

FTY720 Suppresses Liver Tumor Growth and Metastasis by Reducing Circulating Regulating T Cells and Enhancing the Anti-Tumor Effect of Rapamycin

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Chang Xian Li¹
Xin Xiang Yang²
Hong Wei Wang¹
Xiang Cheng Li¹
Kevin Tak-Pan Ng¹ 
Chung Mau Lo²
Kwan Man¹ 

¹Hepatobiliary Center, The First Affiliated Hospital of Nanjing Medical University, Key Laboratory of Living Donor Liver Transplantation, Nanjing, Jiangsu Province, People's Republic of China; ²Department of Surgery, The University of Hong Kong, Hong Kong, People's Republic of China

Background: In this study, we aimed to study the effect of FTY720 treatment in reducing circulating Tregs level and then suppressing liver tumor metastasis after hepatectomy and I/R injury in animal models. Furthermore, we also investigated the synergistic anti-tumor effect of FTY720 combined with rapamycin on hepatocellular carcinoma.

Methods: The effect of FTY720 on suppressing Tregs mobilization and tumor metastasis after hepatectomy was investigated in an orthotopic liver tumor rat model with hepatectomy and hepatic ischemia/reperfusion (I/R) injury. The synergistic anti-tumor effect of FTY720 combined with rapamycin was further explored both in in vitro functional study and in orthotopic liver tumor mouse model.

Results: In rat model, hepatic I/R promoted tumor metastasis and increased circulating Tregs after hepatectomy. The treatment of FTY720 reduced liver tumor metastasis and the number of circulating Tregs. Furthermore, FTY720 enhanced the anti-tumor capacity of rapamycin by inhibiting tumor cell proliferation and migration in vitro and reducing tumor growth in vivo through suppressing hepatic stellate cell activation and tumor angiogenesis.

Conclusion: FTY720 suppressed liver tumor growth and metastasis by reducing the population of circulating Tregs and enhancing the anti-tumor effect of rapamycin. It was suggested that FTY720 single or combined with rapamycin might provide novel insight for suppressing tumor growth and metastasis for HCC patients.

Keywords: FTY720, rapamycin, regulatory T cells, hepatic ischemia/reperfusion, tumor recurrence

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers and the third-leading cause of cancer-related death worldwide. More than 50% of new cases occur in China.¹ Liver surgery such as hepatectomy and liver transplantation are curative treatment options for selected HCC patients.² However, tumor recurrence and metastasis after surgical treatment is quite common, with 5-year recurrence rates reaching 70% after liver resection and 35% post-liver transplantation.³ Hepatic ischemia reperfusion (I/R) injury is an inevitable consequence during liver surgery.⁴ Increasing clinical and experimental evidences have shown that hepatic I/R injury promoted tumor recurrence after liver transplantation or liver resection.⁵⁻⁷ Hepatic I/R injury can trigger a series of inflammatory cascades, which

Correspondence: Kwan Man
Department of Surgery, The University of Hong Kong, L9-55, Faculty of Medicine Building, 21 Sassoon Road, Hong Kong, People's Republic of China
Tel +852 39179646
Fax +852 39179634
Email kwanman@hku.hk

may not only activate the cell signaling pathways regulating tumor cell invasion and migration, but also mobilize the circulating progenitor and immune cells including regulatory T cells (Tregs) to facilitate tumor recurrence and metastasis.^{8,9} Targeting at hepatic I/R injury and reducing Tregs mobilization may be potential therapy for suppressing tumor growth and metastasis after liver surgery for HCC patients.

FTY720 is an immunomodulatory drug and has been approved by the Food and Drug Administration for treating multiple sclerosis through down regulating sphingosine-1 phosphate receptor inhibited lymphocyte egress from lymphoid tissues.¹⁰ Baer et al reported that FTY720 inhibited T cell activation through inhibition of distal TCR signaling.¹¹ FTY720 was also demonstrated to prolong allograft survival in several solid organ transplantation by regulating lymphocytes migration and apoptosis.¹² Several groups have shown that FTY720 can attenuate hepatic I/R injury by ameliorating acute-phase inflammatory response and up-regulating several protective genes including heat shock proteins and anti-apoptotic genes.¹³ Furthermore, FTY720 has been demonstrated a strong anti-tumor effect on liver cancer, breast cancer, bladder cancer and so on.^{14–17} FTY720 enhanced the anti-tumor activity of carboplatin and tamoxifen in a patient-derived xenograft model of ovarian cancer.¹⁸ Our previous data also showed that FTY720 suppressed liver tumor metastasis after hepatectomy and I/R injury through reducing the numbers of circulating endothelial progenitor cells.¹⁹

In this study, we aimed to study the effect of FTY720 treatment in reducing circulating Tregs and then suppressing liver tumor metastasis in an orthotopic rat liver tumor model. Furthermore, we also investigated whether FTY720 treatment can enhance the anti-tumor effect of rapamycin. The significance of this study will hopefully open a novel therapy to inhibit liver tumor growth and metastasis after liver surgery for HCC patients.

Methods

Rat Liver Tumor Model

The rat liver tumor cell line McA-RH7777 purchased from the American Type Culture Collection (number CRL1601, ATCC, Manassas, VA, USA, $5 \times 10^5/100\text{ul}$) was applied for establishing the orthotopic Rat liver tumor model described in our previous paper.¹⁹ In order to investigate the role of hepatic I/R injury in liver tumor metastasis, major hepatectomy (the left lobe with tumor)

was performed with or without hepatic I/R injury (The right portal vein and hepatic artery were clamped for 30 minutes) after two weeks orthotopic liver tumor implantation. Rats were housed in a standard animal laboratory with free activity and access to water and food. The animals were obtained from Animal Lab of The University of Hong Kong and had been licensed according to Animal Ordinance Chapter 340 by the Department of Health, Hong Kong Special Administrative Region (4325–17). The welfare of the laboratory animals was followed by guidelines of The University of Hong Kong committee on the use of animal livers in teaching & research (M.292/108).

In order to explore the role and mechanism of FTY720 in tumor growth and metastasis after major hepatectomy and I/R injury. The treatment of FTY720 (2 mg/kg) or saline water (control group) were administrated through the portal vein before and after I/R injury. Circulating Tregs were detected at days 0, 1, 3, 7, 14, 21 and 28. The Buffalo rats were sacrificed under anesthesia with overdose pentobarbitone (100mg/kg) at day 28 for investigating tumor metastasis and detecting rat bone-marrow Tregs.

Nude Mice Orthotopic Liver Tumor Model

In order to investigate the synergistic anti-tumor effect of FTY720 combined with rapamycin, the nude mice orthotopic liver tumor model was established, which was the same as the rat orthotopic tumor model. One week after tumor implantation, rapamycin alone or combine with FTY720 were used for the treatment of liver cancer. In the single treatment group, rapamycin was given by intraperitoneal injection at 0.8mg/kg/3 days. In the combination treatment group, rapamycin (0.5mg/kg) and FTY720 (2mg/kg) were administrated by intraperitoneal injection every 3 days. The mice were sacrificed at day 20, 30 and 40 after treatment, respectively. The tumor growth, proliferation (Ki67), and apoptosis (TUNEL) were compared among control group, rapamycin alone treatment group and rapamycin combined FTY720 treatment group. Hepatic stellate cell activation and angiogenesis (CD34) in the tumor tissue were compared.

Detection of Circulating and Bone-Marrow Tregs by Flow Cytometry

The numbers of circulating and bone-marrow Tregs were detected by flow cytometry. The details of flow cytometry were described in our previous paper.⁸ Cells were stained

with PE-Cy5-conjugated anti CD4 (eBioscience, San Diego, CA) and PE-conjugated anti CD25 (BD Pharmingen, San Diego, CA), and then permeabilized with fixation/permeabilization working solution and incubated with FITC-conjugated anti-Foxp3 (eBioscience, San Diego, CA). The matched isotype controls were also prepared at the same time.

Hematoxylin and Eosin (H&E), TUNEL and Immunohistochemical Staining

The histological changes and cell TUNEL were detected by H&E staining and TUNEL staining, respectively. The expressions of Foxp3, CD34, ki-67 and α -smooth muscle actin (α -SMA) were detected by immunohistochemical staining (IHC). The details of H & E, TUNEL and IHC staining were described in our previous paper.²⁰

Cell Culture, Colony Formation Assay and Wound Healing Assay

The human liver cancer cell line MHCC97L was obtained from the Liver Cancer institute of Fudan University, Shanghai, the People's Republic of China. The use of this cell line was approved by The University of Hong Kong institutional research ethics committee. In order to explore the effect of FTY720 combined with rapamycin in proliferation and migration of HCC cells, colony formation assay and wound healing assay were performed by treating the cells with 4 different conditions: (1) control group without any treatment; (2) 1 μ g/mL of rapamycin; (3) 1 μ M of FTY720 and (4) 1 μ g/mL of rapamycin plus 1 μ M of FTY720.

Statistics and Data Analyses

Continuous variables were expressed as median with range. Mann-Whitney *U*-test was used for statistical comparison. χ^2 test was used to compare incidence of intrahepatic and lung metastasis after hepatectomy and I/R injury. $P < 0.05$ was considered statistically significant. Calculations were performed by using the SPSS computer software version 15 (SPSS, Chicago, IL).

Results

Hepatic Ischemia-Reperfusion Promoted Liver Tumor Local and Distant Metastasis After Hepatectomy

In order to investigate the effect of ischemia-reperfusion on the liver tumor metastasis after hepatectomy, a rat orthotopic liver tumor model with local and distant

metastatic potentials was established. Tumor metastasis after hepatectomy is presented in Figure 1A. Compared to the control group, hepatic I/R injury promoted liver tumor intrahepatic and lung metastasis after major hepatectomy. The incidence of intrahepatic metastasis was much higher in the I/R injury group [14/22 (63.6%)] compared to that in the control group [5/20 (25%)]. Furthermore, the incidence of lung metastasis increased from 6/20 (30%) to 12/22 (54.5%) after hepatic I/R injury (Figure 1A). Compared to the control group, the infiltrative growth pattern of the tumor and venous invasion was mainly found in I/R injury group (Figure 1B).

Hepatic Ischemia-Reperfusion Increased the Circulating Tregs After Hepatectomy

Tregs can suppress the immune responses against tumors and thereby promote tumor growth. In order to investigate the role of hepatic I/R injury in Tregs mobilization after hepatectomy, we analyzed the number of Tregs at different time points after hepatectomy with or without hepatic I/R injury. Before hepatectomy, there was no difference of circulating Tregs between the I/R group and control group. The results showed that the number of circulating Tregs was reduced at day 1 after the major hepatectomy in both groups. More circulating Tregs were found in the I/R injury group at day 3, 7, 14 and 28 after hepatectomy than those without I/R injury (Figure 1C).

The Treatment of FTY720 Repressed Liver Tumor Metastasis After Hepatectomy and Hepatic I/R Injury

The result showed that compared with control group, the treatment of FTY720 decreased the incidence of intrahepatic metastasis (12/18 (66.7%) vs to 7/19 (36.8%); $P = 0.07$). Similarly, the incidence of lung metastasis was reduced from 10/18 (55.6%) to 6/19 (31.5%) after FTY720 treatment (Figure 2A, B). Intrahepatic and lung metastasis were confirmed by histology examination (Figure 2C).

The Treatment of FTY720 Reduced the Number of Circulating and Bone-Marrow Tregs

Before the hepatectomy and I/R injury, there was no difference in circulating Tregs between the treatment group and control group (Figure 3A). The number of circulating Tregs was significantly decreased after

A

	I/R injury group	Control group	P value
Intrahepatic metastasis	14/22 (63.6%)	5/20(25%)	0.01
Lung metastasis	12/22 (54.5%)	6/20 (30%)	0.1

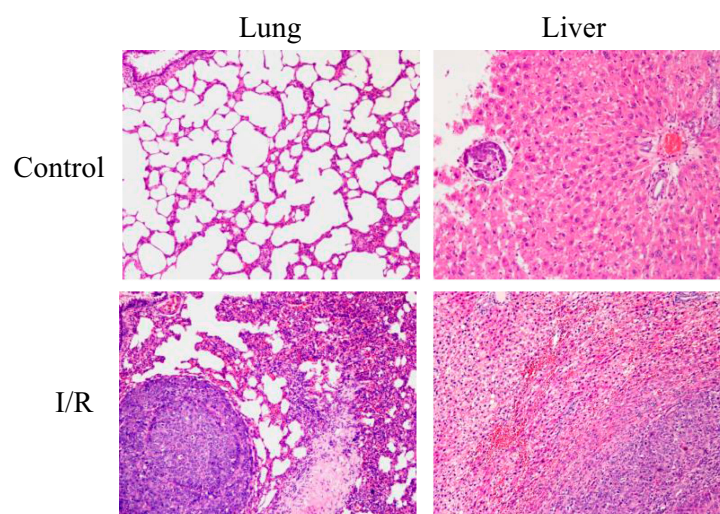
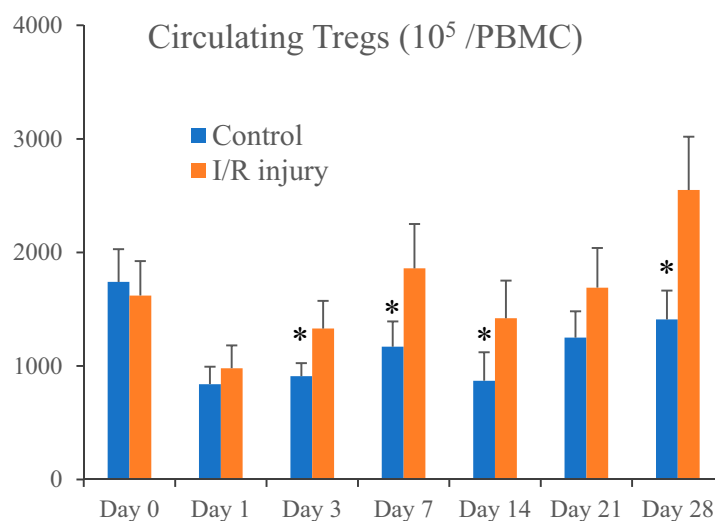
B**C**

Figure 1 Hepatic ischemia-reperfusion promoted tumor metastasis and increased circulating Tregs after hepatectomy. **(A)** Hepatic ischemia-reperfusion promoted tumor metastasis after hepatectomy. **(B)** Histological features of tumor metastasis after hepatectomy with or without hepatic I/R injury. **(C)** Hepatic ischemia-reperfusion increased circulating Tregs after hepatectomy. *P< 0.05.

A

	Control group	Treatment group	P value
Intrahepatic metastasis	12/18 (66.7%)	7/19 (36.8%)	0.07
Lung metastasis	10/18 (55.6%)	6/19 (31.6%)	0.14

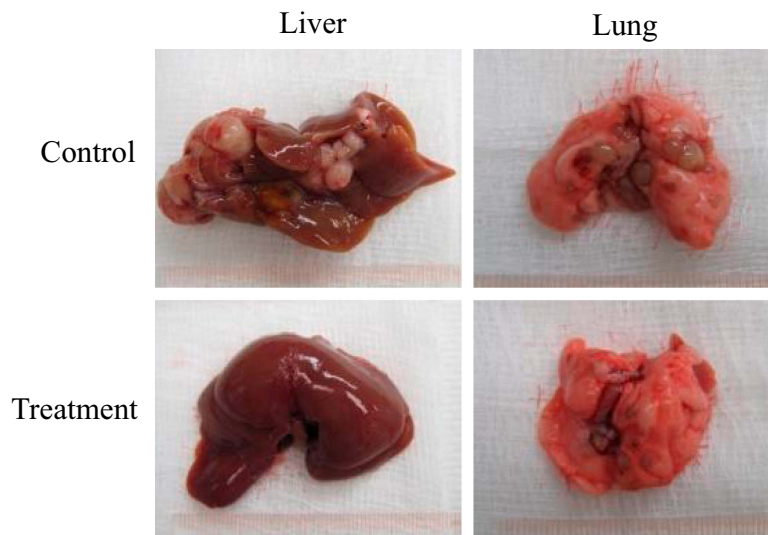
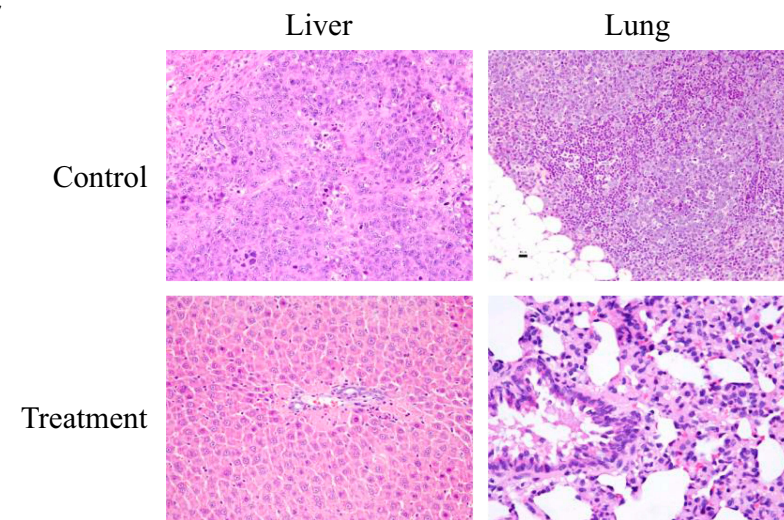
B**C**

Figure 2 FTY720 suppressed liver tumor intrahepatic and lung metastasis after hepatectomy and I/R injury. **(A)** FTY720 suppressed liver tumor intrahepatic and lung metastasis after hepatectomy and I/R injury. **(B)** Intrahepatic and lung metastasis were confirmed in specimen at four weeks after liver resection and I/R injury. **(C)** Histological features of tumor metastasis at four weeks after hepatectomy and I/R injury with and without FTY720 treatment.

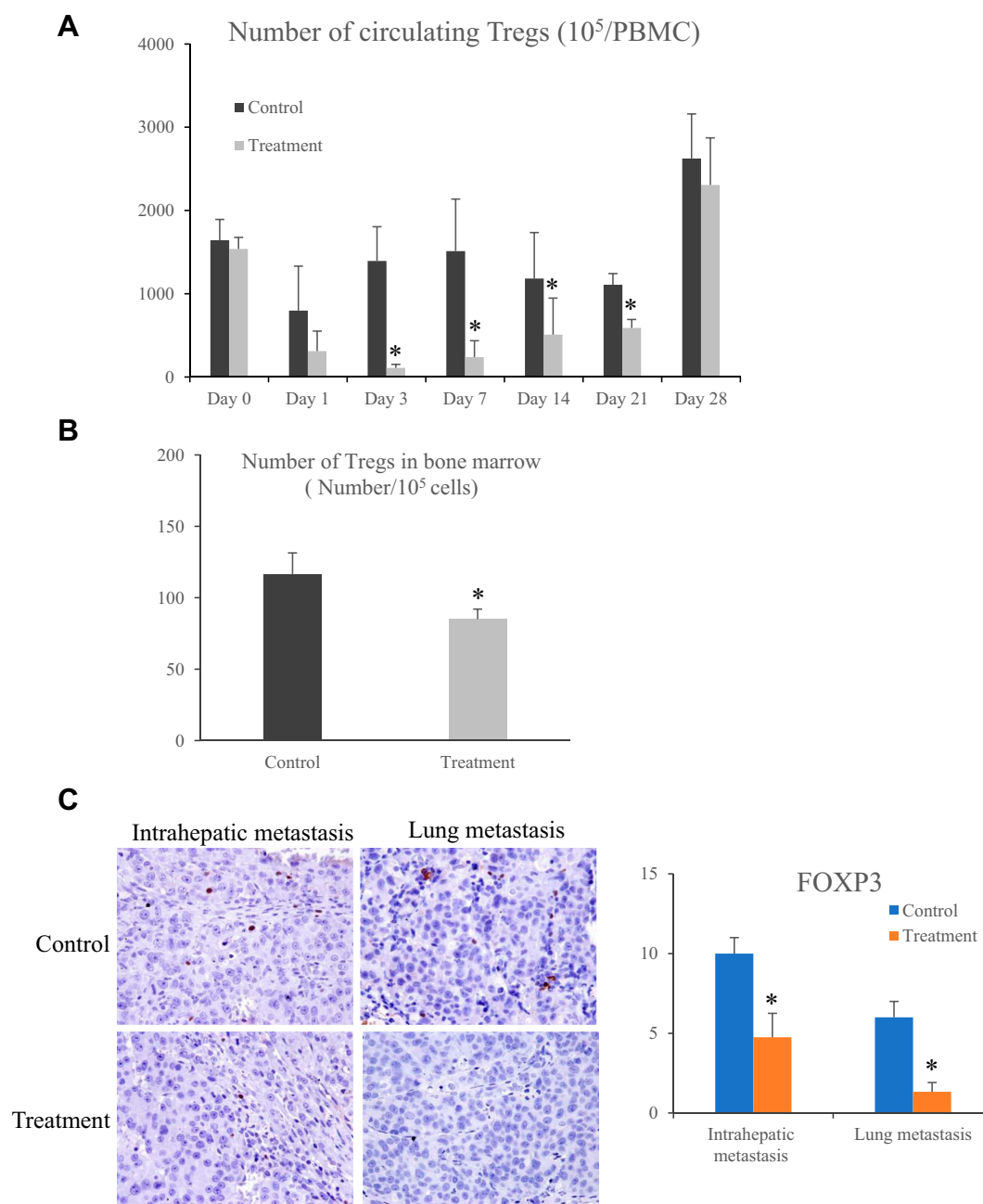


Figure 3 The treatment of FTY720 reduced the number of circulating and bone marrow Tregs. **(A)** Comparison of circulating Tregs at different time points after the major hepatectomy and partial I/R injury. **(B)** The treatment of FTY720 decreased the number of Tregs in bone marrow at day 28 after hepatectomy and I/R injury. **(C)** there were less Foxp3 positive cells in both recurrent liver and lung tumors after FTY720 treatment * $P < 0.05$.

FTY720 treatment at day 3, 7, 14 and 21 after hepatectomy and I/R injury (Figure 3A). Furthermore, the treatment of FTY720 also decreased the number of Tregs in bone-marrow at day 28 after hepatectomy and I/R injury compared to the control group (116.5 vs $85/10^5$ Cells, $P < 0.05$) (Figure 3B). In current study, we also found that there were less Foxp3 positive cells both in recurrent liver and lung tumors after FTY720 treatment (Figure 3C).

FTY720 Enhanced Anti-Tumor Capacity of Rapamycin by Inhibiting Proliferation and Migration

In order to explore whether FTY720 treatment can enhance the anti-tumor effect of rapamycin, colony formation assay and wound healing assay were performed in vitro functional study. In colony formation assay, the result showed that the treatment of FTY720 or rapamycin

alone reduced the colony number and size (Figure 4A). More importantly, the colony number and size were significantly decreased in FTY720 combined with rapamycin group. Furthermore, the combination treatment significantly suppressed migration of HCC cells in wound healing assay (Figure 4B).

FTY720 Combined with Rapamycin Suppressed Tumor Growth and Invasiveness in Mice Liver Cancer Model

In nude mice liver cancer model, the treatment of rapamycin suppressed tumor growth at day 20, 30 and 40 after mice orthotopic liver tumor implantation compared with control group (Figure 5A). More importantly, the treatment of FTY720 combined with rapamycin further suppressed tumor growth compared to rapamycin group. A relatively clear margin between tumor and adjacent non-tumor tissues was found in the FTY720 combined with rapamycin group. On the contrary, invasive patterns of tumor growth were present in control group (Figure 5B). Furthermore, compared to control group, there were more apoptotic cells and less ki-67 positive cells in FTY720 combined with rapamycin group (Supplementary Figure 1). The results suggested that the treatment of FTY720 may enhance the anti-tumor growth and invasiveness capacity of rapamycin.

FTY720 Inhibited Hepatic Stellate Cells Activation and Tumor Angiogenesis

The ability to manipulate the microenvironment is one of the hallmarks of promoting tumor progression, invasion and metastasis.²¹ The effect of FTY720 treatment on tumor-microenvironment was therefore studied from different aspects. Compared to the control group, the number of activated hepatic stellate cells, revealed by immunohistochemical staining of α -SMA protein, was significantly suppressed after FTY720 combined with rapamycin treatment (Figure 6A). In order to study the effect of FTY720 on angiogenesis of HCC, the intratumoral CD34 expression which indicated the formation of new vessels was determined. The result showed that FTY720 combined with rapamycin treatment significantly decreased the CD34 expression and microvessel density (MVD) in tumor compared to control group (Figure 6B).

Discussion

Accumulating evidences showed that hepatic I/R injury promoted tumor recurrence and metastasis after liver transplantation or liver resection.^{5–7} In this study, we confirmed that hepatic I/R injury promoted tumor recurrence and metastasis after hepatectomy in orthotopic rat liver tumor model. Our results also showed that hepatic I/R injury increased the levels of circulating Tregs. Hepatic I/R injury was a typical inflammatory response involving a complex web of interactions between various cellular and molecular signals, which trigger a series of inflammatory cascades such as CXCL10/CXCR3 signaling.^{21–23} CXCL10 can recruit more Tregs trafficking to the liver through their surface receptor CXCR3.^{24,25} Our previous study also demonstrated that CXCL10/CXCR3 signaling induced the mobilization and recruitment of Tregs and endothelial progenitor cells, which further promoted liver tumor growth and recurrence after transplantation.^{8,9,26} Tregs plays important roles in different cancers, which were associated with the mortality.^{27–29} Tregs can help tumor to escape from the host immunosurveillance by their immunosuppressive activity against effector immune cell response.³⁰ Therefore, it is worthwhile to study whether attenuating hepatic I/R injury and reducing Tregs mobilization and recruitment could suppress liver tumor metastasis after surgical resection.

In the present study, the effect of FTY720 on inhibition of liver tumor metastasis after liver resection and I/R injury was confirmed in an orthotopic rat liver tumor model. FTY720, is synthetically derived from myriocin (ISP-1), a metabolite isolated from ascomycete, *Isaria sinclairii*. Several groups have shown that FTY720 can attenuate hepatic I/R injury by ameliorating acute phase inflammatory response and up-regulating several protective genes including heat shock proteins and anti-apoptotic genes.¹³ Our previous study indicated that FTY720 attenuated hepatic I/R injury and down-regulated intracellular mRNA and protein levels of CXCL10 and CXCR3.¹⁹ CXCL10/CXCR3 signaling plays an important role in the mobilization of circulating Tregs. Our previous results showed that the knockout of CXCL10 and CXCR3 decreased circulating and hepatic Tregs after hepatectomy and hepatic I/R injury.⁸ Furthermore, the number of CXCR3+ Tregs recruited into the liver was decreased in CXCL10-/- mice. In the present study, our data showed that FTY720 significantly reduced circulating and bone marrow Tregs after liver resection and hepatic I/R injury.

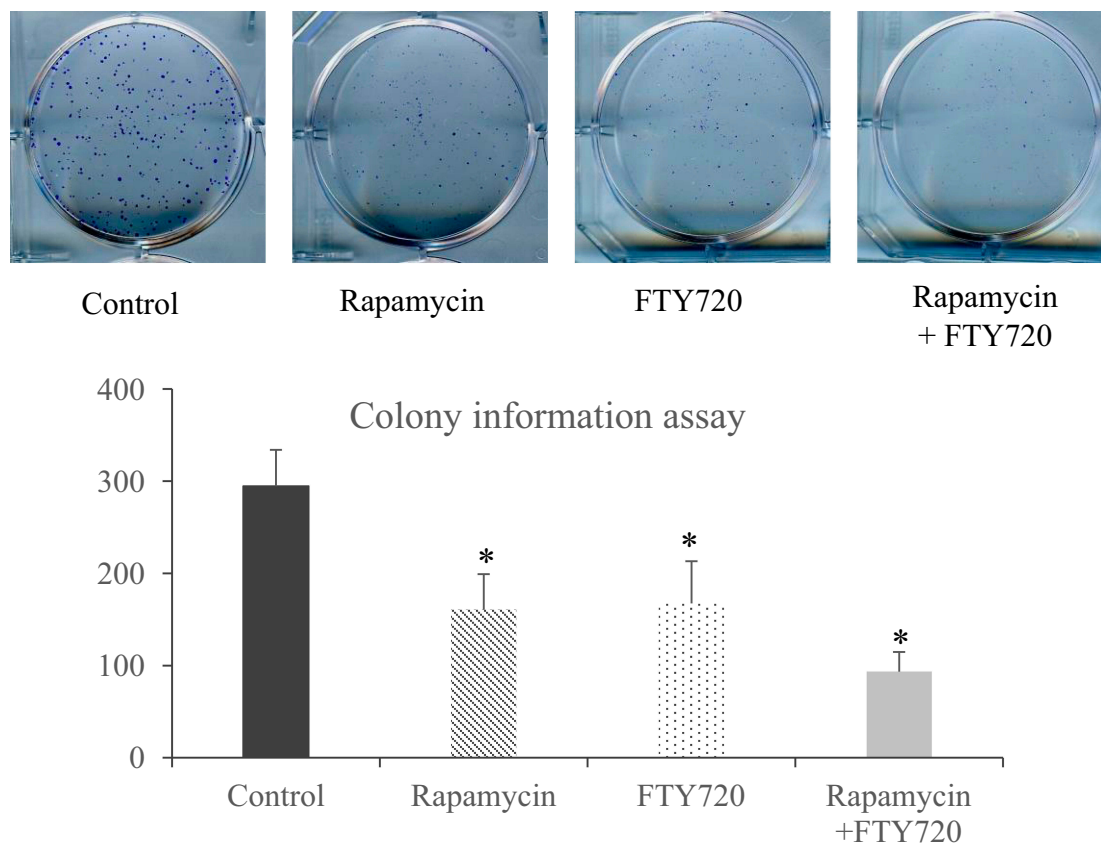
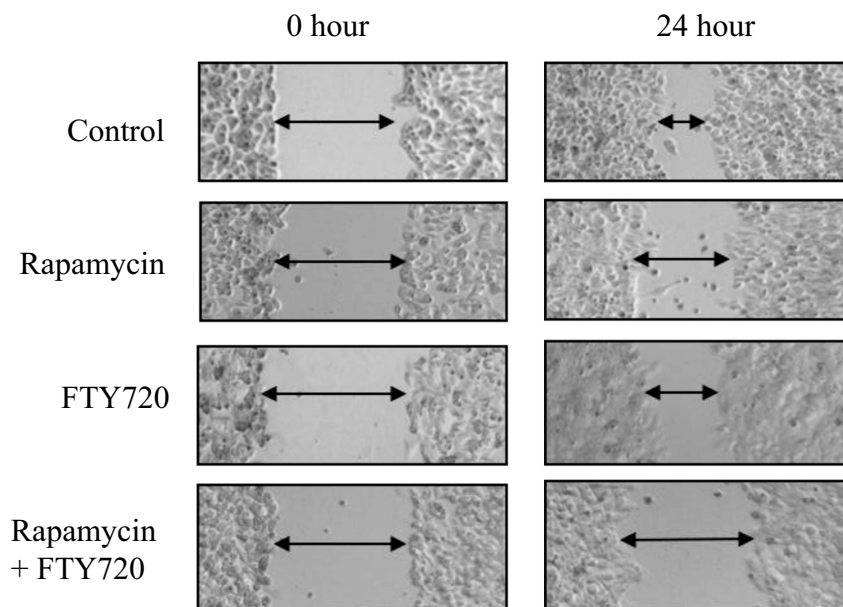
A**B**

Figure 4 FTY720 enhanced the anti-tumor proliferation and migration of rapamycin. **(A)** The colony number and size were lower in FTY720 combined with rapamycin group. **(B)** The treatment of FTY720 combined with rapamycin suppressed migration of HCC cells in wound healing assay. * $P < 0.05$.

These results suggested that FTY720 inhibited liver tumor metastasis after liver resection and I/R injury maybe through attenuating CXCL10/CXCR3 signaling and

decreasing circulatory Tregs. Targeting at hepatic I/R injury and circulating Tregs by FTY720 treatment may effectively decrease tumor recurrence and metastasis

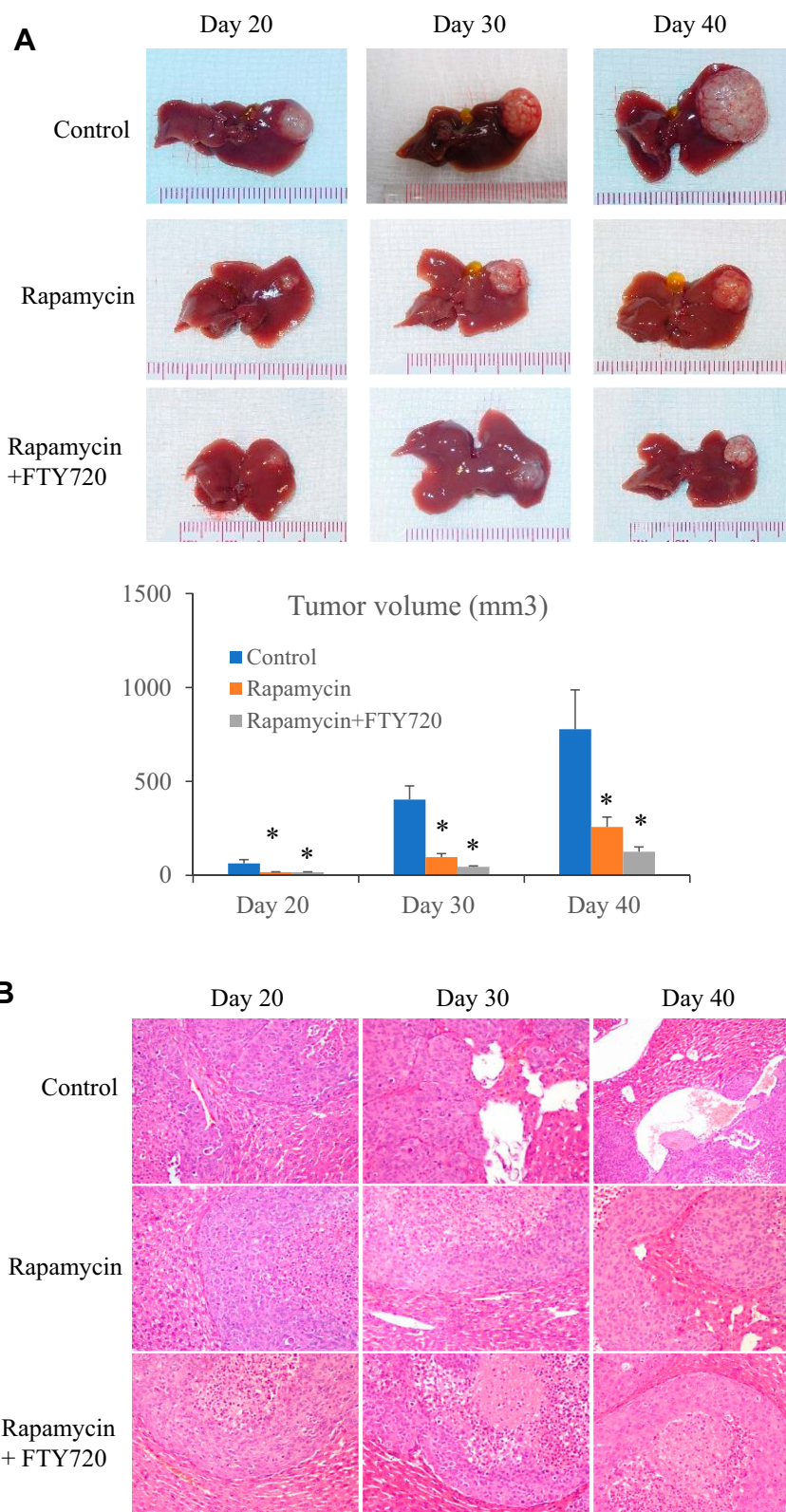


Figure 5 FTY720 combined with rapamycin suppressed tumor growth and invasion in mice liver cancer model. **(A)** The treatment of FTY720 combined with rapamycin further suppressed tumor growth compared to rapamycin group. **(B)** Comparison of histological features of tumor growth after FTY720 combined with rapamycin treatment. * $P < 0.05$.

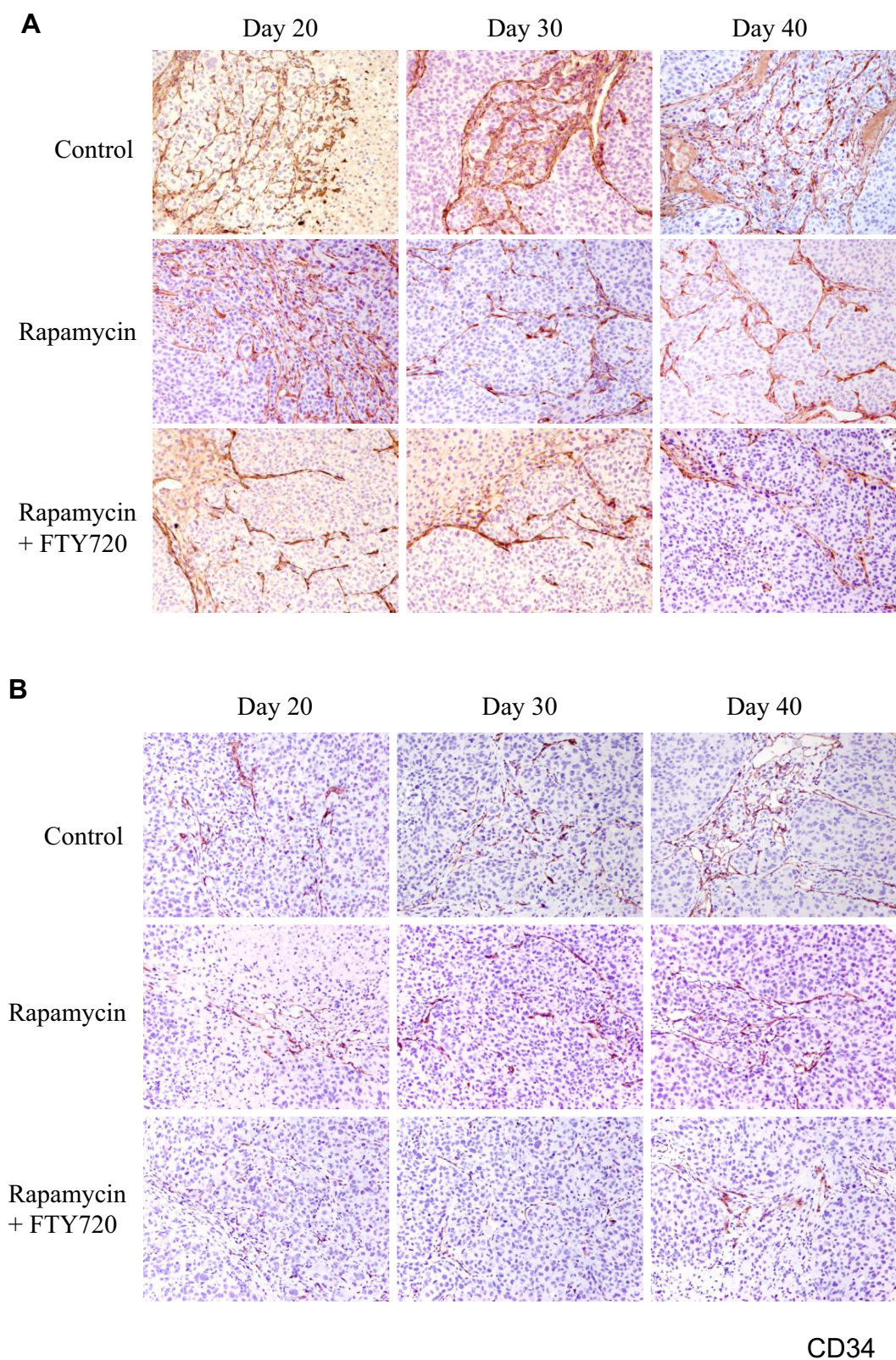


Figure 6 FTY720 inhibited hepatic stellate cell activation and tumor angiogenesis. **(A)** The number of activated hepatic stellate cells was suppressed after FTY720 combined with rapamycin treatment. **(B)** FTY720 combined with rapamycin treatment decreased the expression of CD34 in tumor compared to control group.

after liver surgery. However, the specific mechanisms of these molecules on FTY720-mediated suppression of circulating Tregs need to be further clarified.

In addition to the inhibition of tumor recurrence and Tregs mobilization, we also demonstrated that FTY720 may enhance the anti-tumor effect of rapamycin both in vivo and vitro. Rapamycin is the targeting inhibitor of mammalian target of rapamycin (mTOR), which is a serine/threonine-protein kinase downstream of the phosphoinositide-3-kinase (PI3K)-related kinase family. There is growing evidence to suggest that mTOR is a central regulator of various oncogenic processes including cell growth, proliferation, metabolism, and angiogenesis.³¹ Blockade of mTOR pathways can promote cell autophagy, induce apoptosis and reduce proliferation in HCC cell lines.^{32,33} However, clinical trials that have targeted mTOR with rapamycin or rapamycin analogs lonely have had minimal antitumor impact and its efficacy varies by dose in several contexts.^{34,35} Furthermore, mTOR inhibitors were considered to be attractive immunosuppressants for post-liver transplantation patients with HCC because of its capabilities of effective immunosuppression and concomitant prevention of hepatocellular tumorigenesis.³⁶ Our results showed that FTY720 combined with rapamycin further suppressed tumor growth and invasion compared to rapamycin single treatment. Furthermore, the combination therapy inhibited tumor angiogenesis and hepatic stellate cells activation. These results suggested that FTY720 may enhance the anti-tumor growth and invasiveness capacity of rapamycin.

Conclusions

FTY720 suppressed liver tumor growth and metastasis by reducing the population of circulating Tregs and enhancing the anti-tumor effect of rapamycin ([Supplementary figure 2](#)). It was suggested that FTY720 single or combined with rapamycin might provide novel insight for suppressing tumor growth and metastasis for HCC patients.

Abbreviations

I/R, hepatic ischemia/reperfusion; HCC, hepatocellular carcinoma; Tregs, regulatory T cells; mTOR, mammalian target of rapamycin.

Data Sharing Statement

The datasets supporting the conclusions of this article are included within the article.

Ethics Approval and Consent to Participate

All animal studies were approved by the Department of Health, Hong Kong Special Administrative Region.

Consent for Publication

All authors read and approved the manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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