Original Article

Rapamycin induces differentiation of glioma stem/progenitor cells by activating autophagy

Wen-Zhuo Zhuang, Lin-Mei Long, Wen-Jun Ji and Zhong-Qin Liang

Abstract

Glioma stem/progenitor cells (GSPCs) are considered to be responsible for the initiation, propagation, and recurrence of gliomas. The factors determining their differentiation remain poorly defined. Accumulating evidences indicate that alterations in autophagy may influence cell fate during mammalian development and differentiation. Here, we investigated the role of autophagy in GSPC differentiation. SU-2 cells were treated with rapamycin, 3-methyladenine (3-MA) plus rapamycin, E64d plus rapamycin, or untreated as control. SU-2 cell xenografts in nude mice were treated with rapamycin or 3-MA plus rapamycin, or untreated as control. Western blotting and immunocytochemistry showed up-regulation of microtubule-associated protein light chain-3 (LC3)–II in rapamycin-treated cells. The neurosphere formation rate and the number of cells in each neurosphere were significantly lower in the rapamycin treatment group than in other groups. Real-time PCR and immunocytochemistry showed down-regulation of stem/ progenitor cell markers and up-regulation of differentiation markers in rapamycin-treated cells. Transmission electron microscopy revealed autophagy activation in rapamycin-treated tumor cells in mice. Immunohistochemistry revealed decreased Nestin-positive cells and increased GFAP-positive cells in rapamycin-treated tumor sections. These results indicate that rapamycin induces differentiation of GSPCs by activating autophagy.

Key words Glioma stem/progenitor cells, autophagy, differentiation, rapamycin

Since the cancer stem cell theory was proposed, an increasing number of studies have shown that glioma tissues contain glioma stem/progenitor cells (GSPCs), also called glioma-initiating cells (GICs), with self-renewal, multi-lineage differentiation, and high proliferative potential[1-13]. The cancer stem cell theory contends that GSPCs are seed cells of glioma that are responsible for glioma recurrence and chemotherapy resistance. GSPCs escape the lethal effect of radiation or chemotherapy on the basis of self-renewal without differentiation, thus leading to tumor recurrence after treatment. To use GSPCs as a treatment target of glioma, elucidating the molecular mechanisms underlying their self-renewal and inhibition of differentiation is necessary. Identifying these molecular mechanisms would provide a basis for finding more specific therapeutic targets.

Under normal circumstances, cells degrade long-lived proteins and organelles by autophagy, produce amino acids, nucleic acids, and other small molecules for reuse, and meet their metabolic needs. When cells encounter unfavorable environments, such as nutrient deficiency, autophagy leads to programmed cell death type II (PCD II) through degrading membranes and activating specific genes, which is different from apoptotic cell death. Researchers have proposed many ideas on the relationship between the cellular capacity for autophagy and tumorigenesis and tumor development. It is now generally accepted that autophagy plays a dual role in these processes. On one hand, autophagy and the consequent cell death program can inhibit tumorigenesis and tumor development. When

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autophagy is down-regulated, cells have less protein loss during starvation, which improves the protein balance and promotes tumor cell survival. In contrast, other studies have shown that autophagy is a self-protective mechanism that prevents damage to tumor cells by nutrient deprivation, ionizing radiation, and chemotherapy. Reduced autophagy can lead to genomic instability by inducing cellular DNA damage, gene amplification, chromosomal aneuploidy, and other changes that increase the rate of carcinogenic mutations and promote tumorigenesis[11-13].

mTOR inhibitor rapamycin has been shown to initiate autophagy. In this study, we sought to determine the role of autophagy in GSPC differentiation using a human GSPC line and an orthotopic transplant nude mouse model.

Materials and Methods

Materials

Glioma stem/progenitor cell line SU-2 was isolated from a surgical specimen of a patient with mixed tumors containing anaplastic astrocytoma and ependymal cells. According to protocols in the literature [14], CDI33+ cells were isolated and cultured in DMEM/F12 medium containing 20 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), and N2 supplement at 37℃ in an atmosphere with 5% CO_2. DMEM/F12 medium, bFGF, and N2 supplement were from GIBCO. EGF was from Invitrogen. Rapamycin (cat #R0395) and 3-methyladenine (3-MA) were from Sigma. LC3 antibody (cat #PD014 and #PD015) was from MBL. β-actin antibody (clone EP1123Y) was from Millipore. E64d was from Sigma. CD133 (Miltenyi Biotec, Bergisch Gladbach, DE, AC133), Nestin (Millipore, Temecula, CA, clone 10C2), GFAP (Therom, Rockford, USA; clone GFA-02), and βIII-tubulin (Millipore, Temecula, CA, clone 2G10) were used for immunocytochemistry. Corresponding secondary antibodies Cy3-conjugated anti-mouse IgG (Cat: 715-165-150), Cy3-conjugated anti-rabbit IgG (Cat: 715-165-152), FITC-conjugated anti-mouse IgG (Cat: 715-165-150), and HRP-conjugated anti-mouse IgG (Cat: 715-035-1500) were from Jackson Immunoresearch.

Drug treatment

To determine whether rapamycin-facilitated GSPC differentiation is due to autophagy, SU-2 cells were divided into 4 groups and treated with 200 nmol/L rapamycin, or 10 mmol/L 3-MA for 10 min followed by 200 nmol/L rapamycin, or 10 μg/mL E64d (a lysosomal enzyme inhibitor) for 10 min followed by 200 nmol/L rapamycin, or untreated as control.

Western blotting

After treatment, cells were collected in 1.5-mL Eppendorf tubes and incubated with lysis buffer for 10 min on ice, followed by ultrasonic cell disruption on ice until lysates were no longer viscous. Cell lysates were centrifuged at 4℃ at 12 000 r/min for 10 min, and the supernatants were stored at –70℃. Protein concentration of the collected lysates was detected using the BCA Protein Assay Kit (Pierce). After adding 5× loading buffer, protein samples were boiled at 95℃ for 5 min for denaturation, after which the protein was separated using SDS-PAGE and then transferred onto a PVDF membrane. The membrane was blocked in 50 g/L nonfat dry milk, incubated with primary antibody overnight, and then membranes were washed in 0.2% Tween-20 (TBST), incubated with corresponding secondary antibody for 1 h. After washing, the membrane was developed using ECL kit (Amersham, Arlington Heights, Illinois). The levels of protein expression were quantitatively analyzed with SigmaScan Pro 5.

Immunofluorescence

At 72 h after treatment, cells were washed twice with PBS. Pre-treated slides were coated with 10 μL PBS, which was spread evenly by a blood smear method. Slides were fixed for 10 min in cold methanol at –20℃ and blocked in 1% bovine serum albumin (BSA) blocking buffer, then incubated with primary antibody overnight at 4℃. Slides were washed in PBS and then incubated with secondary antibody for 1 h at 4℃ in the dark. The slides were sealed using fluorescent mounted liquid containing DAPI (vector: w0212). The results were observed under confocal microscope.

Detection of GSPC self-renewal ability

GSPCs were digested with trypsin to make a single-cell suspension, inoculated at a density of 10 cells/well in 24-well plates, and cultured in DMEM/F12 medium containing bFGF, EGF, and N2 supplement. After 7 days, the number of neurospheres in suspension in each well was counted to calculate the neurosphere formation rate: neurosphere formation rate = (number of neurospheres / number of inoculated cells) × 100%. After digestion with trypsin, single cells were counted to calculate the number of cells in each neurosphere.

Detecting the mRNA levels of differentiation markers by quantitative real-time PCR

At 1, 2, and 3 days after different treatments, total RNA of cells was extracted using Trizol reagent. cDNA products of reverse transcription were used as templates

for quantitative real-time PCR (Primescript™ RT Reagent Kit, Takara), in which SYBR Green I was used as the dye and β-actin was used as an internal control. The 25 μL of PCR mixture was composed of 0.5 μL of ROX Dye (SYBR Green), 12.5 μL of 2× Premix, 1 μL of upstream primer, 1 μL of downstream primer, 1 μL of template cDNA, and 9.5 μL H_2O. The primers for the differentiation markers glial fibrillary acidic protein (GFAP), Tuj1, Olig2, and β-actin were designed by Primer Premier 5.0 software and were synthesized by Shanghai Sangon Biotech Co., Ltd. (Table 1). Amplification conditions were as follows: 95℃ for 10 s, 95℃ for 5 s, and 60℃ for 60 s for a total of 40 cycles. The mRNA levels of GFAP, Tuj1, and Olig2 were measured in each group. Real-time quantitative PCR was performed in an iCycler 5 (Bio-Rad, Hercules, California).

Establishing orthotopic xenografts of glioma in nude mice

BALB/c nu/nu mice of both genders aged between 5 to 7 weeks and weighing between 18 to 20 g, with Animal Certification Number SYXK (SU) 2007-0035, were intraperitoneally injected with 10% chloral hydrate at a dose of 200 mg/kg. The head skin was cleaned by Anerdian II after anesthesia, then cut (0.5 cm) vertically in the middle of the head to expose the skull. Hydrogen peroxide was applied to the skull to expose the bregma, sagittal suture, and coronal suture. Using a skull drill with a diameter of 1.0 mm, a hole (1.0 mm before the bregma and 2.5 mm at the right side from the middle line) was drilled to the cranial dura mater. A total of 1 × 10^5 cells in 15 μL of suspension in a microsyringe were injected into the right brain caudate nucleus via the skull hole. The needle was drawn back slowly and the hole was sealed by bone wax. The scalp was sutured using 0 # silk thread before natural revival. One week after inoculation, the tumor-bearing mice were randomly divided into three groups with 6 mice in each group: (1) the negative control group was injected in situ with 15 μL

saline; (2) the rapamycin group was injected in situ with 3 nmol of rapamycin; (3) the 3-MA plus rapamycin group was injected in situ with 200 nmol 3-MA followed by 3 nmol rapamycin. The mice were injected every 3 days, and were killed at 4 weeks after tumor development. In each group, the brains of 3 mice were paraffin-embedded for immunohistochemical analysis, whereas the brains from other 3 mice were used for transmission electron microscopy (TEM).

Transmission electron microscopy (TEM)

Brain tissues were fixed with 2.5% glutaraldehyde at 4℃ for 2 h. After washing with PBS, the cells were fixed with 1% osmium tetroxide for 1 h, followed by ethanol dehydration, infiltration, embedding, and ultrathin sectioning by diamond knives. The slides were observed by TEM with Philips CM-120 electron microscope at the voltage of 100 kV.

Immunohistochemical SABC method

The SABC detection kit (vector Vectastain) was used according to the instructions. DAB staining and hematoxylin counterstaining were performed. Clear brown staining in the cell cytoplasm was considered a positive result.

Statistical analyses

SPSS13.0 software was used for Student's t-test and the chi-square (χ^2) test. Values of $P < 0.05$ were considered statistically significant. Experiments were repeated at least 3 times.

Results

Effect of rapamycin on autophagy of GSPCs

Although microtubule-associated protein light chain

Table 1. Real-time PCR primer sequnces

Gene	Primer sequences
GFAP	Forward: 5′–CCAACCTGCAGATTCGAGA–3′
	Reverse: 5′–TCTTGAGGTGGCCTTCTGAC–3′
Tuj1	Forward: 5′–CAACAACCGCTATGTATTTCGT–3′
	Reverse: 5′–GGTTTTTATGTACTACAGGTGGTCAA–3′
Olig2	Forward: 5′–CCTCAAATCGCATCCAGAGT–3′
	Reverse: 5′–CTGTCCCTGGGATCTAGGC–3′
β-actin	Forward: 5′–TGTGACGTTGGACATCCGCAAAG–3′
	Reverse: 5′–TGGAAGGTGGACAGCGAGGC–3′

3 (LC3) has several homologs in mammals, LC3-II is most commonly used for autophagy assays. LC3-II or the protein tagged at its N terminus with a fluorescent protein has been used to monitor autophagy through indirect immunofluorescence. Another approach is to detect LC3 conversion (LC3-I to LC3-II) by immunoblot analysis because the amount of LC3-II is clearly correlated with the number of autophagosomes[15,16]. We used immunofluorescent microscopy and Western blotting to detect the expression levels of LC3 in GSPCs. Weak fluorescence of LC3 was observed in untreated GSPCs, indicating that autophagy activity was very low. In contrast, LC3 expression was up-regulated in cells treated with rapamycin alone or in combination with E64d, but was down-regulated in cells treated with rapamycin in combination with 3-MA. In some cells, LC3 expression was scattered as small green granules near cell membranes, indicating the formation of autophagosomes with LC3-II (Figure 1A, B). Western blotting showed that the ratio of LC3-II/LC3-I was significantly higher in cells treated with rapamycin alone

or in combination with E64d than in untreated cells (P < 0.05), but was not significantly higher in cells treated with 3-MA plus rapamycin than in untreated cells (P > 0.05) (Figure 1C, D). These results suggest that the expression levels of LC3 were up-regulated and that autophagy was activated after rapamycin treatment.

Effect of rapamycin on GSPC self-renewal ability

Self-renewal is a key feature of stem cells. The self-renewal ability of cancer stem cells is essential for tumorigenesis and tumor development. We tested the formation of neurospheres in each group. The neurosphere formation rate was significantly lower in the rapamycin group than in the control group and the 3-MA plus rapamycin group (25.9% vs. 65.3% and 53.5%, P < 0.05) (Figure 2). The number of cells in each neurosphere was significantly lower in the rapamycin group than in control group and 3-MA plus rapamycin group (1.2 × 10^4 cells vs. 5.1 × 10^4 cells and 5.3 × 10^4 cells, P < 0.05), suggesting that rapamycin was able to
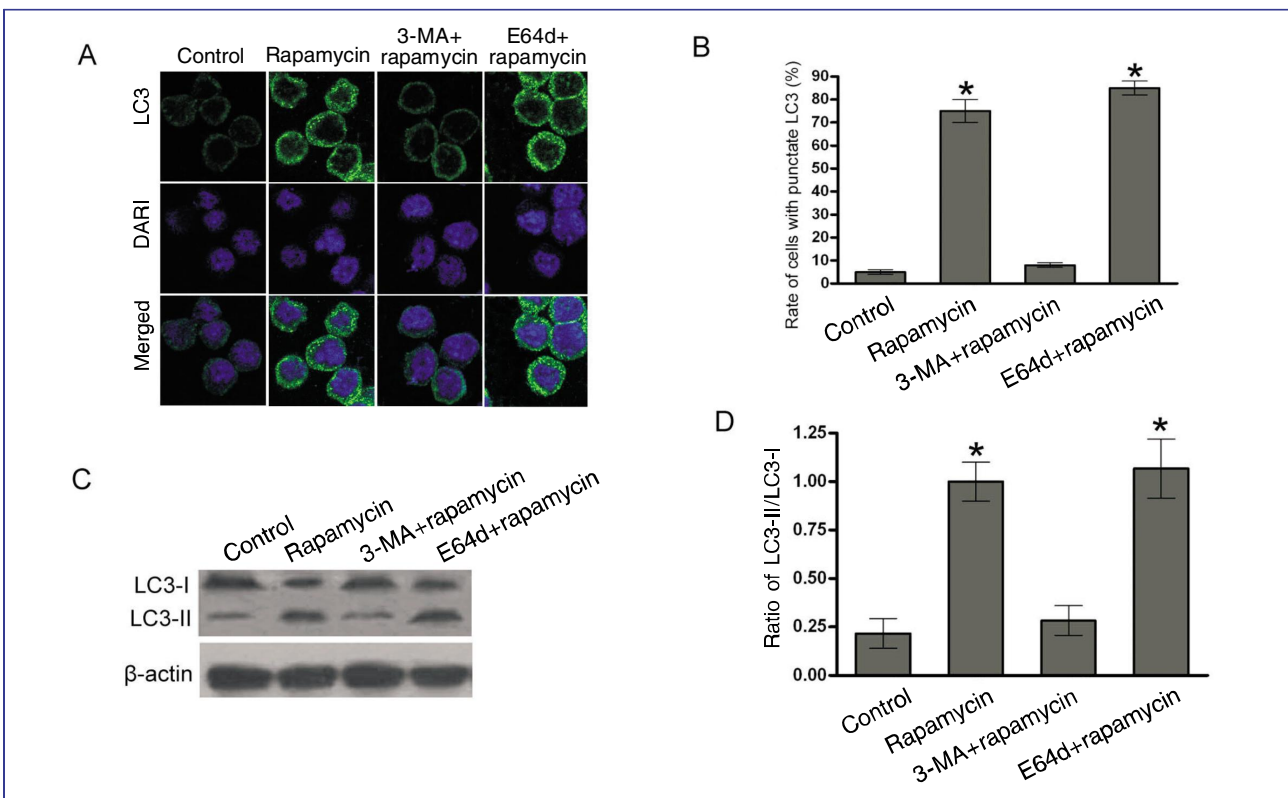
Figure 1. Autophagy detection in glioma stem/progenitor cells (GSPCs) after treatment. SU-2 cells were treated with rapamycin, 3-methyladenine (3-MA) plus rapamycin, E64d plus rapamycin, or untreated as control. A, immunofluorescent microscopy shows changes in the localization of LC3 in GSPCs after indicated treatments. B, quantification of LC3 expression in GSPCs after indicated treatments. The positive rate of LC3 was significantly higher in cells treated with rapamycin alone or in combination with E64d than in untreated cells (P < 0.05). C, western blotting shows up-regulation of LC3-II in cells treated with rapamycin alone or in combination with E64d as compared with untreated cells. D, quantification of the ratio of LC3-II/LC3-I. *P < 0.05, vs. control. Points, mean of triplicate experiments; bars, standard deviation (SD).
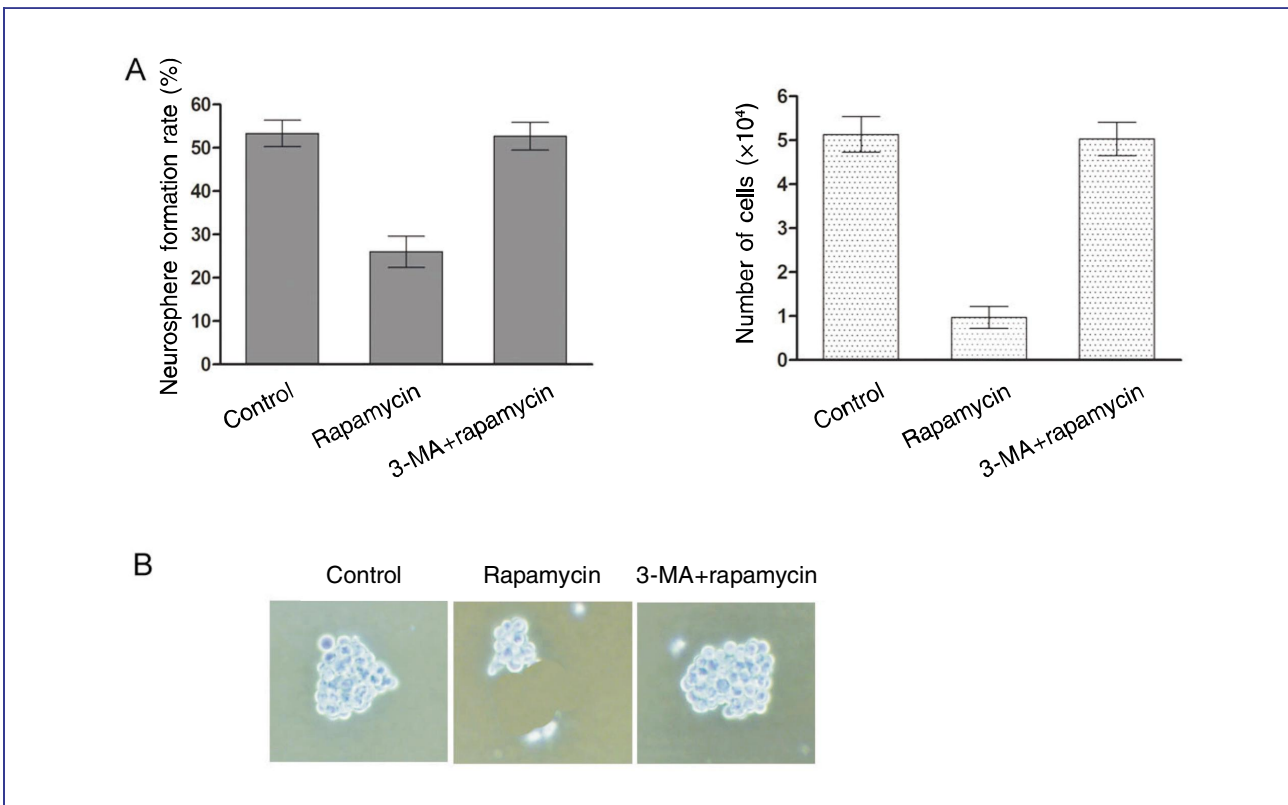
Figure 2. Effect of rapamycin on GSPC self-renewal. A, rapamycin attenuated the efficiency of neurosphere formation of GSPCs. The percentage of neurosphere-forming cells and the total number of cells were determined. B, representative images of SU-2 neurospheres treated as indicated. The neurosphere is significantly smaller in the rapamycin group than in control group and 3-MA plus rapamycin group, suggesting that rapamycin reduced the self-renewal ability of GSPCs and that 3-MA blocked this effect.

reduce the self-renewal ability of GSPCs except when GSPCs were pretreated with 3-MA. These results indicate that rapamycin can activate autophagy in GSPCs and thereby reduce their self-renewal ability.

Effect of rapamycin on GSPC differentiation

To determine the effects of rapamycin on the differentiation of GSPCs, we detected the mRNA levels of cell differentiation markers *GFAP*, *Tuj1*, and *Olig2* in each group using quantitative RT-PCR (Figure 3A). The mRNA levels of these markers significantly increased in the rapamycin group compared to the control group and 3-MA plus rapamycin group since the first day after treatment ($P < 0.05$), whereas no significant change was observed in the latter two groups. These results suggest that rapamycin treatment can increase the expression levels of cell differentiation markers and promote GSPC differentiation.

To further test the effects of rapamycin on the differentiation of GSPCs, we used immunofluorescent microscopy to detect changes of expression levels of

stem/progenitor cell markers CD133 and Nestin as well as differentiation markers βIII-tubulin and GFAP. The expression of CD133 and Nestin were obviously weaker and the expression of βIII-tubulin and GFAP were obviously stronger in the rapamycin group than in the control and 3-MA plus rapamycin groups (Figure 3B), suggesting that rapamycin treatment activated autophagy in GPSCs and led to GSPC differentiation.

The effects of rapamycin on autophagy of GSPCs *in vivo*

To determine the effects of rapamycin on autophagy of GSPCs *in vivo*, we observed the morphology of cells from the brains of tumor-bearing mice after indicated treatments under TEM. The nuclear membrane appeared intact, the chromatin structure was normal, and the distribution and number of mitochondria in the cytoplasm were normal in both the control and 3-MA plus rapamycin groups. There were no autophagosomes and no increase in the number of lysosomes in these groups. However, the autophagosomes significantly increased
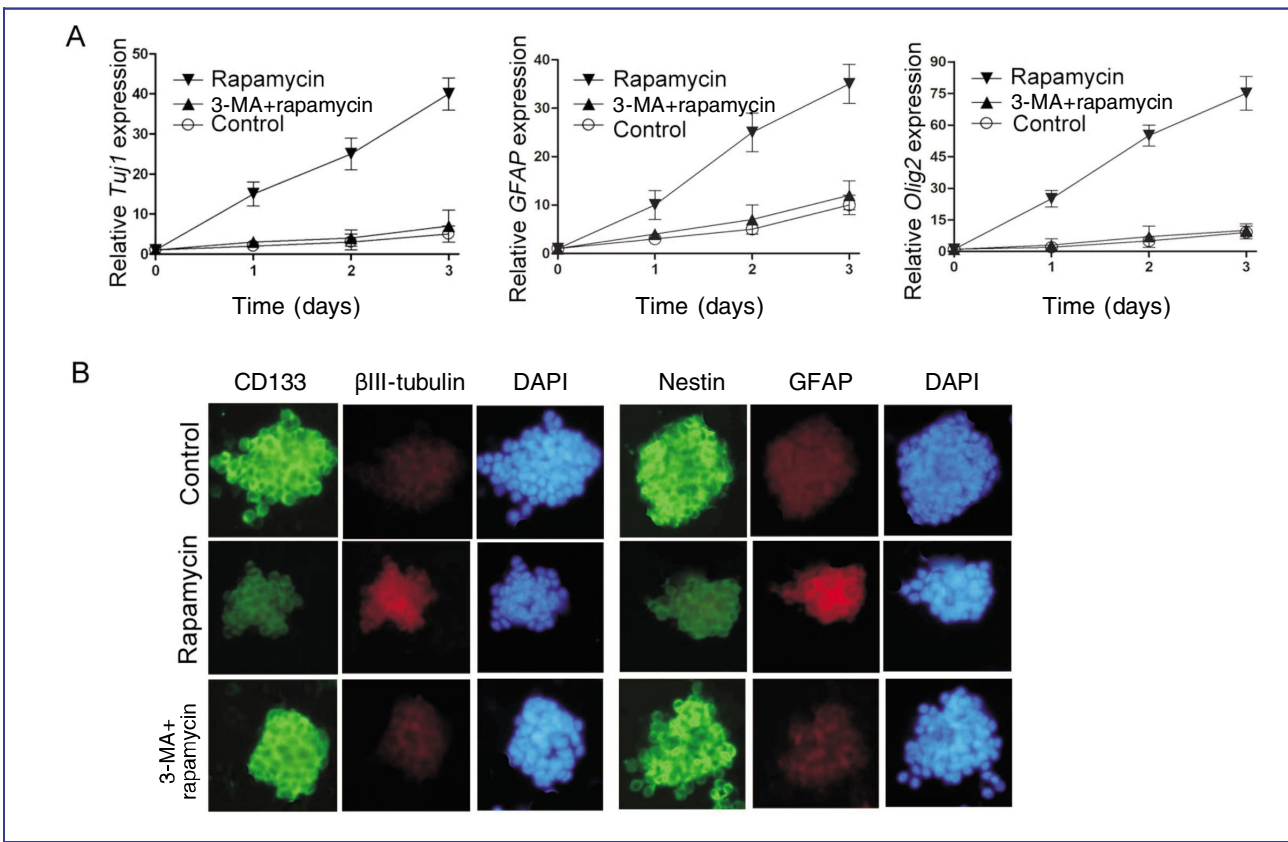
Figure 3. Effect of rapamycin on GSPC differentiation. A, quantitative RT-PCR analysis was performed to determine the mRNA levels of the differentiation markers *GFAP, Tuj1*, and *Olig2* in SU-2 neurospheres at 3 days after the indicated treatments. β-actin was used as an internal normalization control. Error bars represent mean ± SD. B, immunocytochemistry for the indicated proteins was performed in SU-2 neurospheres at 3 days after the indicated treatments on poly-L-lysine-coated coverslips. Nuclei were counterstained with DAPI.

and mitochondria was surrounded by autophagosomes in the rapamycin group (Figure 4), suggesting that rapamycin can activate tumor cell autophagy *in vivo*.

### The effects of rapamycin on differentiation of GSPCs *in vivo*

To determine the effects of rapamycin on differentiation of GSPCs *in vivo*, we observed the expression of the GSPC marker Nestin and the differentiation marker GFAP in tumor tissues from tumor-bearing mice after indicated treatments using immunohistochemistry. The expression level of Nestin was significantly reduced and the expression level of GFAP was significantly increased in xenografts of the rapamycin group than in the control and 3-MA plus rapamycin groups ($P < 0.05$) (Figure 5), suggesting that rapamycin promoted differentiation of GSPCs *in vivo*.

Discussion

In recent years, how to reduce oncogenicity and increase sensitivity of GSPCs to radiation by inducing their differentiation has been widely investigated [7-9,12,13]. Most researchers believe that many factors, including Notch, Wnt, tumor growth factor-β (TGF-β), leukemia inhibitory factor (LIF), Sonic hedgehog (SHH), and bone morphogenic protein (BMP), are involved in the self-renewal maintenance and differentiation inhibition of GSPCs. Current researches in this area mainly focus on specific signal transduction pathways, namely on selectively targeting to a signaling molecule and inhibiting one of the signaling pathways by gene regulation or drugs to promote GSPC differentiation and apoptosis. However, many studies have shown that multiple signaling pathways are involved in GSPC differentiation. Thus, the inhibition of GSPC self-renewal and differentiation is due to the synergistic effects of multiple signaling pathways[7-9,12,13,17]. At present, no common factor leading to dysregulation of multiple signaling pathways in GSPCs has been found.

Autophagy has recently attracted attention as a novel response to chemotherapy and radiation in tumor cells. In the process of autophagy, cells degrade their
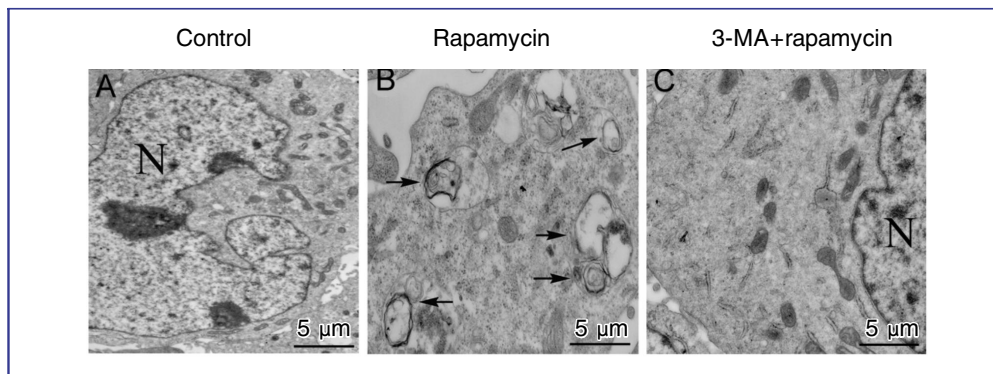
Figure 4. Effect of rapamycin on autophagy of GSPCs *in vivo*. Representative electron micrograph images show increased autophagosomes following rapamycin treatment in an SU-2 xenograft model. Three mice from each group and ten fields for each mouse were examined and displayed similar morphologic changes. TEM showed intact nuclear membranes as well as normal chromatin structure and mitochondrial numbers and distribution in cases from the untreated group. TEM also showed a remarkable increase of double-membrane autophagosomes (as indicated by arrows) in the rapamycin group and in the 3-MA plus rapamycin group. N, nucleus.
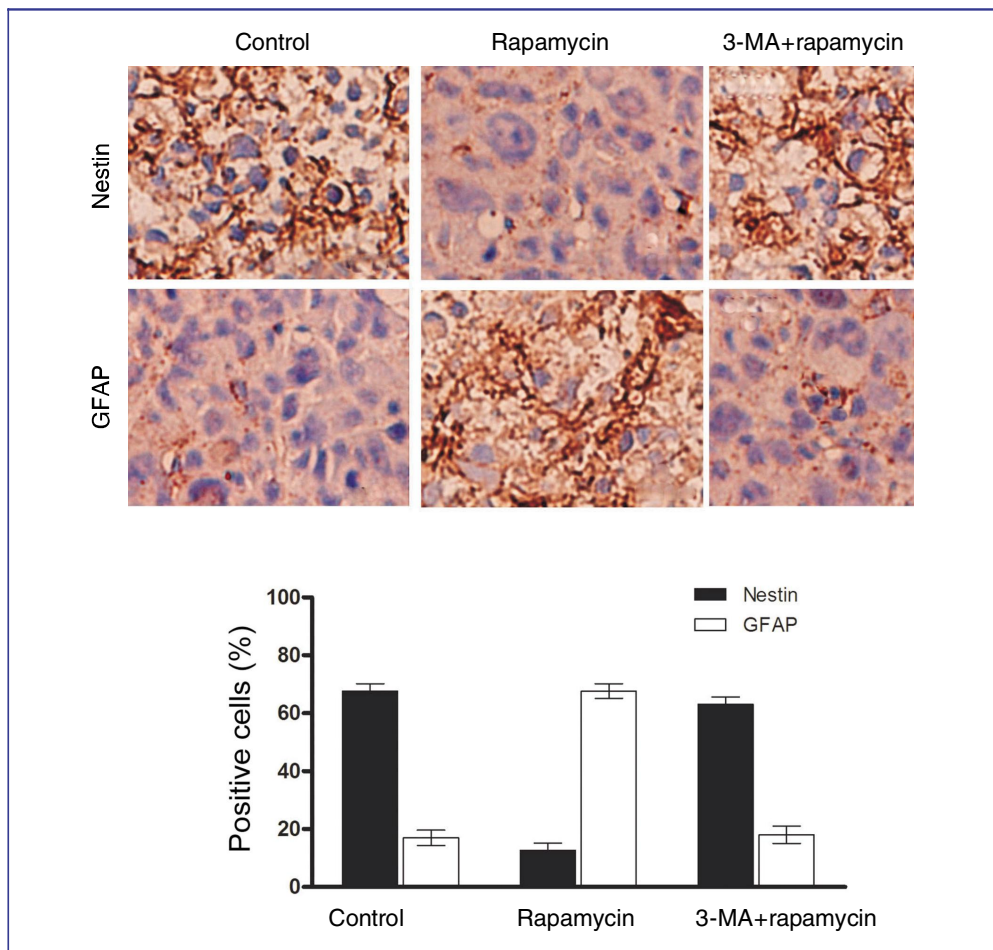
Figure 5. Effect of rapamycin on GSPC differentiation *in vivo*. A, representative images of immunohistochemical staining of GFAP and Nestin in tumor sections at 35 days after indicated treatments. The expression of Nestin is weaker and the expression of GFAP is stronger in the rapamycin group than in the control and 3-MA plus rapamycin groups. B, positive rate of GFAP is significantly higher and that of Nestin is significantly lower in the rapamycin group than in the control and 3-MA plus rapamycin groups.

own damaged organelles and macromolecules by lysosomes and recycle the products. Unique to eukaryotic cells, autophagy is an important regulatory mechanism for cell growth, maturation, and death and is related to a variety of human diseases including cancer[18,19]. Autophagy has been found to play a complex role in antitumor therapy[20-24]. Recent studies have shown that in some cells, autophagy neither has a protective effect nor induces programmed cell death, but plays an important role in differentiation and development [25,26]. Differentiation of several cell types requires autophagy[26-28], whereas inhibition of differentiation is considered a prominent feature of GSPCs[1]. Therefore, the relationship between low autophagic activity and differentiation inhibition of GSPCs warrants investigation.

In our study, we found that the expression levels of GSPC markers were reduced while differentiation markers were increased in the rapamycin group, suggesting that rapamycin treatment promotes GSPC differentiation. This effect of rapamycin was inhibited by the autophagy inhibitor 3-MA, indicating that rapamycin-promoted GSPC differentiation is closely related to autophagy. In the orthotopic transplant nude

mouse model, we observed cell differentiation in rapamycin-treated xenografts, which is consistent with the results *in vitro*.

Based on our results, we speculate that low autophagic activity is one of the reasons that differentiation is inhibited in GSPCs. The autophagy activator rapamycin induces autophagy in GSPCs and promotes GSPC differentiation. Taken together, these results have provided not only a new understanding of autophagy but, more importantly, new clues about the mechanisms underlying the inhibition of GSPC differentiation.

Acknowledgements

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