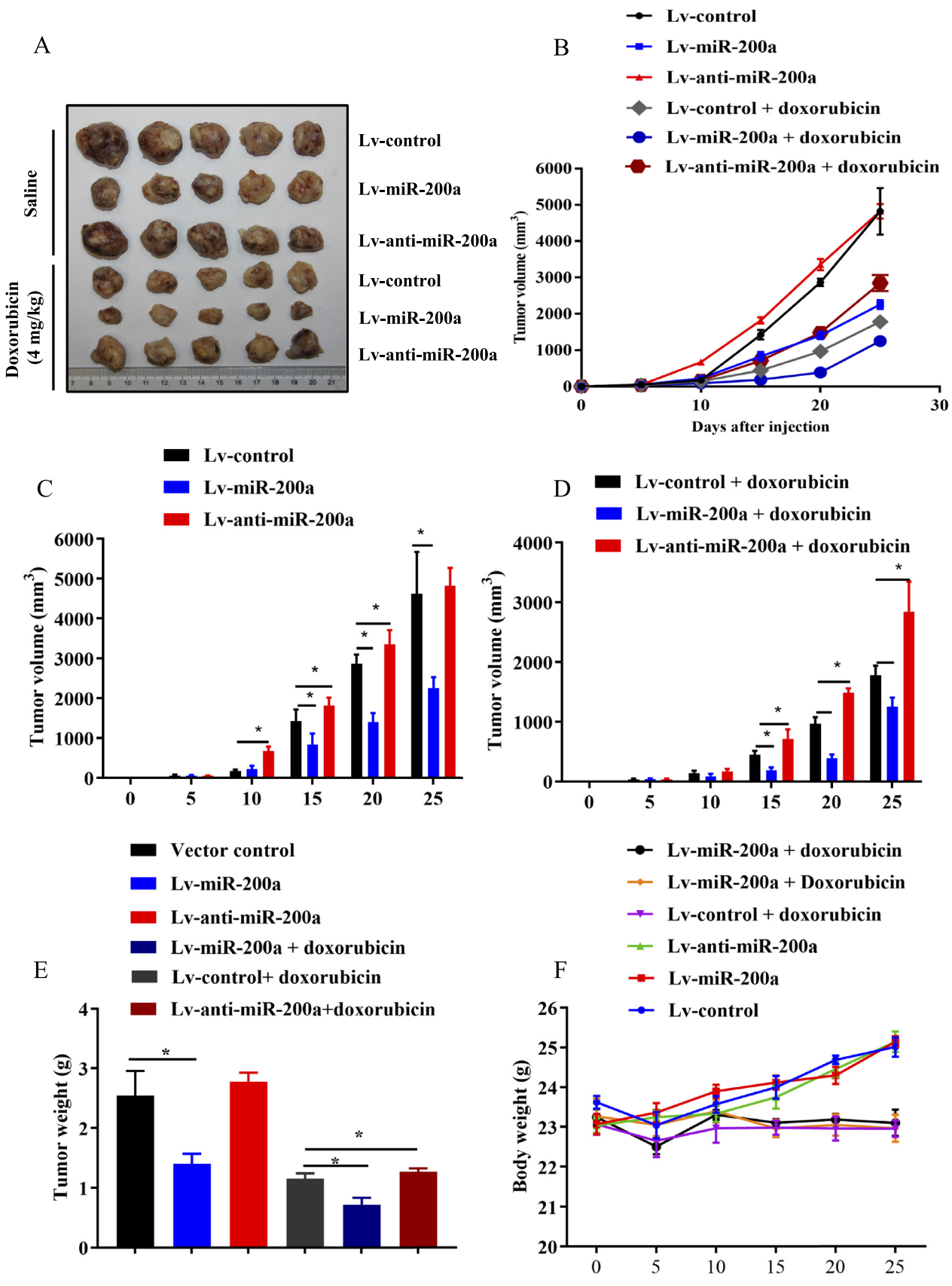
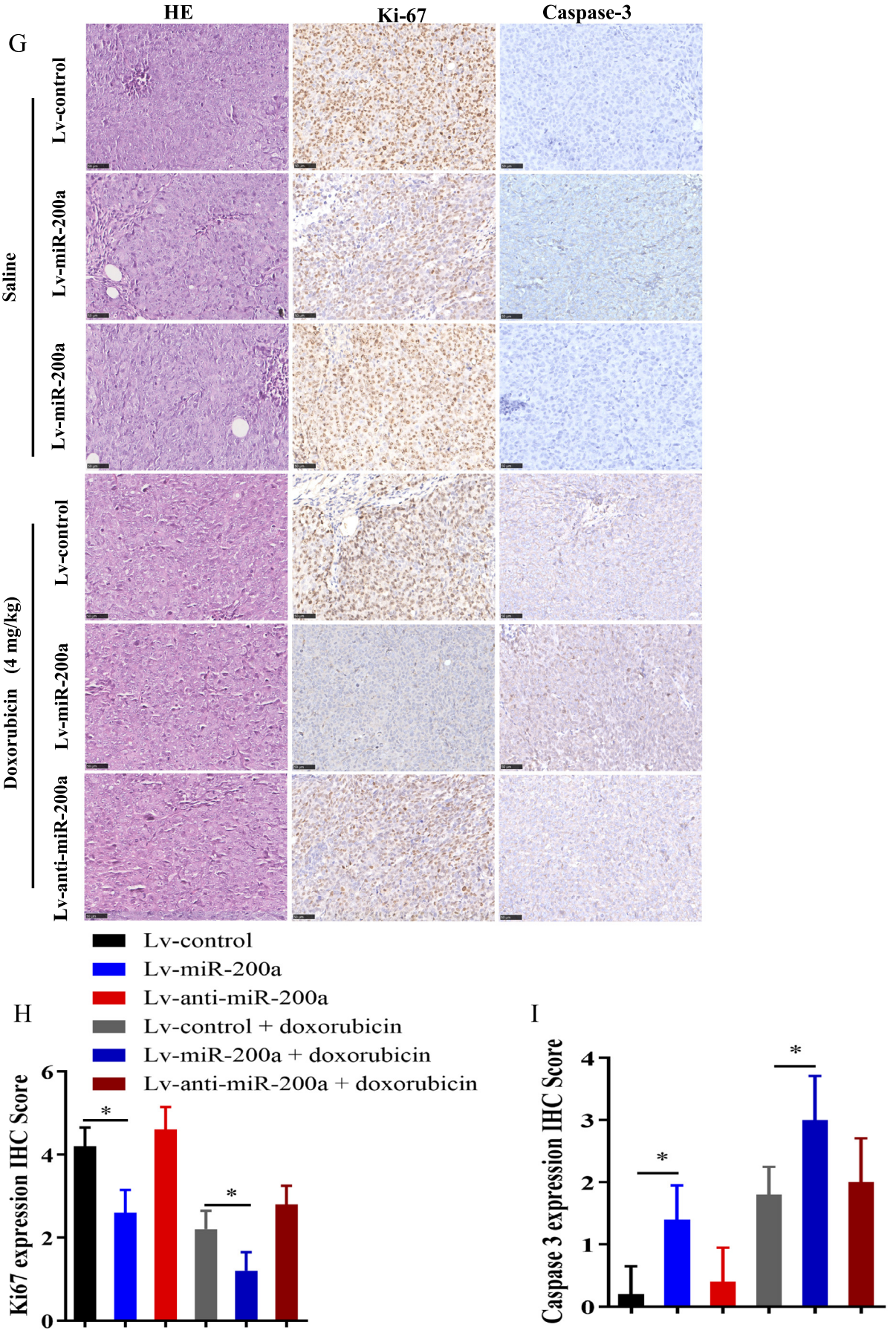


Figure 4. *In vivo*, miR-200a inhibited xenograft HCC tumor growth and enhanced doxorubicin-mediated effects. (A, B, C) miR-200a inhibited HCC growth size and weight in comparison to control, while reducing endogenous miR-200a promoted HCC growth. (D, E) Increasing miR-200a expression further suppressed tumor growth in combination with doxorubicin. Reducing miR-200a counteracted the inhibition effects caused by doxorubicin. (F) No significant weight loss was observed between different groups treated with doxorubicin. (G, H, I) Increasing miR-200a expression enhanced caspase 3 expression and downregulated Ki-67. Decreasing endogenous miR-200a further enhanced repression of Ki-67 and upregulation of caspase-3 expression induced by doxorubicin.



← **Figure 4.** (*continued.*)

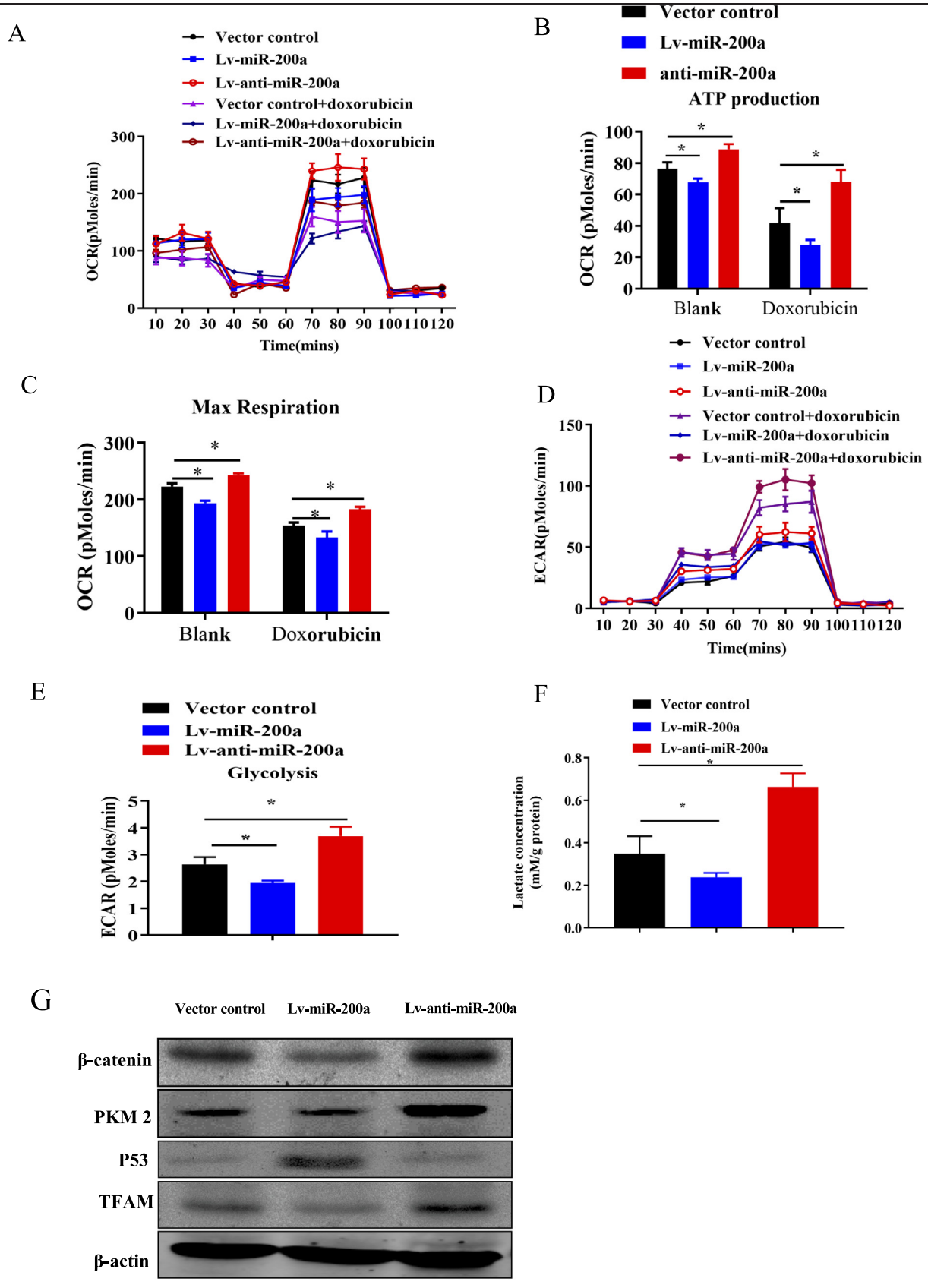


Figure 5. MiR-200a negatively regulated mitochondrial metabolism in HCC when treated with doxorubicin. (A) Modulating miR-200a regulated OXPHOS detected by OCR. (B, C) MiR-200a did not change basal respiration and ATP production in HCC, but in combination with doxorubicin, miR-200a significantly suppressed them. (D, E) The effects of modulating miR-200a in glycolysis process in Huh7 cells by measuring ECR. MiR-200a inhibited glycolysis, while down-regulating miR-200a increased glycolytic capacity. (F) MiR-200a regulated lactate expression in Huh7 cells. (G) MiR-200a downregulated the expression of β-catenin, PKM2, and TFAM and increased P53 expression in Huh7 cells.

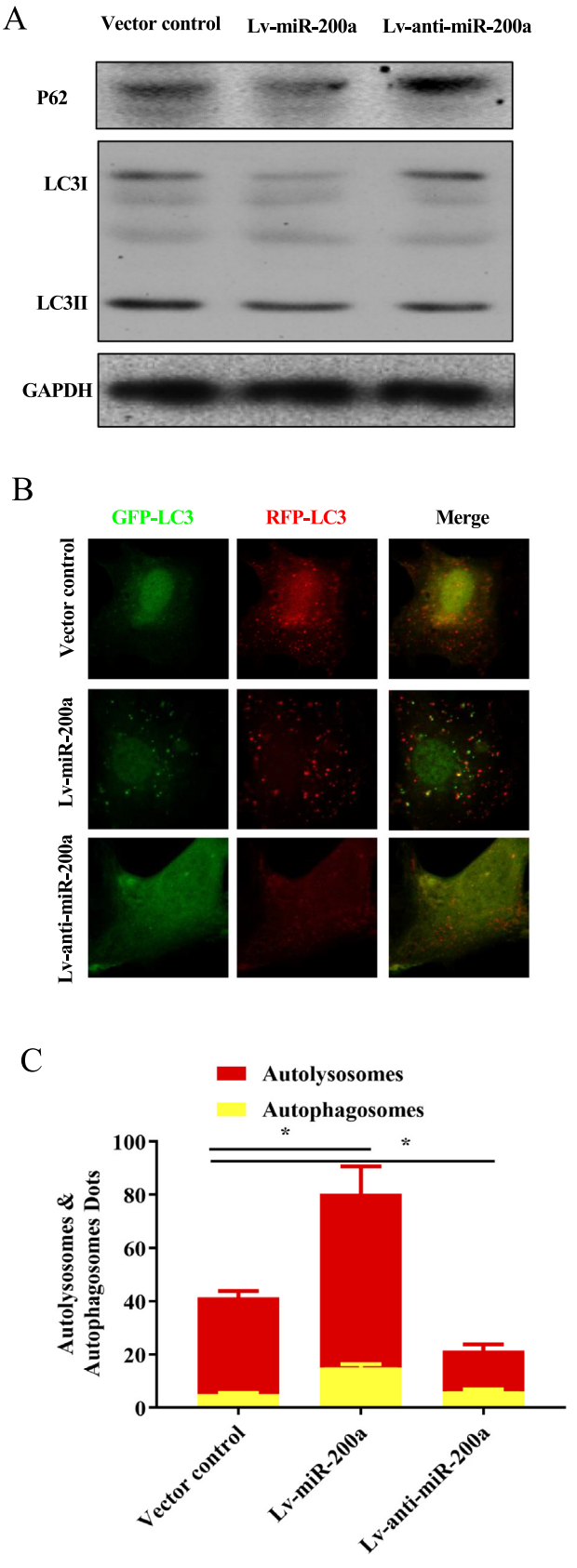


Figure 6. Endogenous miR-200a increased autophagy flux. (A) Increasing miR-200a decreased P62 expression and increased LC3II/I ratio expression. (B, C) MiR-200a increased the autolysosomes (red dots), while inhibiting endogenous miR-200a reduced them.

inhibited HCC growth and synergized with the antitumor effects of doxorubicin; 3) inhibiting endogenous miR-200a promoted tumor growth and chemotherapeutic resistance; 4) increasing miR-200a expression inhibited tumor metabolism (ATP production, mitochondrial respiration, glycolysis), while inhibition of endogenous miR-200a reversed these effects; 5) miR-200a expression increased autophagy and synergized with doxorubicin-mediated cytotoxicity.

Doxorubicin is cytotoxic agent commonly used in treating HCC that acts by inducing mitochondrial dysfunction and inhibiting cell metabolism [22]. Our findings showed that miR-200a suppressed tumor cell metabolism capacity through targeting OXPHOS and glycolysis process and inhibited lactate secretion. We confirmed that β-catenin, TFAM, p53, and PKM2 (modulators of mtDNA transcription) were regulated by miR-200a expression. Accumulating evidence shows that β-catenin has an important role in maintaining mitochondrial metabolism in liver tumor cells dependent on PKM2 by enhancing Warburg effects [14,23]. Reducing endogenous PKM2 in tumors reduced glucose uptake and suppressed aerobic glycolysis and production of lactate acid, while increasing PKM2 reversed the inhibitory effects [24]. Similarly, TFAM is a direct target of miR-200a and maintained the stability of mtDNA by activating p53 binding [25]. Decreasing TFAM expression changed tumor metabolism and reduced ATP production [15]. Recombinant human TFAM increased the mitochondrial biogenesis and increased OXPHOS [26]. We observed that miR-200a suppressed mitochondrial metabolism partly by targeting β-catenin and TFAM expression.

Autophagy is a homeostatic recycling mechanism in which cells face metabolic stress [27]. Autophagy can help cancer cells with high metabolic requirements to survive and resist cellular stress from antitumor therapy. However, noncytoprotective and cytotoxic autophagy either kills cells of its own or acts as a precursor to apoptosis [27]. In our findings, miR-200a increased autophagy flux. As a target protein of miR-200a, β-catenin bridged metabolism, autophagy, and chemotherapy resistance: it is not only a negative regulator of autophagy by directly modulating LC3 degradation and suppressing P62 transcription but also an efficient regulator in reducing sensitivity of chemotherapy by activating Wnt/β-catenin signal [28,29]. Inhibiting β-catenin renders HCC cells sensitive to doxorubicin [30]. Here, we found that miR-200a reduced β-catenin expression, increased LC3II/LC3I ratio, and reduced P62 expression, which positively regulated the autophagy process and enhanced sensitivity to doxorubicin. These effects were reversed by reducing miR-200a expression. The inhibition of growth in HCC cells resulting from doxorubicin treatment was further enhanced by miR-200a. Enhanced inhibition effects caused by increasing miR-200a expression under doxorubicin were suppressed by autophagy inhibitor 3-MA. These results suggest that miR-200a synchronizes antitumor effects with doxorubicin partly through regulating autophagy.

With regards to clinical correlation and HCC tumor features, mir-200a has been shown to be correlated with tumor differentiation, metastasis, and liver cirrhosis in several clinical studies [31–33]. MiR-200a is recognized to have an antifibrotic role in the progression of cirrhosis [34]. By targeting ZEB1/ZEB2, miR-200a reverted the epithelial-mesenchymal transition process which initiates transdifferentiation of epithelial cells that induce progression of fibrosis [35]. In addition, miR-200a also suppressed liver fibrogenesis through inhibiting hepatic stellate cell activation by targeting TGF-β2/TGF-β2 and β-catenin [36]. The significant alteration of miR-200a expression was noted during the process of liver carcinogenesis, including cirrhosis, low-/high-grade dysplastic nodule, and HCC [37].

Regulating microRNA expression in cancer therapy is a promising strategy. It has been reported that metformin (a common diabetes medication) also reprograms cell metabolism in tumor cells and cancer stem cells in part by directly upregulating miR-200a expression [38,39]. The antitumor effects of metformin have been well recognized in many malignant tumors. So, metformin could be a candidate agent to regulate miR-200a expression in clinical practice. This approach might require large animal studies before clinical trials.

In conclusion, miR-200a inhibited tumor mitochondrial metabolism and induced autophagy which enhanced apoptosis and suppression of

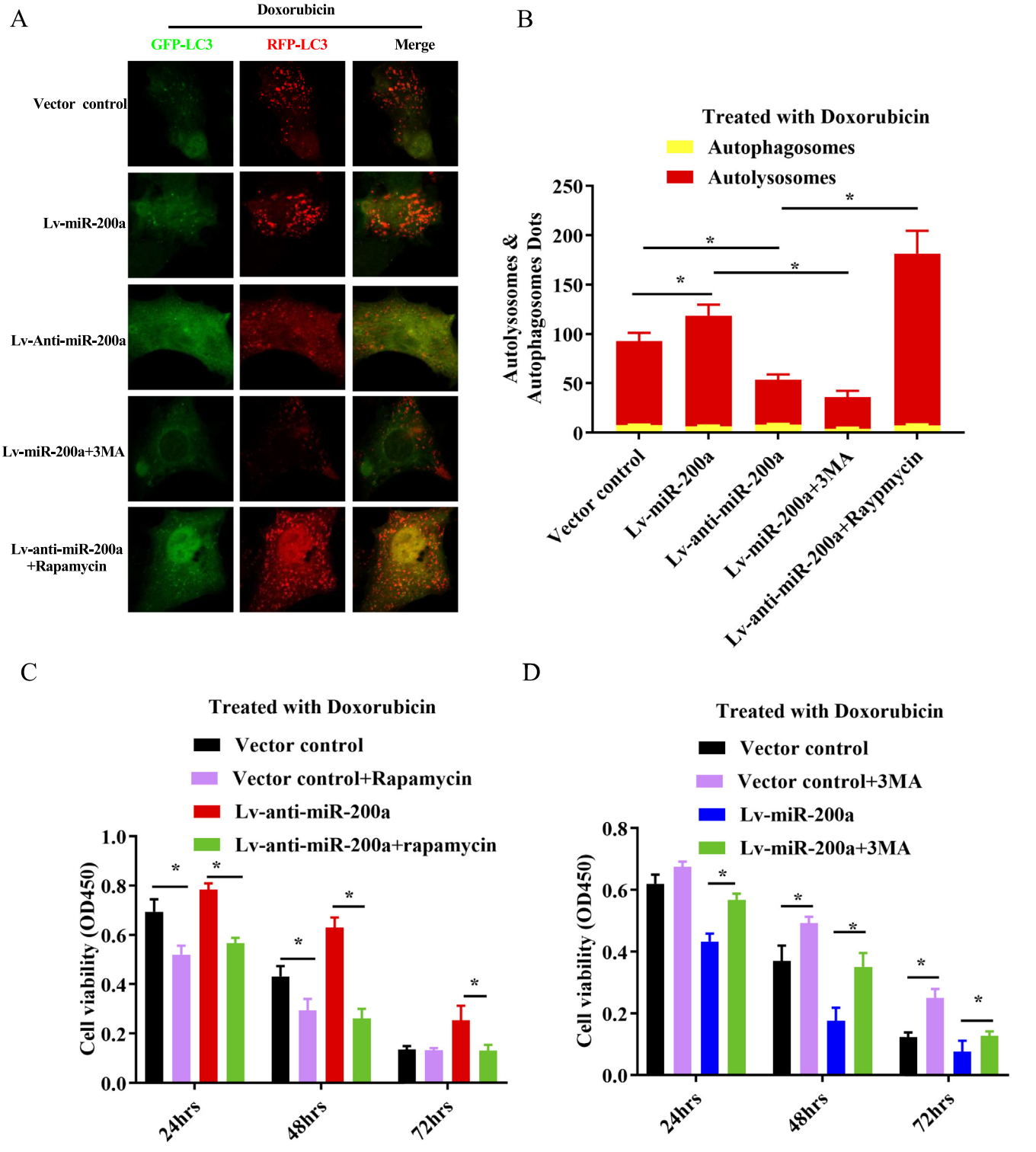


Figure 7. Increasing autophagy synergized the antitumor effects of miR-200a in doxorubicin treatment. (A, B) In combination with doxorubicin, miR-200a strengthened autophagy flux. (C) Rapamycin enhanced the antiproliferation effects of doxorubicin when endogenous miR-200a expression was inhibited. (D) Decreased cell viability caused by miR-200a was antagonized by 3-MA.

proliferation induced by doxorubicin in HCC. It may serve as a potential target for improved doxorubicin chemotherapy.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Design: Xiao Cui, David. A. Geller.
Methodology: Xiao Cui. Dachen Zhou, Qiang Du, Hui Hou,
Clinical and animal data: Xiao Cui, Qiang Du, Dachen Zhou, Peiqi Wan.
Data analysis: Xiao Cui, Kun Dong, Dachen Zhou, David. A. Geller.
Writing and reviewing manuscript: Xiao Cui, David. A. Geller.
Technique support: Qiang Du.
Study supervision: David. A. Geller and Hui Hou.
Hui Hou is considered as co-correspondence author.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100805.

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