

**Evidence That Curcumin Suppresses the Growth of Malignant Gliomas *In Vitro* and *In Vivo* Through Induction of Autophagy: Role of Akt and ERK Signaling Pathways**

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**Running Title:** Curcumin-induced autophagy in glioma

**Abbreviations:** mTOR, mammalian target of rapamycin; ERK1/2, extracellular signal-regulated kinases; NF- $\kappa$ B, nuclear factor  $\kappa$ B; p70S6K, p70 ribosomal protein S6 kinase

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## ABSTRACT

Autophagy is a response of cancer cells to various anticancer therapies. It is designated as programmed cell death type II and characterized by formation of autophagic vacuoles in the cytoplasm. The Akt/mammalian target of rapamycin (mTOR)/p70S6K and the extracellular signal-regulated kinases 1/2 (ERK1/2) pathways are two major pathways which regulate autophagy induced by nutrient starvation. These pathways are also frequently associated with oncogenesis in a variety of cancer cell types, including malignant gliomas. However, few studies have examined both of these signal pathways in the context of anticancer therapy-induced autophagy in cancer cells, and autophagy's effect on cell death remains unclear. Here, we examined the anticancer efficacy and mechanisms of curcumin, a natural compound with low toxicity in normal cells, in U87-MG and U373-MG malignant glioma cells. Curcumin induced G<sub>2</sub>/M arrest and non-apoptotic autophagic cell death in both cell types. It inhibited the Akt/mTOR/p70S6K pathway and activated the ERK1/2 pathway, resulting in induction of autophagy. Interestingly, activation of the Akt pathway inhibited curcumin-induced autophagy and cytotoxicity, whereas inhibition of the ERK1/2 pathway inhibited curcumin-induced autophagy and induced apoptosis, thus resulting in enhanced cytotoxicity. These results imply that the effect of autophagy on cell death may be pathway specific. In the subcutaneous xenograft model of U87-MG cells, curcumin inhibited tumor growth significantly ( $P < 0.05$ ) and induced autophagy. These results suggest that curcumin has high anticancer efficacy *in vitro* and *in vivo* by inducing autophagy and warrant further investigation toward possible clinical application in patients with malignant glioma.

## Introduction

Malignant glioma is the most common primary malignant tumor in the brain. In spite of the combination of surgery, chemotherapy, and radiotherapy, the median survival time of patients with glioblastoma multiforme, the most malignant type of malignant glioma, is less than one year from diagnosis (Ohgaki, 2004). Consequently, there is an urgent need to develop new therapeutic strategies.

Accumulating evidence shows that a natural product, curcumin (diferuloylmethane), has a potent anticancer effect both *in vitro* and *in vivo* on a variety of cancer cell types, such as leukemia, breast cancer, prostate cancer, and pancreatic cancer (Aggarwal et al., 2003; Shishodia et al., 2005). However, the efficacy of curcumin for malignant glioma cells *in vitro* and *in vivo* is not yet fully determined. As expected from the fact that curcumin is an active ingredient of the spice turmeric, it caused no serious toxicity in animal studies (up to 5 g/kg; Wahlstrom and Blennow, 1978) and it was safely administered to humans without major toxicity in phase I clinical studies (up to 12 g per day; Sharma et al., 2001; Sharma et al., 2004; Loa et al., 2006). However, these findings also underscore that we need to overcome the low absorption and bioavailability of curcumin outside the colon to use it as systemic cancer preventive agent.

Several mechanisms by which curcumin exerts its anticancer effect have been reported. First, curcumin inhibits a transcription factor, nuclear factor  $\kappa$ B (NF- $\kappa$ B), by inhibiting inhibitor of  $\kappa$ B kinase and subsequent I $\kappa$ B $\alpha$  phosphorylation (Singh and Aggarwal, 1995; Bharti et al., 2003; Aggarwal et al., 2004; Aggarwal et al., 2006). As a result, curcumin down-regulates the expression of NF- $\kappa$ B-regulated gene products such as Bcl-2, Bcl-X<sub>L</sub>, cyclin D1, matrix metalloproteinase-9, cyclooxygenase-2, and interleukin-6, resulting in cell cycle arrest, suppression of proliferation, and induction of apoptosis (Bharti et al., 2003; Aggarwal et al.,

2004; Aggarwal et al., 2006; Mukhopadhyay et al., 2002). Second, curcumin inhibits the Akt/mammalian target of rapamycin (mTOR) pathway and phosphorylation of p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein, resulting in inhibition of proliferation and induction of apoptosis (Aggarwal et al., 2006; Woo et al., 2003; Bava et al., 2005; Beevers et al., 2006). Other mechanisms of curcumin's antitumor effect include down-regulation of transcription factors AP-1 (Nakamura et al., 2002; Prusty and Das, 2005; Tomita et al., 2006) and Egr-1 (Chen et al., 2006).

Recently, autophagy has attracted the interest of scientists in the field of cancer research because it is designated as programmed cell death type II, while apoptosis is well known as programmed cell death type I (Bursch et al., 2000). Autophagic cell death is characterized by numerous autophagic vacuoles in the cytoplasm, while the nucleus remains intact until the late stage of cell death. In contrast, apoptosis is manifested by DNA condensation and fragmentation. We have reported that malignant glioma cells are very resistant to apoptosis but that they undergo autophagy in response to anticancer therapies such as radiation, temozolomide, and ceramide (Kanzawa et al., 2004; Daido et al., 2004; Daido et al., 2005; Ito et al., 2005). Autophagy is basically a protein degradation system of the cell's own lysosomes (Klionsky and Emr, 2000). It is a process that maintains ATP level and is typically activated on amino acid deprivation (Meijer and Codogno, 2004; Keleker, 2006). Conversely, amino acids and ATP are negative regulators of autophagy. As a sensor of amino acids and ATP, mTOR negatively regulates autophagy through the mTOR/p70S6K pathway by activating this pathway in response to amino acids and ATP (Blommaert et al., 1995; Shigemitsu et al., 1999). Further, PTEN and Akt are upstream regulators of the mTOR pathway: PTEN induces autophagy, and Akt inhibits autophagy (Arico et al., 2001). The Raf-1/MEK1/2/ERK1/2 pathway is another pathway that

mediates signals stimulated by amino acids: amino acids inhibit this pathway and autophagy. ERK phosphorylates G  $\alpha$ -interacting protein, which accelerates the rate of GTP hydrolysis by the G<sub>oi3</sub> protein, resulting in induction of autophagy (Ogier-Denis et al., 2000; Pattingre et al., 2003). Although it is established that the Akt/mTOR/p70S6K pathway and the Raf-1/MEK1/2/ERK1/2 pathway are involved in regulating autophagy, their roles in autophagy in cancer are not yet fully determined.

In the present study, we investigated the anticancer effect of curcumin on U87-MG and U373-MG human malignant glioma cells *in vitro* and *in vivo*. We found that curcumin efficiently inhibited growth of these cell types by inducing non-apoptotic autophagic cell death. Further, we examined the signal pathways of curcumin-induced autophagy and investigated the role of the pathways in cell death. To the best of our knowledge, this is the first study to demonstrate that curcumin induces autophagy, which is regulated by simultaneous inhibition of the Akt/mTOR/p70S6K pathway and stimulation of the ERK1/2 pathway.

## Materials and Methods

**Reagents.** Curcumin with a purity greater than 95% was kindly supplied by Sabinsa Corporation (Piscataway, NJ) and dissolved in DMSO (Sigma) to produce a 100 mM stock solution. Acridine orange was purchased from Polysciences (Warrington, PA). 3-Methyladenine (3-MA), a phosphatidylinositol 3-phosphate kinase (PI3K) inhibitor, was purchased from Sigma. PD98059, a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEK1) inhibitor, was purchased from Cell Signaling Technology (Beverly, MA). Paclitaxel (Taxol) was purchased from Bristol-Myers Squibb (Princeton, NJ). A recombinant full-length human active Akt1 protein (rAkt1) was purchased from Upstate (Temecula, CA).

**Cell Culture.** U87-MG and U373-MG human malignant glioma cells with PTEN mutation and KBM-5 human leukemia cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10–15% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/ml of penicillin (Invitrogen), and 2.5 µg/ml of antimycotic (Fungizone; Invitrogen) at 37°C in 5% CO<sub>2</sub>.

**Cell Viability Assay.** The cytotoxic effect of curcumin was determined by using the cell proliferation reagent WST-1 (Roche Applied Science, Indianapolis, IN), as described previously (Ito et al., 2006). Briefly, U87-MG and U373-MG cells were seeded at  $3 \times 10^3$  cells/well in 96-well flat-bottomed plates and incubated at 37°C overnight. After cells were treated with 0, 10, 30, 50, 70, or 90 µM curcumin for 72 hours, they were exposed to 10 µl of the WST-1 reagent for 1 hour at 37°C. The absorbance at 450 nm was measured using a microplate reader. The viability of untreated cells was considered to be 100%.

**Cell Cycle Analysis.** Tumor cells treated with curcumin (0, 20, and 40 µM) for 72 hours were trypsinized, fixed with ice cold 70% ethanol, stained with propidium iodide by using a cellular DNA flow cytometric analysis reagent set (Roche), and analyzed for DNA content by FACScan (Becton Dickinson, San Jose, CA). Data were analyzed by Cell Quest software (Becton Dickinson). At least 100,000 cells were analyzed for each sample. Paclitaxel (5 nM) was used as a positive control to induce apoptosis (Kondo and Kondo, 2006).

**Apoptosis Detection Assay.** Tumor cells were seeded on Lab-Tek chamber slides (Nunc, Rochester, NY) and incubated overnight, and then were treated with 40 µM curcumin for 72 hours and stained with the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) technique using an ApopTag apoptosis detection kit (Chemicon) as described previously (Takeuchi et al., 2005). Two hundred cells were counted and scored for the incidence

of positive staining under a microscope. Paclitaxel (5 nM) was used as a positive control to induce apoptosis.

**Clonogenic Assay.** Tumor cells were diluted serially and seeded into the 6-well plates in triplicate per data point. Twenty-four hours after seeding, cells were treated with different concentrations of curcumin as indicated. Two weeks after treatment, cells were fixed and stained with 0.5 % crystal violet (Sigma) in methanol for 5 minutes. And then colonies consisting 50 or more cells were counted.

**Electron Microscopy.** To detect the induction of autophagy morphologically in curcumin-treated tumor cells, we performed ultrastructural analysis. Cells were grown on glass coverslips, treated with 40  $\mu$ M curcumin for 48 hours, and then fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 hour. After fixation, the samples were postfixated in 1%  $\text{OsO}_4$  in the same buffer for 1 hour and then subjected to electron microscopic analysis. Representative areas were chosen for ultrathin sectioning and viewed with a JEM 1010 transmission electron microscope (JEOL, Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained with an AMT imaging system (Advanced Microscopy Techniques, Danvers, MA).

**Detection and Quantification of Acidic Vesicular Organelles with Acridine Orange staining.** Autophagy is the process of sequestering cytoplasmic proteins into the lytic component and is characterized by the formation and promotion of acidic vesicular organelles (AVOs), as described previously (Paglin et al., 2001). To detect the development of AVOs, we treated cells with 20 and 40  $\mu$ M curcumin for 72 hours and then performed vital staining with acridine orange. To quantify the development of AVOs, the cells were stained with acridine orange (1  $\mu$ g/ml) for 15 minutes, removed from the plate with trypsin-EDTA (Invitrogen), and analyzed using a

FACScan flow cytometer and CellQuest software. To inhibit autophagy, 2.0 mM 3-MA was added 24 hours after addition of curcumin.

**GFP-LC3 Dot Assay.** The green fluorescent protein (GFP)-tagged microtubule-associated protein 1 light chain 3 (LC3) expression vector was kindly provided by Dr. Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). LC3 is recruited to the autophagosomal membrane during autophagy (Kabeya et al., 2000). Therefore, GFP-tagged LC3-expressing cells have been used to demonstrate induction of autophagy (Kanzawa et al., 2004; Kabeya et al., 2000; Mizushima, 2004). GFP-LC3 cells present a diffuse distribution under control conditions, whereas a punctate pattern of GFP-LC3 expression (GFP-LC3 dots) is induced by autophagy. Cells were transiently transfected with the GFP-LC3 vector using Fugene 6 transfection reagent (Roche). After overnight culture, cells were treated with 20 and 40  $\mu$ M curcumin for 72 hours, fixed with 4% paraformaldehyde, and examined under a fluorescence microscope. To quantify autophagic cells after curcumin treatment, we counted the number of autophagic cells demonstrating GFP-LC3 dots ( $\geq 10$  dots per cell) among 200 GFP-positive cells.

**Western Blotting.** Soluble proteins were isolated from untreated and curcumin-treated cells. For the detection of LC3, poly(ADP-ribose) polymerase (PARP), and NF- $\kappa$ B p65, culture medium with 10% FBS was used. For the detection of signal pathway molecules phospho-Akt, phospho-p70S6K, and phospho-ERK, culture medium with low serum (0.5% FBS) was used for up to 24 hours to exclude the effects of growth factors contained in the serum. Equal amounts of protein were separated by 10% or 15% SDS-PAGE gel (Bio-Rad, Richmond, CA) and transferred to a Hybond-P membrane (GE Healthcare, USA). The membranes were treated with primary antibodies overnight at 4°C and incubated for 1 hour with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:3,000 dilution; GE Healthcare) at

room temperature for 1 hour. Bound antibody complexes were detected using an enhanced chemiluminescence reagent (GE Healthcare) according to the manufacturer's instructions. Anti-LC3 antibody against a synthetic peptide corresponding to the N-terminal 14 amino acids of isoform B LC3 and an additional cysteine (PSEKTFKQRRTFEQC) was prepared by immunization of rabbit and was affinity purified on an immobilized peptide-Sepharose column (Covance, Denver, PA). We purchased anti- $\beta$ -actin (Sigma), anti-phospho-Akt at Ser<sup>473</sup>, anti-total Akt, anti-phospho-p70S6K at Thr<sup>389</sup>, anti-total p70S6K, anti-phospho-ERK1/2 at Thr<sup>202</sup>/Tyr<sup>204</sup>, anti-total ERK1/2, anti-phospho-PP1 $\alpha$  at Thr<sup>320</sup>, anti-total PP1 $\alpha$ , and anti-PARP antibodies from Cell Signaling Technology. Anti-phospho-PP2A at Thr<sup>307</sup> and anti-total PP2A antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). To inhibit MEK1, 25  $\mu$ M PD98059 was added 1 hour before curcumin treatment.

**PI3K Activity Assay.** PI3K activity was measured using PI 3-Kinase ELISA kit (Echelon Bioscience, Inc., Salt Lake City, UT) according to the manufacture's instructions. This kit evaluates PI3K activity to detect the conversion of PI(4,5)P2 into PI(3,4,5)P3. Briefly, cell culture and treatments were the same as described above. The cells were rinsed three times with ice cold Buffer A (137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 0.1 mM sodium orthovanadate) and harvested with ice cold Lysis Buffer (Buffer A plus 1 % NP-40 and 1 mM PMSF). The cellular proteins were extracted by centrifugation. PI3K was isolated from equal amounts (100  $\mu$ g) of the cellular protein by immunoprecipitation using anti-PI3K antibody (Upstate Biotechnology, Lake Placid, NY). The immuno complexes bound onto protein A-agarose beads were incubated in the reaction buffer containing PI(4,5)P2 substrate and ATP, and the kinase reaction was stopped by pelleting the beads by centrifugation. The reaction mixtures were used for the following detection reaction. The absorbance of final solution was

measured by a microplate reader at 450nm. PI3K activity was calculated from the standard curve using various concentrations of PI(3,4,5)P3.

**Electrophoretic Mobility Shift Assay (EMSA) for NF- $\kappa$ B.** NF- $\kappa$ B activation was analyzed by EMSA as described previously (Takada et al., 2004). Cells were incubated with or without 0.1 nM tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) for 30 minutes and then treated with 50  $\mu$ M curcumin for 2 hours. Eight  $\mu$ g of nuclear extracts was incubated with  $^{32}$ P end-labeled 45-mer double-stranded NF- $\kappa$ B oligonucleotide from human immunodeficiency virus 1 long terminal repeat (5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3'; boldface sequence indicates NF- $\kappa$ B binding site) for 15 minutes at 37°C, and the DNA-protein complex was resolved in a 6.6% native polyacrylamide gel. The radioactive bands from the dried gels were visualized and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with ImageQuant software (Molecular Dynamics).

**NF- $\kappa$ B p65 siRNA Transfection.** SignalSilence NF- $\kappa$ B p65 siRNA was purchased from Cell Signaling Technology. siCONTROL non-targeting siRNA purchased from Dharmacon (Chicago, IL) was used as a control siRNA. Cells were transfected with siRNA using Oligofectamine transfection reagent (Invitrogen) for 72 hours according to the manufacturer's instructions. The final concentration of siRNA was 100 nM. To confirm the efficacy of NF- $\kappa$ B p65 siRNA, Western blotting using anti-NF- $\kappa$ B p65 antibody (Cell Signaling Technology) was performed as described above. The cytotoxic effect of NF- $\kappa$ B p65 siRNA on U87-MG and U373-MG cells were determined using the WST-1 assay as described above.

**Animal Studies.** Adult nude mice (five mice per treatment group) were anesthetized with ketamine and xylazine as described previously (Ito et al., 2006). U87-MG cells ( $1.0 \times 10^6$  cells in 20  $\mu$ l of serum-free DMEM) were inoculated subcutaneously into the right flank of mice. Tumor

growth was measured daily with calipers. Tumor volume was calculated as  $(L \times W^2)/2$ , where L is the length in millimeters and W is the width in millimeters, as described previously (Ito et al., 2006). When the tumors reached a mean volume of 50–70 mm<sup>3</sup>, a 10- $\mu$ l intratumoral injection of curcumin (100 mg/kg/20  $\mu$ l in DMSO/PBS) or the same dose of DMSO/PBS was given (day 0). Mice were euthanized by exposure to CO<sub>2</sub> 16 days after the initiation of treatment. Tumors were then removed, frozen rapidly, and used for Western blotting and immunohistochemical staining for LC3. All animal studies were performed in the veterinary facilities of The University of Texas M. D. Anderson Cancer Center in accordance with all institutional, state, and federal ethical regulations for experimental animal care.

**Statistical Analysis.** The data were expressed as means  $\pm$  standard deviation (SD). Statistical analysis was performed with the two-tailed Student's *t* test. The criterion for statistical significance was set at  $P < 0.05$ .

## Results

**Curcumin Induces G<sub>2</sub>/M Arrest but Not Apoptosis in U87-MG and U373-MG Cells.** To examine the effect of curcumin on cell proliferation, we treated U87-MG and U373-MG cells with different concentrations of curcumin for 72 hours and measured cell viability using the WST-1 assay. Cell viability decreased in a dose-dependent manner in both cell types (Fig. 1A). The 50% inhibitory concentration was about 35  $\mu$ M for both cell types.

We then examined the effect of 20 or 40  $\mu$ M curcumin on the cell cycle of U87-MG and U373-MG cells. Treatment with curcumin for 72 hours induced G<sub>2</sub>/M cell cycle arrest in a dose-dependent manner in both cell types (Fig. 1B). However, the percentage of the sub-G<sub>1</sub> population, which is indicative of apoptosis, did not increase much even after treatment with 40  $\mu$ M

curcumin: from 0.4% to 2.2% in U87-MG cells and from 0.3% to 3.7% in U373-MG cells. In contrast, treatment with 5 nM paclitaxel, which is known to induce apoptosis in malignant glioma cells (Kondo and Kondo, 2006), increased the percentage of the sub-G<sub>1</sub> population to 22.1% in U87-MG cells and to 15.7% in U373-MG cells.

To further examine whether curcumin induces apoptosis using an assay more specific for apoptosis, we performed TUNEL staining of U87-MG and U373-MG cells after treatment with curcumin. Treatment with 40  $\mu$ M curcumin did not increase TUNEL-positive cells in either cell type (Fig. 1C and D). In contrast, treatment with 5 nM paclitaxel for 72 hours induced apoptosis in 17% of U87-MG cells and 13% of U373-MG cells. These results indicate that curcumin induces the G<sub>2</sub>/M arrest, but not apoptosis, in U87-MG and U373-MG cells.

To evaluate long term efficacy of curcumin on cell survival, clonogenic assay was performed. U373-MG cells were treated with different concentrations of curcumin in 6-well plates 24 hours after seeding. Two weeks after treatment, colonies were counted. Curcumin suppressed the surviving fraction of U373-MG cells in a dose-dependent manner (Fig. 1E), indicating the cell killing effect of curcumin.

**Curcumin Induces Autophagy in U87-MG and U373-MG Cells.** Recently, an increasing number of studies have shown that cancer cells, including malignant glioma cells, undergo autophagy in response to various anticancer therapies (Ogier-Denis and Codogno, 2003; Gozuacik and Kimichi, 2004; Kondo et al., 2005). Thus, we examined whether curcumin induces autophagy in U87-MG and U373-MG cells. Electron microscopic analysis showed that autophagic vacuoles containing cellular material or membranous structures increased in U373-MG cells treated with 40  $\mu$ M curcumin for 48 hours compared with untreated control cells (Fig. 2A). To quantify the incidence of curcumin-induced autophagy, we performed the following

assays. First, to quantify AVOs, which include autophagic vacuoles and lysosomes, we used acridine orange staining. Cells with AVOs showed enhanced red fluorescence that increased after treatment with curcumin in a dose-dependent manner (Fig. 2B). Moreover, addition of the autophagy inhibitor 3-MA inhibited the increase in the percentage of cells with enhanced AVOs after treatment with curcumin.

Second, we determined induction of autophagy by localizing an autophagosome-specific protein LC3 by using the GFP-LC3 plasmid (Kabeya et al. 2004). When autophagy is induced, exogenous LC3 distributes to the membrane of autophagosomes as endogenous LC3 does and shows characteristic GFP-LC3 dots. We transiently transfected U87-MG and U373-MG cells with the GFP-LC3 plasmid for 24 hours and then treated them with 40  $\mu$ M curcumin for 48 hours and determined the localization of LC3 using fluorescence microscopy. In untreated tumor cells, GFP-LC3 was distributed homogeneously in the cytoplasm, whereas the cells treated with curcumin showed GFP-LC3 dots (Fig. 2C). Quantitative analysis showed that the percentage of cells with GFP-LC3 dots increased after treatment with curcumin in a dose-dependent manner in both cell types: from 5.6%  $\pm$  1.9% to 33.3%  $\pm$  10.0% after treatment with 20  $\mu$ M curcumin and to 47.8%  $\pm$  5.1% after treatment with 40  $\mu$ M curcumin in U87-MG cells; from 3.3%  $\pm$  3.3% to 30.0%  $\pm$  12.0% after treatment with 20  $\mu$ M curcumin and to 45.6%  $\pm$  8.4% after treatment with 40  $\mu$ M curcumin in U373-MG cells.

Additionally, we examined the expression of LC3-I and LC3-II using Western blot analysis, because LC3-II is closely associated with the membrane of autophagosomes (Kabeya et al. 2004). Expression of LC3-II increased in U87-MG and U373-MG cells treated with curcumin in dose- and time-dependent manners (Fig. 2D). Collectively, these results indicate that curcumin induces autophagy in U87-MG and U373-MG cells.

It is still debating whether cancer treatment-induced autophagy is a protective response or an anticancer effect (Kondo et al., 2005). To assess the role of curcumin-induced autophagy, we determined whether inhibition of autophagy by 3-MA affects the cytotoxicity of curcumin. When 3-MA inhibited curcumin-induced autophagy (Fig. 2B), the decreased viability of U87-MG and U373-MG cells treated with curcumin was reversed ( $P < 0.05$ , respectively). These results suggest that curcumin-induced autophagy is an antitumor effect, not a protective response to curcumin.

**Curcumin Inhibits the Akt/mTOR/p70S6K Pathway and Activates the ERK Pathway in U87-MG and U373-MG Cells.** Because the Akt/mTOR/p70S6K pathway is the main regulatory pathway that negatively regulates autophagy (Blommaert et al., 1995; Shigemitsu et al., 1999; Arico et al., 2001), we examined the effect of curcumin on it using Western blotting. Treatment with curcumin decreased phosphorylated Akt effectively for a period of 15 minutes to 6 hours in both U87-MG and U373-MG cells (Fig. 3A), whereas the PI3K activity was not affected by curcumin (Fig. 3B). These results suggest that the upstream pathway of Akt was not influenced by curcumin treatment. Treatment with curcumin also decreased phosphorylated p70S6K gradually for 15 minutes to 6 hours in U87-MG cells and for 1 to 6 hours in U373-MG cells (Fig. 3A). Because the ERK pathway positively regulates autophagy in cancer cells on starvation (Ogier-Denis et al., 2000; Pattingre et al., 2003), we also examined this pathway after curcumin treatment. Treatment with curcumin increased phosphorylated ERK1/2 for 15 minutes to 3 hours in U87-MG cells and for 15 minutes to 6 hours in U373-MG cells (Fig. 3A). These results indicate that curcumin inhibited the Akt/mTOR/p70S6K pathway and activated the ERK pathway and suggest that both changes mediate curcumin-induced autophagy.

The reversible phosphorylation of proteins regulated by protein kinases and protein phosphatases is a key mechanism that controls a wide variety of cellular processes (Garcia et al.,

2003). Because the serine/threonine protein phosphatases type -1 (PP1) and type-2A (PP2A) are key players in the complexity of phosphorylation and dephosphorylation in these cellular processes, we determined whether PP1 or PP2A is involved in curcumin treatment. As shown in Figure 3C, the phosphorylation of PP1 in U87-MG and U373-MG cells was remarkably inhibited by curcumin, whereas curcumin treatment reduced only slightly the phosphorylation of PP2A in tumor cells. These results suggest that the down-regulation of PP1 might stimulate the ERK phosphorylation in curcumin treatment.

**Activation of the Akt Pathway Inhibits Curcumin-Induced Autophagy and Cytotoxicity in U87-MG and U373-MG Cells.** We and others have shown that the Akt/mTOR/p70S6K pathway mediates autophagy induced by some anticancer therapies (Blommaert et al., 1995; Shigemitsu et al., 1999; Takeuchi et al., 2005). Thus, we used rAkt1 to activate the Akt pathway as described previously (Takeuchi et al., 2004) so we could examine the role of this pathway in curcumin-induced autophagy. U87-MG cells were treated with 80  $\mu$ M curcumin, 500 ng/ml of rAkt1, or both for 3 hours for Western blotting. The addition of rAkt1 inhibited the curcumin-induced decrease in phosphorylated Akt and phosphorylated p70S6K in U87-MG cells (Fig. 4A); we obtained similar results with U373-MG cells (data not shown). The addition of rAkt1 significantly decreased curcumin-induced autophagy in both cell types ( $P < 0.05$ ; Fig. 4B). Furthermore, the addition of rAkt1 significantly inhibited curcumin-induced cytotoxicity in both cells ( $P < 0.05$ ; Fig. 4C). These results indicate that curcumin-induced inactivation of the Akt/mTOR/p70S6K pathway plays a role in the induction of autophagy and suggest that the autophagy negatively regulated by this pathway may be associated with cell death.

**Inhibition of the ERK Pathway Inhibits Curcumin-Induced Autophagy and Induces Apoptosis in U87-MG and U373-MG Cells.** The involvement of the ERK pathway in

regulating autophagy induced by nutrient starvation in cancer cells is well documented (Ogier-Denis et al., 2000; Pattingre et al., 2003). However, the role of this pathway in autophagy in response to anticancer therapy is not clear. Thus, to determine whether activation of the ERK pathway is involved in curcumin-induced autophagy, we used a MEK1 inhibitor, PD98059. Pretreatment of the cells with PD98059 effectively inhibited phosphorylated ERK in a dose-dependent manner (Fig. 5A). Expression of LC3-II increased in U373-MG cells treated with curcumin but decreased to some extent in the cells pretreated with PD98059 and then treated with curcumin for 72 hours.

We next determined the extent of the inhibitory effect of PD98059 on curcumin-induced autophagy in U87-MG and U373-MG cells by examining GFP-LC3 localization. The percentage of cells with GFP-LC3 dots decreased significantly in U87-MG and U373-MG cells treated with PD98059 and curcumin compared with that in the cells treated with curcumin alone ( $P < 0.05$ ; Fig. 5B). The ERK pathway is known as an antiapoptosis pathway (Xia et al., 1995), although prolonged activation of the ERK pathway has been shown to induce apoptosis. (Cagnol et al., 2006; Lee et al., 2003). Then we examined whether its inhibition induces apoptosis in curcumin-treated tumor cells. PARP was clearly cleaved in U373-MG cells treated with both PD98059 and curcumin, whereas it was not in the untreated cells or in those treated with PD98059 or curcumin alone (Fig. 5C). Similar results were obtained using U87-MG cells (data not shown). Interestingly, curcumin-induced cytotoxicity was significantly enhanced in the cells treated with PD98059 ( $P < 0.05$  in both cell types; Fig. 5D).

These results indicate that blocking the ERK pathway negatively impacts the curcumin effect and therefore, suggest that the ERK pathway is involved in curcumin-induced autophagy in U87-MG and U373-MG cells and this pathway protects these cells from apoptosis, at least to some

extent, although we need more direct evidence.

**NF- $\kappa$ B Inhibition is Not Involved in the Cytotoxicity of Curcumin in U87-MG and U373-MG Cells.** Many studies showed that curcumin inhibits NF- $\kappa$ B activity and induces apoptosis in various cancer cell types (Bharti et al., 2003; Aggarwal et al., 2004; Aggarwal et al., 2006). Thus, we examined whether inhibition of NF- $\kappa$ B was involved in the anticancer effect of curcumin in U87-MG and U373-MG cells. First, we examined active NF- $\kappa$ B using EMSA in cells with no treatment and cells treated with curcumin, TNF, or both. We also used KBM-5 leukemia cells as a positive control for TNF-activated NF- $\kappa$ B (Takada et al., 2004). Both U87-MG and U373-MG cells showed very little or no active NF- $\kappa$ B (Supplementary Fig. S1A). When cells were treated with 0.1 nM TNF for 30 minutes, U373-MG and U87-MG cells showed, respectively, moderate and modest levels of active NF- $\kappa$ B, whereas KBM-5 cells showed a very high level of active NF- $\kappa$ B (Supplementary Fig. S1A). Treatment with 50  $\mu$ M curcumin for 2 hours inhibited TNF-induced active NF- $\kappa$ B by 50% in U373-MG cells only.

Next, to determine whether inhibition of NF- $\kappa$ B is involved in curcumin-induced cytotoxicity in these cell types, we used siRNA for *NF- $\kappa$ B p65* to knock down this gene. Treatment of U373-MG cells with 100 nM *NF- $\kappa$ B p65* siRNA for 24 hours suppressed the expression of NF- $\kappa$ B p65 to an undetectable level (Supplementary Fig. S1B). Similar results were obtained using U87-MG cells (data not shown). However, knockdown of *NF- $\kappa$ B p65* did not reduce the viability of either cell type (Supplementary Fig. S1C). Furthermore, knockdown of *NF- $\kappa$ B p65* did not affect curcumin-induced AVO formation in either cell type (Supplementary Fig. S1D).

These results indicate that U87-MG and U373-MG cells have very little, if any, active NF- $\kappa$ B constitutively and that curcumin-induced cytotoxicity and autophagy in these cell types are not

likely due to curcumin's inhibitory effect on NF- $\kappa$ B.

**Curcumin Inhibits Growth of Subcutaneous Tumors by Inducing Autophagy.** Finally, we determined whether curcumin inhibits growth and induces autophagy in malignant glioma *in vivo*. Nude mice were inoculated subcutaneously with  $1 \times 10^6$  U87-MG cells. When tumors reached 50–70 mm<sup>3</sup> in volume, intratumoral injections of curcumin (100 mg/kg in DMSO in PBS) or vehicle (DMSO in PBS) were administered every 24 hours for 7 days, and tumor growth was observed until 16 days after the initiation of treatment. On day 16, tumor growth was inhibited significantly in tumors treated with curcumin compared with the control-treated tumors ( $3.5 \pm 2.8$  fold vs.  $12.5 \pm 5.9$  fold;  $P < 0.05$ ) (Fig. 6A). To examine whether curcumin induces autophagy *in vivo*, we examined the expression of LC3, especially LC3-II, using Western blotting and immunohistochemical staining. The expression of LC3-II increased remarkably in tumors treated with curcumin compared with that in the control-treated tumors (Fig. 6B). The expression of total LC3 in the cytoplasm detected by immunohistochemical staining using an anti-LC3 antibody also increased in the tumors treated with curcumin compared with the control-treated tumors (Fig. 6C).

These results together with the *in vitro* findings with 3-MA (Fig. 2E) suggest that curcumin inhibits tumor growth *in vivo* by inducing autophagy.

## Discussion

The results of this study showed that curcumin induced G<sub>2</sub>/M cell cycle arrest and autophagy, but not apoptosis, in U87-MG and U373-MG cells. Regarding signal pathways, curcumin inhibited the Akt/mTOR/p70S6K pathway and activated the ERK pathway, resulting in autophagy. The autophagy regulated by these two different pathways influenced curcumin's

cytotoxicity differently. Furthermore, curcumin effectively inhibited tumor growth and induced autophagy in the xenograft tumor model of U87-MG cells. These results demonstrate that curcumin may be a promising agent for the treatment of patients with malignant glioma. To the best of our knowledge, this study is the first to demonstrate that curcumin induces autophagy in cancer cells *in vitro* and *in vivo*.

This study clearly demonstrated that curcumin inhibits the Akt/mTOR/p70S6K pathway and activates ERK signaling, resulting in induction of autophagy (Figs. 2 and 3). Several other studies have also shown that curcumin inhibits the Akt/mTOR/p70S6K pathway in various cancer cells including leukemia, renal cancer, breast cancer, and prostate cancer cells (Aggarwal et al., 2006; Woo et al. 2003; Bava et al., 2005; Beevers et al., 2006). Some investigators reported curcumin's effect on ERK signaling, but with different results. Woo et al. (Woo et al., 2005) showed that curcumin repressed the phorbol ester–induced activation of ERK, whereas Collett and Campbell (Collett and Campbell, 2004) found no effect of curcumin on ERK. Because curcumin modulates many pathways (Aggarwal et al., 2006; Shishodia et al., 2005), its detailed mechanisms may vary depending on the cancer cell type. In the context of induction of autophagy, Ellington et al. (Ellington et al., 2006) showed that the natural products triterpenoid B-group soyasaponins induced autophagy by inhibiting Akt signaling and enhancing ERK activity, in accord with our findings. This combination of Akt inhibition and ERK activation may be one of the common mechanisms of autophagy induction by anticancer agents.

Because the extent of autophagy increased in dose- and time-dependent manners, we concluded that autophagy is a response of U87-MG and U373-MG cells to curcumin. Our results clearly indicated that curcumin's cytotoxic effect on these cells is due to autophagy, but not apoptosis. Curcumin's overall effect is as an anticancer agent both *in vitro* and *in vivo*, as shown

in other cancer cells (Aggarwal et al., 2006; Shishodia et al., 2005). Although autophagy is designated programmed cell death type II, whether autophagy actually leads cells to death or protects them from death has been a controversial issue (Takada et al., 2004; Gozuacik et al., 2004). Some investigators knocked down *autophagy-related* (*Atg*) genes using siRNA and specifically inhibited autophagy but reached opposite conclusions depending on their experimental system. For example, in an apoptosis-defective system where *Bax* and *Bak* were both knocked out (i.e., *Bax/Bak*<sup>-/-</sup>), siRNA for *Beclin 1* or *Atg5* inhibited etoposide-induced autophagy and led cells to survival, whereas siRNA for *Atg5* or *Atg7* inhibited autophagy caused by interleukin-3 deprivation and killed more cells (Shimizu et al., 2004; Lum et al., 2005). One possibility is that autophagy kills or protects cells depending on how autophagy is induced. That is, interleukin-3 deprivation-induced autophagy is supposed to be a survival mechanism, so inhibition of this autophagy leads to death: etoposide induces cell death, so inhibition of this autophagy saves cells from death. In this study, we examined the role of autophagy by manipulating the regulatory pathways individually, because the Akt and ERK pathways are known to regulate autophagy, but with opposite effects: the Akt pathway regulates autophagy negatively, whereas the ERK pathway regulates it positively. Activation of the Akt pathway using rAkt1 inhibited curcumin-induced autophagy and cytotoxicity (Fig. 4). On the other hand, inhibition of the ERK pathway using PD98059 inhibited autophagy and induced apoptosis, thus enhancing cytotoxicity (Fig. 5). These results implicate that autophagy's role on cell death is pathway specific. That is, the autophagy the Akt pathway inhibits confers cell death, and the autophagy the ERK pathway induces confers cell survival. This hypothesis can explain the double effect autophagy has on cell death, and it is worth being evaluated in different experimental systems.

NFκB is one of the main targets of curcumin for its anticancer effect (Singh and Aggarwal, 1995; Bharti et al., 2003; Aggarwal et al., 2004). However, we found that U87-MG and U373-MG cells had very little or no constitutively active NFκB. Furthermore, when we completely knocked down *NFκB p65*, the viability of these cell types did not change (Supplementary Fig. S1). These results indicate that the anticancer effect of curcumin and the autophagy we detected in these cell types are not due to inhibition of NFκB. However, curcumin can be used to inhibit active NFκB that is induced by chemokines or other anticancer treatments as shown in leukemia and cervical cancer cells (Bava et al., 2005; Xia et al., 1995). For example, radiation induces NFκB activity in malignant glioma cells, which implies a resistant mechanism (Raju et al., 1997). Thus, curcumin may need to be used in combination with radiation or other chemotherapeutic agents for treating malignant glioma to demonstrate its inhibitory effect of NFκB.

Our results showed that curcumin significantly inhibited the growth of malignant glioma both *in vitro* and *in vivo*. An increasing number of studies have shown curcumin's anticancer efficacy in preclinical and clinical settings. In subcutaneous animal models of various cancer cell types, curcumin effectively inhibited the growth of tumors (Aggarwal et al., 2006; Shishodia et al., 2005). Further, a recent study reported that curcumin suppressed lung metastasis of breast cancer cells when used as a single agent or in combination with paclitaxel (Aggarwal et al., 2005). Several phase I clinical studies have demonstrated that curcumin was well tolerated up to 12 g per day without major adverse effects (Sharma et al., 2001; Sharma et al., 2004; Lao et al., 2006). A phase II clinical trial for patients with pancreatic cancer is on-going at our institute. However, absorption and bioavailability of curcumin outside the colon is very problematic. Therefore, the intratumoral injection of rather large concentrations of curcumin that we used in this study might be not very useful clinically for human gliomas. Recently, convection-enhanced delivery has

been developed as a new technique of direct injection to increase drug uptake and distribution to large regions of the brain tumor by applying a pressure gradient (Lopez et al., 2006). With this method, curcumin can be delivered to malignant gliomas directly and efficiently while limiting toxicity to surrounding normal tissues.

In summary, we have shown for the first time that curcumin induces autophagy in malignant glioma cells both *in vitro* and *in vivo*. The Akt/mTOR/p70S6K and ERK1/2 pathways are involved in curcumin-induced autophagy. Our results suggest that autophagy's effect on cell death may be dependent on its regulatory pathways. We recommend that the use of curcumin as a new anticancer agent for malignant glioma should be pursued further because of its prominent effect and its new anticancer mechanism of inducing autophagy.

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## Footnotes

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## Figure Legends

**Figure 1.** Curcumin induces G<sub>2</sub>/M arrest, but not apoptosis, in U87-MG and U373-MG cells. A, the cytotoxic effect of curcumin as measured using the WST-1 assay. Cells were treated with different concentrations of curcumin for 72 hours and subjected to the WST-1 cell proliferation assay. The viability of untreated cells was considered 100%. Data are the means of triplicate experiments; *error bars*, SD. B, cell cycle analysis after treatment with curcumin. Cells were treated with 20 μM or 40 μM curcumin or 5 nM paclitaxel for 72 hours and subjected to cell cycle analysis. Results shown are representative of three independent experiments. C, representative micrographs of the cells stained with TUNEL. Cells were treated with 40 μM curcumin or 5 nM paclitaxel for 72 hours and subjected to TUNEL staining. *Scale bars*, 50 μm. D, quantitation of TUNEL-positive cells. Cells were treated as described above and subjected to TUNEL staining. TUNEL-positive cells were counted among 100 cells in an area, and more than three areas were selected at random. Data are the means of triplicate experiments; *error bars*, SD. E, curcumin effect on clonogenic survival 14 days after addition of curcumin. Clonogenic survival is expressed as a surviving fraction of non-treatment control for each treatment and is plotted on a semi-logarithmic scale. *Points*, mean of triplicate experiments; *bars*, SD.

**Figure 2.** Curcumin induces autophagy in U87-MG and U373-MG cells. A, representative electron micrographs of U373-MG cells treated with DMSO (control) or 40 μM curcumin for 48 hours. *Arrows*, autophagic vacuoles; *scale bar*, 2 μm. B, quantification of cells with enhanced red fluorescence using flow cytometry after acridine orange staining. Cells were treated with DMSO (control) or the indicated concentrations of curcumin for 72 hours and subjected to acridine orange staining. To inhibit autophagy, 2 mM 3-methyladenine (3-MA) was added 24

hours after addition of curcumin. Results shown are representative of three independent experiments. C, representative micrographs of cells that show green fluorescent protein (GFP)-LC3 localization. Cells were transiently transfected with the GFP-LC3 plasmid for 24 hours and treated with DMSO (control) or 40  $\mu$ M curcumin for 72 hours. D, Western blot analysis of LC3-I and LC3-II. Cells were treated with the indicated concentrations of curcumin for 48 hours (top) or with 40  $\mu$ M curcumin for the indicated times (bottom) and subjected to Western blotting. Anti- $\beta$ -actin antibody was used as a loading control. Results shown are representative of two independent experiments. E, the effect of autophagy inhibition on the cytotoxicity of curcumin. After U87-MG and U373-MG cells were treated with 0, 20, or 40  $\mu$ M curcumin with or without 2 mM 3-MA for 72 hours, the cell viability was measured by WST-1 cell proliferation assay. The viability of untreated cells was considered 100%. Data are the means of triplicate experiments; *error bars*, SD.  $P < 0.05$  for curcumin-treated cells with and without 3-MA.

**Figure 3.** Curcumin inhibits the Akt/mTOR/p70S6K pathway and activates the ERK pathway in U87-MG and U373-MG cells. A, Western blot analysis for the Akt/mTOR/p70S6K and ERK pathways. Cells were treated with 80  $\mu$ M curcumin for the indicated times and subjected to Western blotting. Relative levels of phosphorylated Akt (phospho-Akt) to total Akt, those of phosphorylated p70S6K (phospho-p70S6K) to total p70S6K, and those of phosphorylated ERK1/2 (phospho-ERK1/2) to total ERK1/2 are indicated below the corresponding bands. The data shown are representative of three independent experiments. B, the change of PI3K activity by curcumin. PI3K activity was determined by measuring the conversion of PI(4,5)P2 into PI(3,4,5)P3 using ELISA kit. U87-MG and U373-MG cells were treated with 80  $\mu$ M curcumin for indicated minutes. Data are the means of triplicate experiments; *error bars*, SD. C, Western

blot analysis of protein phosphatase phosphorylation. U87-MG and U373-MG cells were treated with 80  $\mu$ M curcumin up to 3 hours, and then subjected to Western blotting for detection of phosphorylated PP1 and PP2A and total PP1 and PP2A. Relative levels of phosphorylated PP1 (phospho-PP1) to total PP1 and those of phosphorylated PP2A (phospho-PP2A) to total PP2A are indicated below the corresponding bands. The data shown are representative of three independent experiments.

**Figure 4.** Activation of the Akt pathway by rAkt inhibits curcumin-induced autophagy and cytotoxicity in U87-MG and U373-MG cells. A, Western blot analysis of the Akt/p70S6K pathway. U87-MG cells were treated with 80  $\mu$ M curcumin, 500 ng/ml of rAkt1, or both for 3 hours and subjected to Western blotting. Relative levels of phosphorylated Akt (phospho-Akt) to total Akt and those of phosphorylated p70S6K (phospho-p70S6K) to total p70S6K are indicated below the corresponding bands. The data shown are representative of two independent experiments. B, quantification of the cells with GFP-LC3 dots. Cells were transfected with the GFP-LC3 plasmid for 24 hours and then treated with 40  $\mu$ M curcumin, 500 ng/ml of rAkt1, or both for 72 hours, and the percentage of cells with GFP-LC3 dots among the total number of cells with GFP expression was determined. Data are the means of triplicate experiments; *error bars*, SD;  $**P < 0.01$ . C, cytotoxic effect measured using the WST-1 assay. Cells were treated with different concentrations of curcumin, 500 ng/ml of rAkt1, or both for 72 hours and subjected to the WST-1 cell proliferation assay. The viability of untreated cells was considered 100%. Data are the means of triplicate experiments; *error bars*, SD;  $*P < 0.05$ .

**Figure 5.** Inhibition of the ERK pathway by PD98059 (PD) inhibits curcumin-induced

autophagy and induces apoptosis in U87-MG and U373-MG cells. A, Western blot analysis of the ERK pathway and LC3. U373-MG cells were pretreated with the indicated concentrations of PD98059 for 1 hour, treated with 80  $\mu$ M curcumin for 1 hour, and then subjected to Western blotting for detection of phosphorylated ERK1/2 (phospho-ERK1/2) and total ERK1/2. For the detection of LC3, cells were pretreated with PD98059 for 1 hour and then treated with 40  $\mu$ M curcumin for 72 hours. B, quantitation of cells with GFP-LC3 dots among cells treated with 40  $\mu$ M curcumin only or 40  $\mu$ M curcumin and 25  $\mu$ M PD98059. Cells were transiently transfected with the GFP-LC3 plasmid for 24 hours and then treated as described for 72 hours. The number of cells with GFP-LC3 dots was counted, and their percentage among the total number of cells expressing GFP was determined. Data are the mean of triplicate experiments; *error bars*, SD; \*\* $P < 0.01$ ; \* $P < 0.05$ . C, Western blot analysis for poly(ADP-ribose) polymerase (PARP) cleavage. U373-MG cells were treated with curcumin, PD98059, or both, as described above, and subjected to Western blotting 72 hours later. Anti- $\beta$ -actin antibody was used as a loading control. Results shown are representative of two independent experiments. D, cytotoxic effect measured using the WST-1 assay. Cells were treated with 40  $\mu$ M curcumin only or 40  $\mu$ M curcumin and 25  $\mu$ M PD98059, and subjected to the WST-1 assay 72 hours later. Data are the means of triplicate experiments; *error bars*, SD; \*\* $P < 0.01$ .

**Figure 6.** Curcumin inhibits the growth of malignant glioma cells *in vivo* by inducing autophagy.

A, tumor growth on day 16 after the initiation of curcumin treatment. U87-MG cells ( $1 \times 10^6$ ) were inoculated subcutaneously into the nude mice. When the tumors reached 50–70 mm<sup>3</sup> in volume, intratumoral injections of curcumin (100 mg/kg in DMSO/PBS) or DMSO/PBS (control) were administered every 24 hours for 7 days. B, Western blot analysis of excised

tumors for LC3. Tumors were removed on day 16, and the proteins were isolated and subjected to Western blotting. Anti- $\beta$ -actin antibody was used as a loading control. Results shown are representative of two independent experiments. C, representative micrographs of immunohistochemical staining using anti-LC3 antibody. Tumor-bearing animals were treated and the tumors were removed as described above. Tumor samples were snap frozen, sliced to 10  $\mu$ m thickness, and subjected to immunohistochemical staining. *Scale bars*, 50  $\mu$ m.

Figure 1

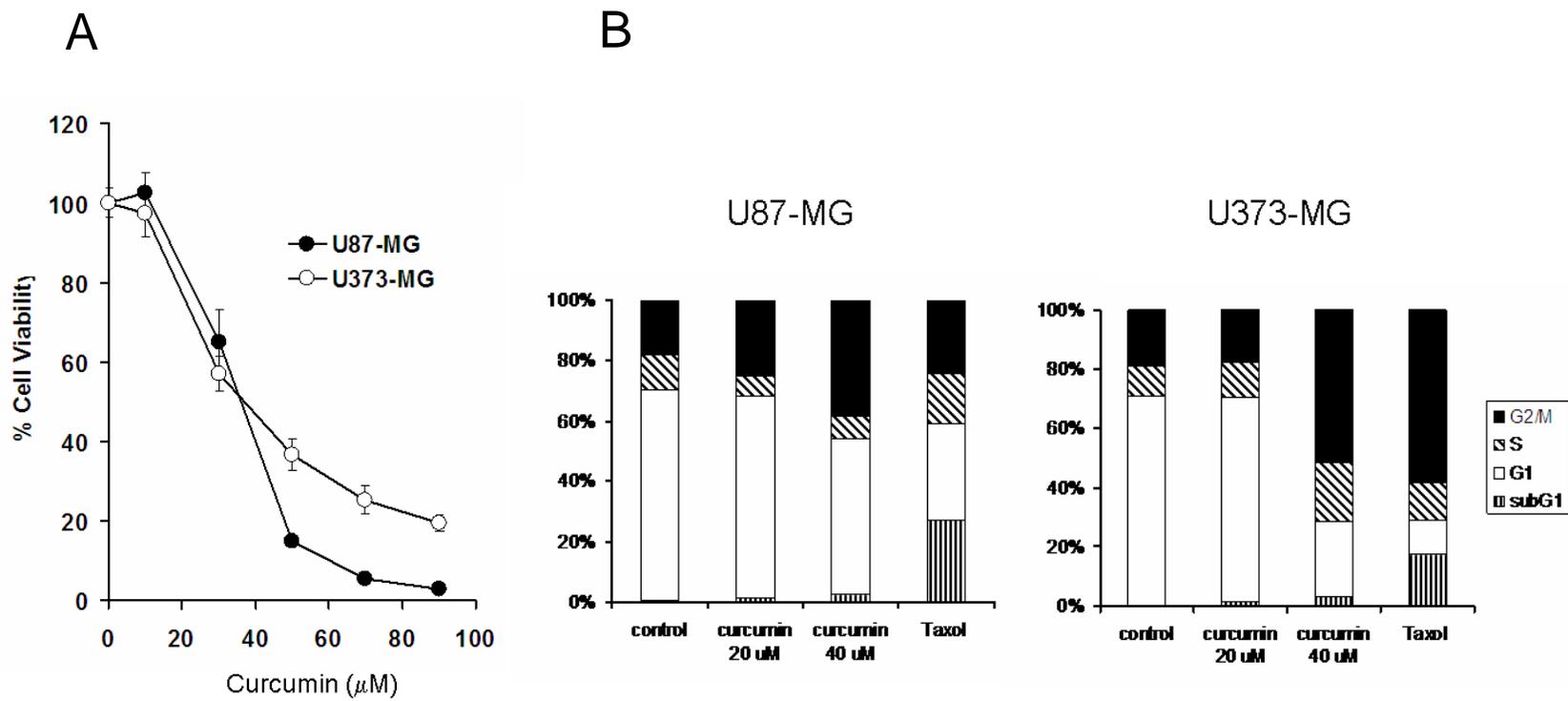


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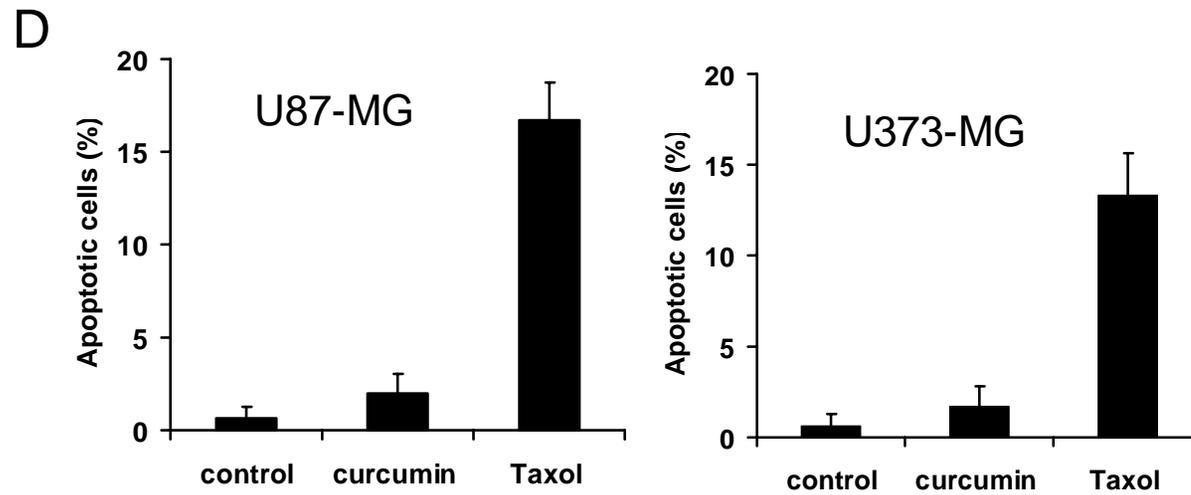
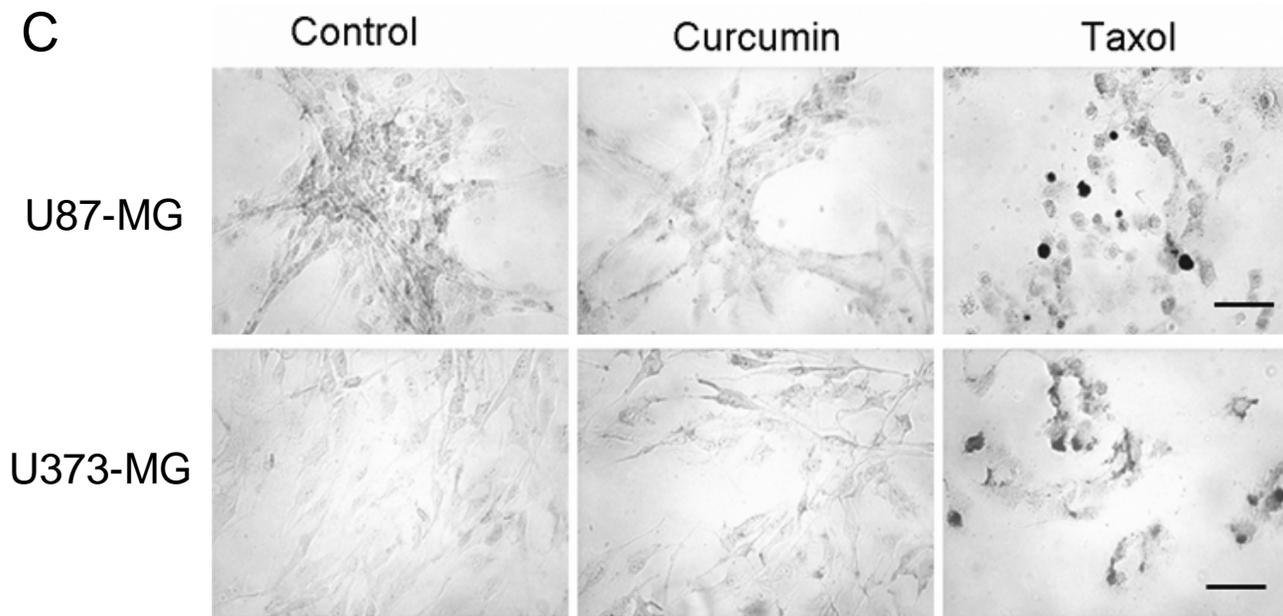


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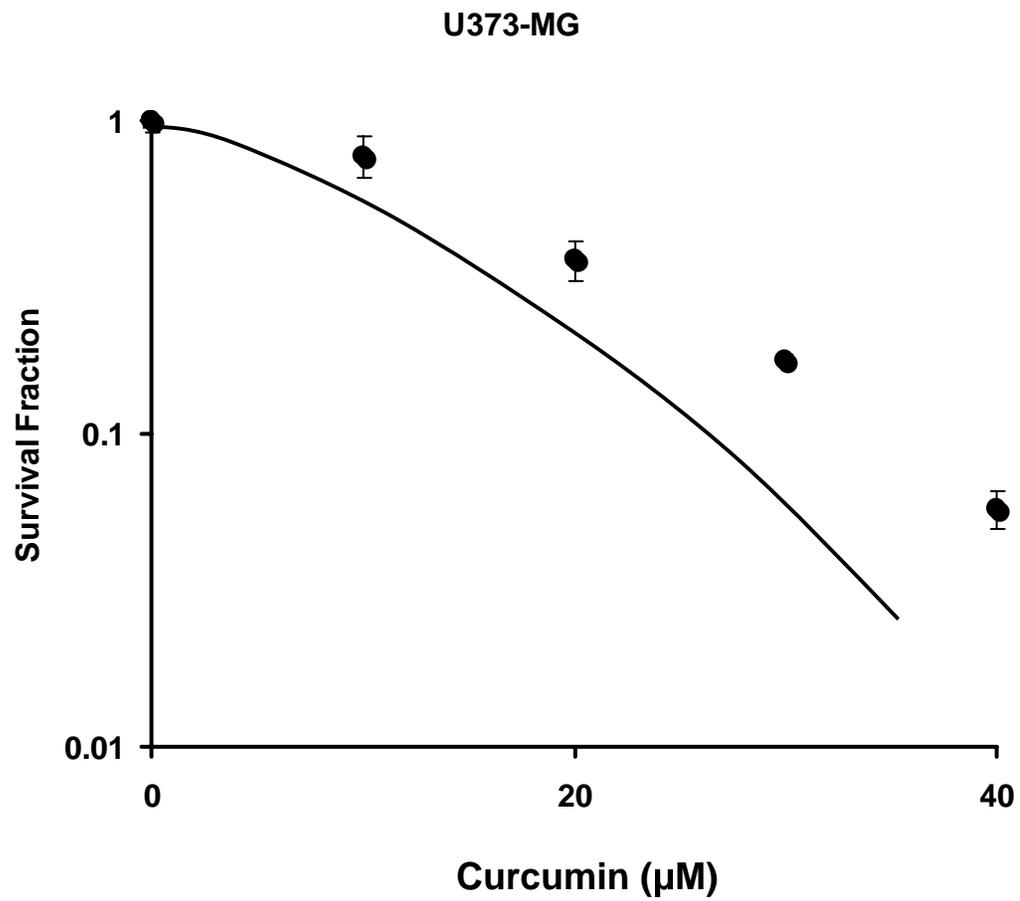


Figure 2

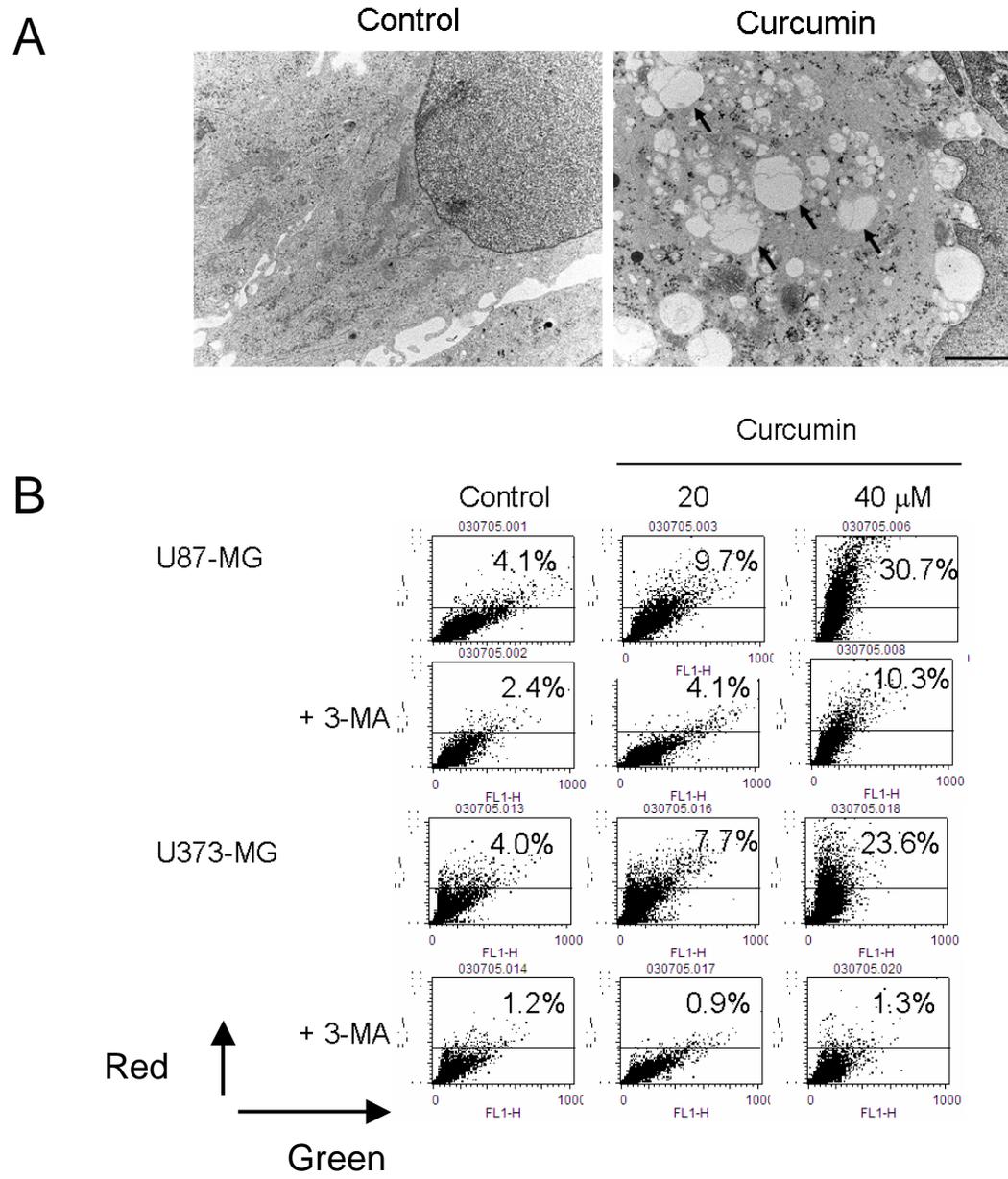


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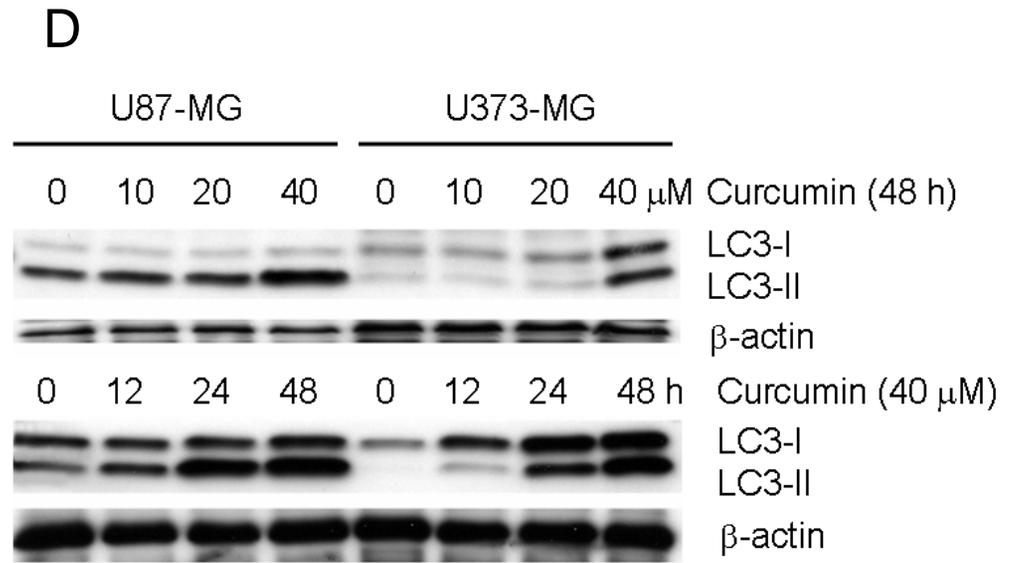
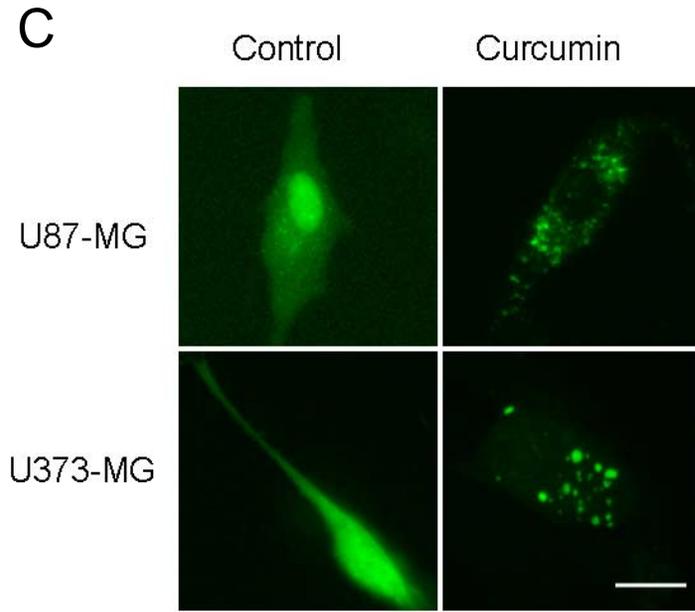


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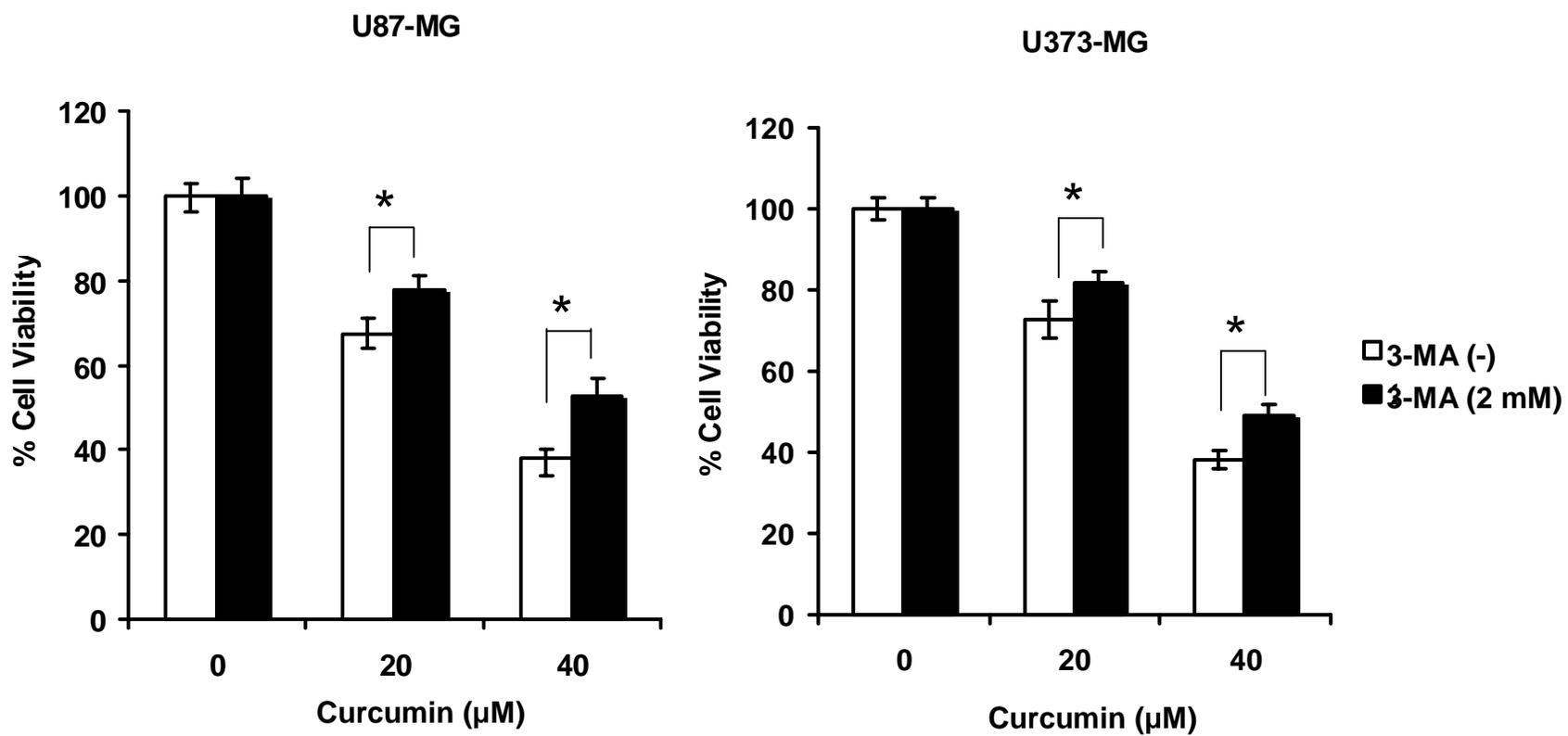


Figure 3

A

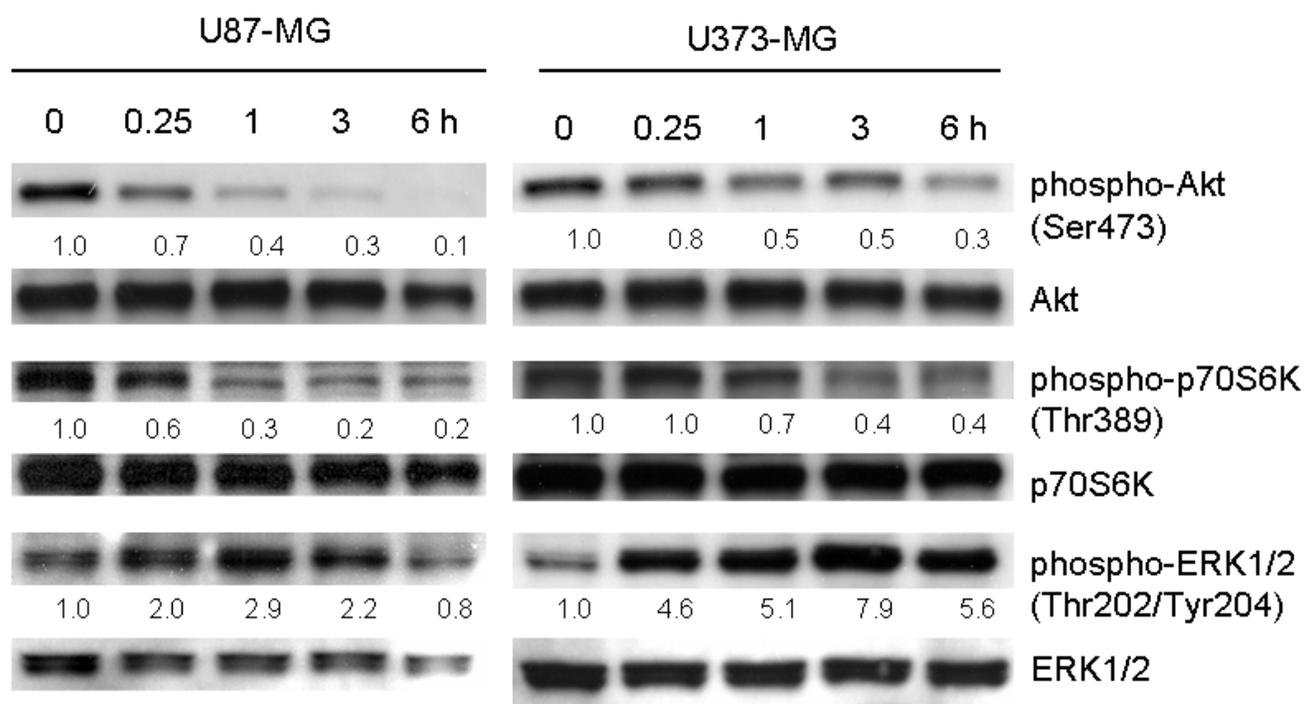


Figure 3 (Continued)

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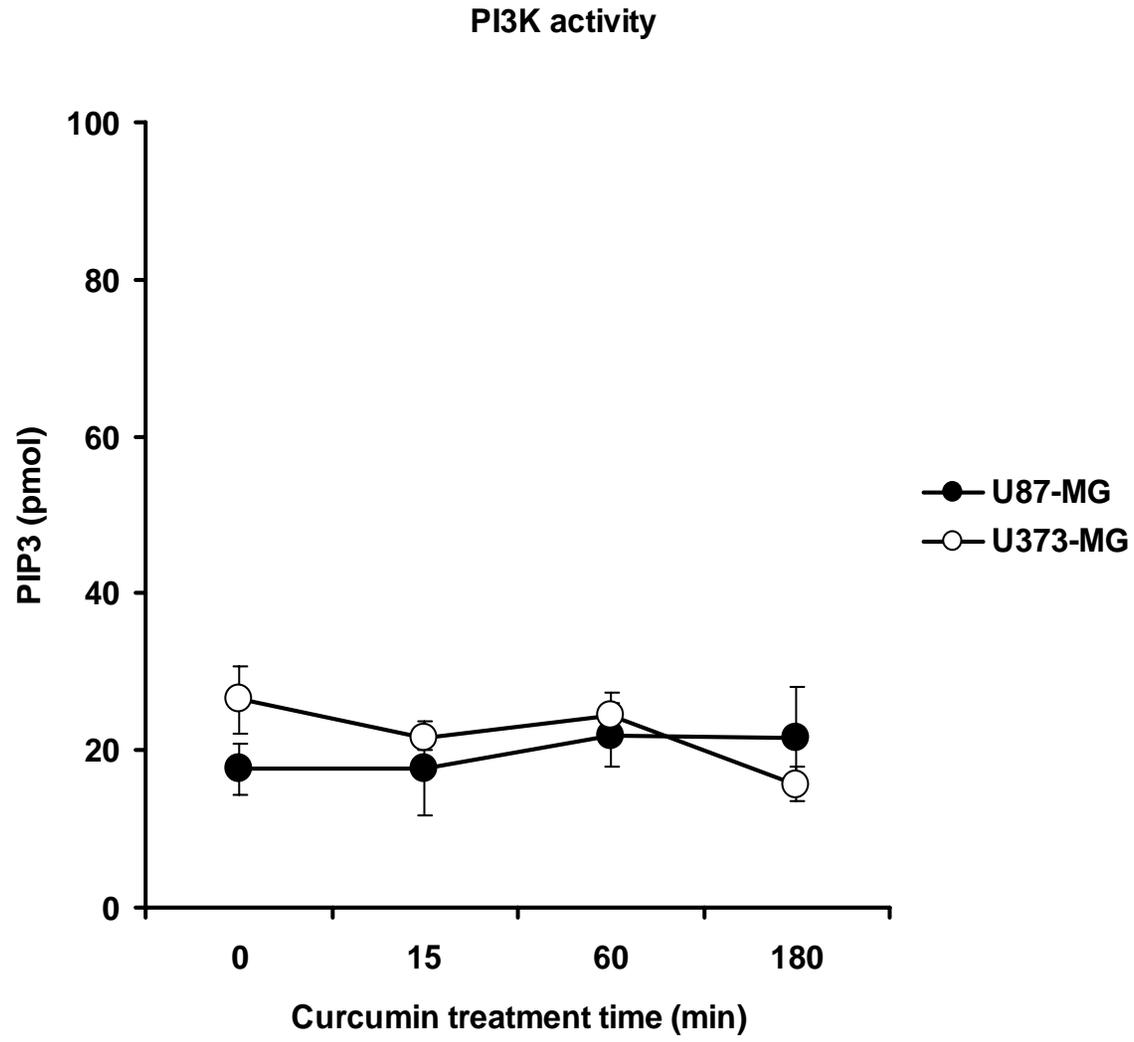


Figure 3 (Continued)

C

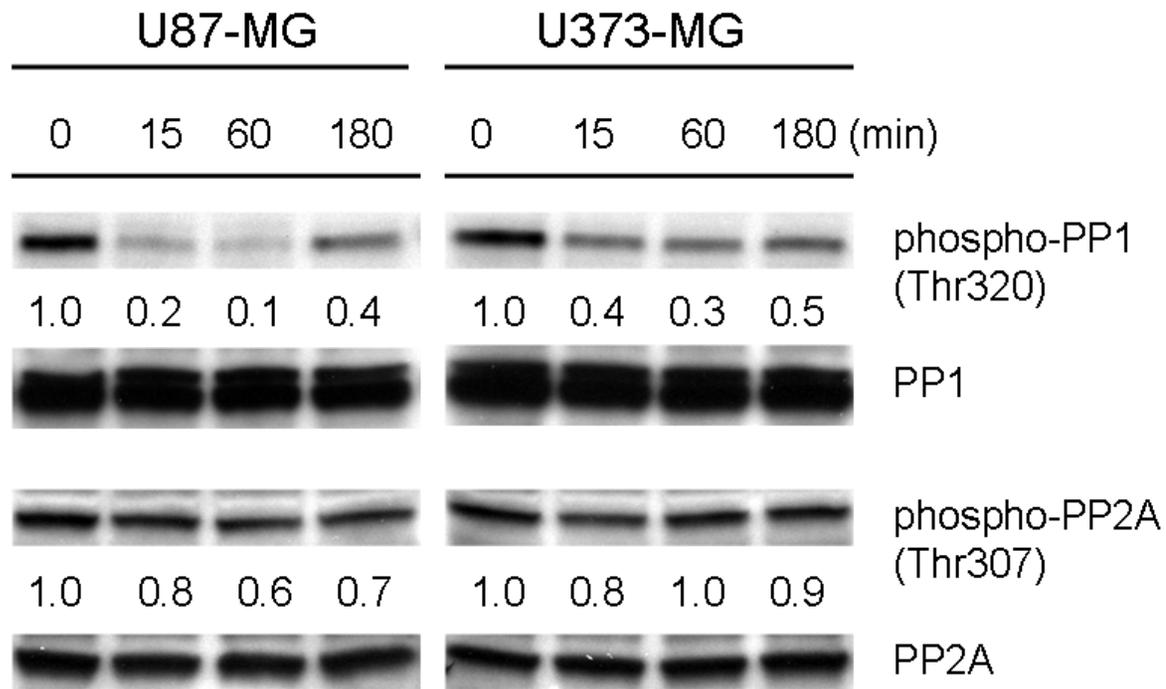


Figure 4

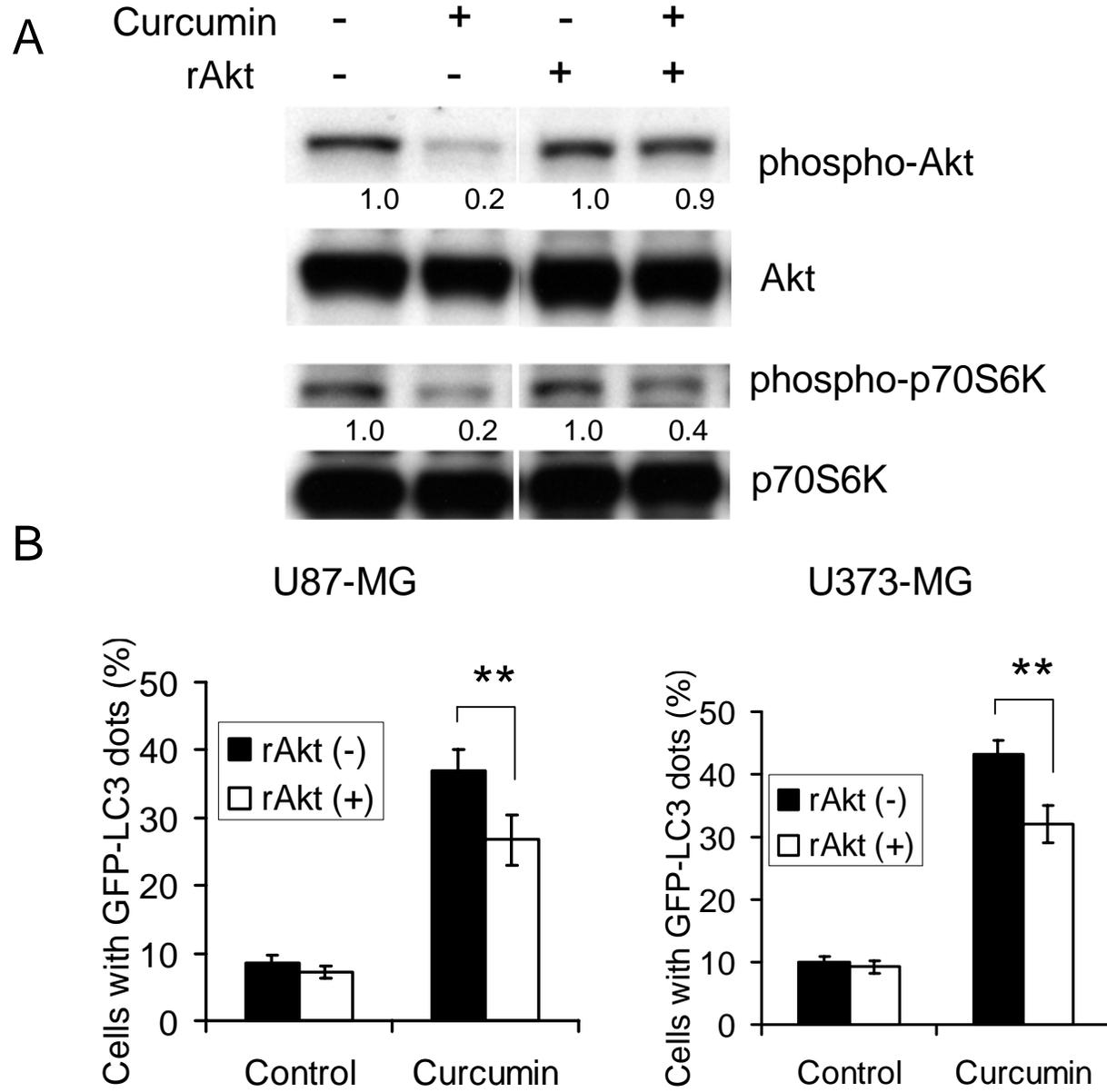


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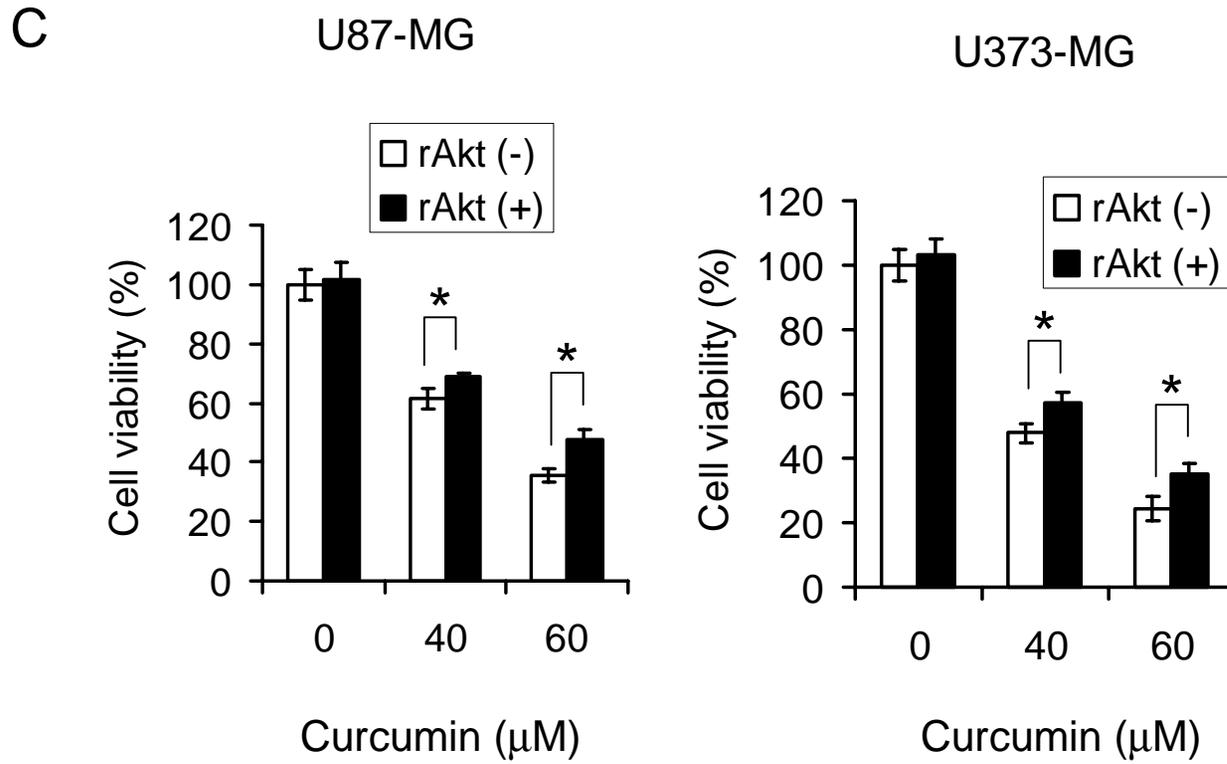


Figure 5

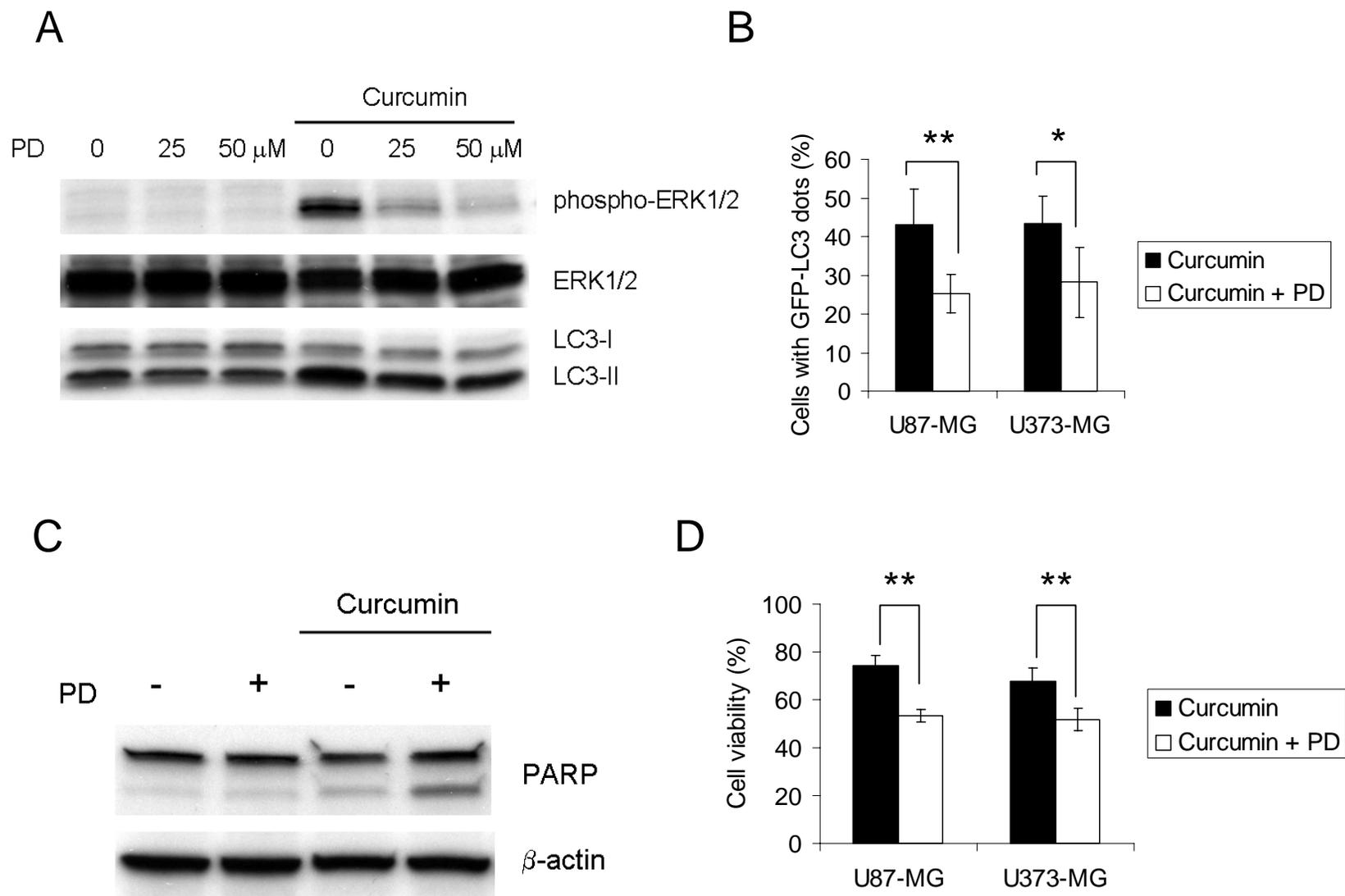


Figure 6

