Curcumin suppresses stem-like traits of lung cancer cells via inhibiting the JAK2/STAT3 signaling pathway

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Abstract. Tumor recurrence and drug resistance are the main obstacles blocking effective treatment of cancer patients. Cancer stem cells (CSCs) have been demonstrated to be highly related to tumor recurrence and drug resistance. Thus, eliminating CSCs may be an alternative for cancer therapy. Tumor sphere formation is a functional assay to enrich the CSC-like cells. In the present study, we tested the effects of curcumin on lung cancer stem-like cells and report that in addition to inhibition on the proliferation and colony formation of lung cancer cells, curcumin reduces tumor spheres of H460 cells. Moreover, by molecular docking analysis and tumor sphere assay we discover that curcumin was able to inhibit JAK2 activity and reduce tumor spheres via inhibiting the JAK2/STAT3 signaling pathway. In a lung cancer xenograft nude mouse model, curcumin strongly repressed tumor growth. These results imply curcumin may be a potential drug in lung CSC elimination and cancer therapy.

Introduction

Lung cancer is the most deadly malignant tumor (1). There are two major types, non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) which comprise 85 and 15% of all cases, respectively. Tumor-initiating cells, also termed cancer stem cells (CSCs), are a rare population of cancer cells which possess the ability to self-renew and differentiate (2). CSCs were first isolated in 1997 in leukemia (3). Subsequently, CSCs have been identified in solid tumors, including breast (4), brain (5), prostate (6), melanoma (7), colon (8,9) and lung cancer (10,11). Since the isolation of CSCs in 1997, a growing body of literature has demonstrated that tumor initiation, recurrence and drug resistance are highly related to CSCs (12-14). Thus, CSCs is likely a plausible target for cancer therapy (15). CSCs were identified using flow cytometry-based cell sorting of specific surface markers or tumor sphere forming assay. Tumor spheres, which could reflect tumor cell stem-like property, are multicellular three-dimensional clones enriching CSCs (2,3). Suppressing tumor spheres may impair cancer cell stem-like property.

The JAK2/STAT3 pathway mediates the effects of a spectrum of cytokines and growth factors. It can be transiently activated in normal cells upon stimulation while in cancer cells, it is constitutively active (16,17). JAK2/STAT3 signaling pathway plays a crucial role in various cancer initiation and development stages, and active STAT3 is commonly associated with a worse prognosis (18-21). It is also reported that JAK2/STAT3 signaling pathway is involved in CSCs in many malignant, including lung CSCs and aberrant expression of JAK2/STAT3 signaling pathway in CSCs can promote cancer initiation (22-24). Thus, JAK2/STAT3 pathway could be a targetable pathway for CSC elimination.

Curcumin (diferuloylmethane, Fig. 1A), a phenolic compound isolated from the plant *Curcuma longa*, has been used in traditional medicines in China for thousands of years. It exhibits anticancer effects by induction of growth inhibition, cell cycle arrest and apoptosis in various types of cancer (25-28). It is also reported that curcumin effectively prevented emergence of chemoresistance and eliminate CSCs in breast, glioblastoma, pancreatic and colon cancer (29-33). The antiproliferation effects of curcumin on lung cancer have been reported to induce apoptosis in A549 and NCI-H460 cells through ER stress and caspase cascade- and mitochondria-dependent pathways (34-36), however, the effects of curcumin on lung cancer stem-like cells remain obscure. In the present study, we investigated the underlying mechanisms of curcumin on lung tumor spheres.

Materials and methods

Cell culture and reagents. The lung cancer cell line NCI-H460 was purchased from American Type Culture Collection (ATCC) and cultured in RPMI-1640 supplemented with

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10% fetal bovine serum (FBS). Curcumin and Taxol were purchased from Sigma. MTT was purchased from Amresco. The antibodies used in the present study were as follows: anti-β-actin (A5441; Sigma); p-JAK2 (#3776), JAK2 (#3230), p-STAT3 (#9138), STAT3 (#4904) (all from CST); C-myc (sc-40; Santa Cruz), anti-myc (#MA1-21316-D680; Invitrogen), cyclin D1 (ab16663; Abcam). The growth factors used in the present study were: N2 supplement (Gibco), FGF, EGF (R&D Systems), and STAT3 siRNA (#6582; CST).

Cell viability and clonogenic assay. For cell viability assay, $8x10^4$ cells were seeded into 12-well plates. Then, different concentrations of curcumin and dimethyl sulfoxide (DMSO) were added into plates for indicated times. Viable cells were counted using trypan blue dye exclusion analysis. For the clonogenic assay, NCI-H460 cells were suspended in 1 ml RPMI-1640 containing 0.3% low melting point agarose (Amresco) and 10% FBS, and plated on a bottom layer containing 0.6% agarose and different doses of curcumin in 35 mm plates (1,000 cells/plate). Ten days later, cells were stained with 0.005% crystal violet and clones >50 cells were counted.

Tumor sphere assay. NCI-H460 cells were cultured in tumor sphere medium consisting of serum-free DMEM/F12 medium, 10 ng/ml human recombinant fibroblast growth factor-basic (FGF), 10 ng/ml epidermal growth factor (EGF), and N2 supplement. Then cells suspended in sphere medium containing different doses of curcumin and Taxol were plated into ultra low-attachment 24 well-plate at a density of 1,000/well. Five days later, tumor sphere formation was observed and photographed by a microscope.

Western blotting assay. NCI-H460 cells were suspended in tumor sphere medium to form tumor spheres. Then medium was replaced with fresh tumor sphere medium containing different doses of curcumin and DMSO and tumor spheres were cultured for indicated time points. Cell lysates of tumor spheres treated with curcumin or DMSO at indicated times or different doses were subjected to SDS-PAGE to detect related protein expression.

STAT3 overexpression and RNAi knockdown. STAT3 was overexpressed by transient transfection with pcDNA3.0-myc-STAT3 plasmids in H460 cells (vector pcDNA3.0-myc as control). To downregulate STAT3 expression in NCI-H460 cells, specific stat3 RNAi (CST) was used. After transfection, cells were suspended in tumor sphere medium in ultra low attachment 24-well plates. One week later, the formed tumor spheres were photographed under a microscope.

Murine models. All animal studies were conducted according to the protocols approved by the Animal Ethics Committee of Guangxi Medical University and maintained under conventional conditions. NCI-H460 derived tumor spheres were digested into single cells and injected into each mouse (100 cells for each) by subcutaneously inoculation in the right flank of nude mice. When tumor volume reached 50 mm^3, mice were randomized into 3 groups (n=5 for each group) and treated with curcumin (40 mg/kg), Taxol (5 mg/kg) or vehicle control (polyoxyethylenated castor oil: ethanol=1:1) for 2 weeks every 2 days with intraperitoneal injection (i.p). Tumor growth and mouse body weight were monitored every other day for 15 days. Tumor volume was calculated using the formula: $V = 0.5 \times L \times W^2$, where L and W represented the long and short diameter of the tumor, respectively. At the time of the animal sacrifice, tumors were excised; cells were separated and lyzed for western blotting.

Molecular docking. Computational docking test was performed using MOE2008.10 (Molecular Operating Environment) to investigate the interaction between JAK2 and curcumin at a molecular level. X-ray crystal structures of JAK2 (PDB ID: 5AEP) and its ligand was obtained from Protein Data Bank (http://www.rcsb.org). Water molecules were manually removed from the protein structures. Docking process was as described previously (39).

Statistical analysis. Differences between data groups were evaluated using Student's t-test. P-values <0.05 were considered to indicate a statistically significant result. Data are presented as the mean ± SD unless otherwise noted.

Results

Curcumin inhibits proliferation and colony formation of NCI-H460 lung cancer cells. The effects of curcumin on lung cancer cell proliferation was determined using trypan blue dye exclusion analysis. The results demonstrated that curcumin significantly inhibited the growth of NCI-H460 cells at 20 and 40 μM (Fig. 1B). The soft agar assay showed that curcumin markedly reduced colony number of NCI-H460 cells in a dose-dependent manner (Fig. 1C and D). Curcumin significantly inhibited the colony forming activity of NCI-H460 cells at a concentration of 10 μM.

Curcumin impairs tumor sphere formation of NCI-H460 lung cancer cells in vitro. To determine the effects of curcumin on lung tumor spheres, we used NCI-H460 lung cancer cells as a *in vitro* model and carried out tumor sphere assays. The results suggested that curcumin reduced NCI-H460 tumor spheres in a dose-dependent manner (Fig. 2A). Compared with the traditional chemotherapy drug Taxol, which could not decrease tumor spheres (37), curcumin significantly reduced tumor spheres at concentrations of 20 and 40 μM (Fig. 2B). These data implied curcumin suppressed tumor spheres growth *in vitro*.

Curcumin inhibits JAK2/STAT3 signaling pathway activity. Considering the significant role of JAK2/STAT3 signaling pathway in lung CSCs development (23,38), we carried out western blotting to detect whether curcumin disturbed the JAK2/STAT3 signaling pathway. The results show that under curcumin treatment, p-JAK2 and p-STAT3 were downregulated in a time- and dose-dependent manner. The downstream molecules of the JAK2/STAT3 pathway, cyclin D1 and C-myc were also downregulated (Fig. 3A and B). Moreover, by molecular docking analysis (39), we discovered that curcumin could potently bind to JAK2. The three-dimensional structure revealed that curcumin can be perfectly embedded into the

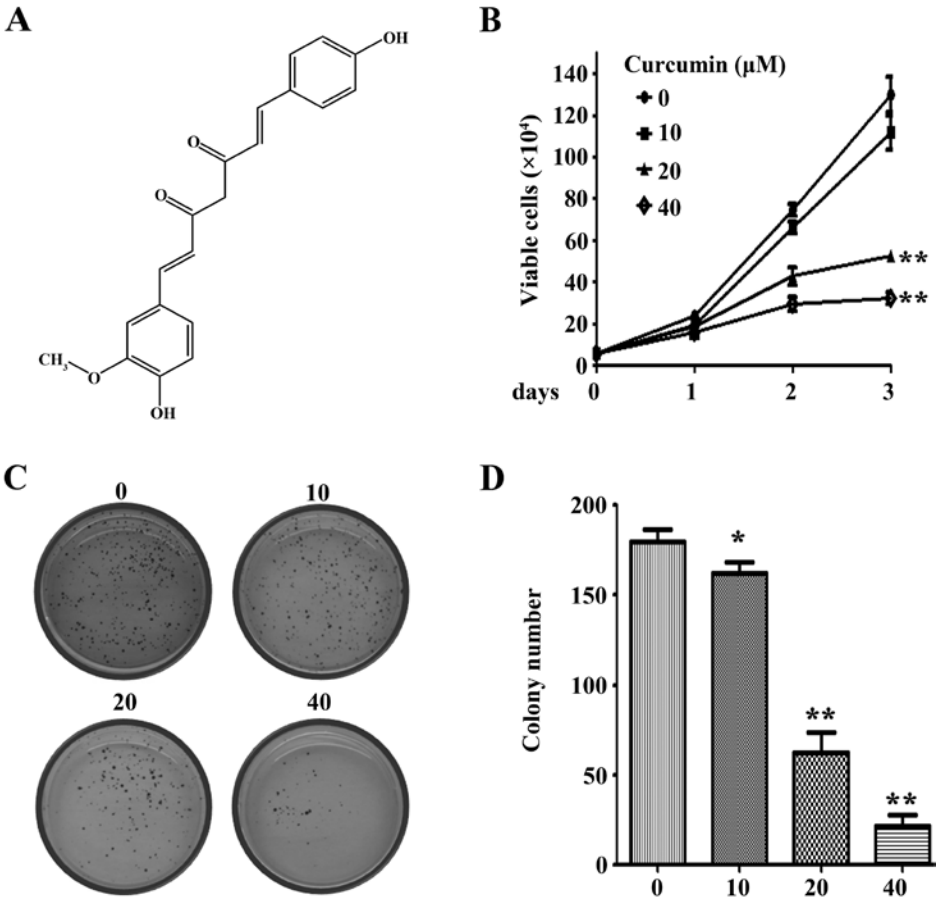

Figure 1. Curcumin inhibits proliferation and colony formation of NCI-H460 lung cancer cells. (A) Chemical structure of curcumin. (B) NCI-H460 cells were treated with curcumin for indicated time points and assessed by trypan blue exclusion analysis. (C) Soft-agar colony formation assay for NCI-H460 cells treated with or without curcumin. (D) Quantitation of foci counting. The data are presented as the mean ± standard deviation with three independent experiments. *P<0.05, **P<0.01, compared with the control group.

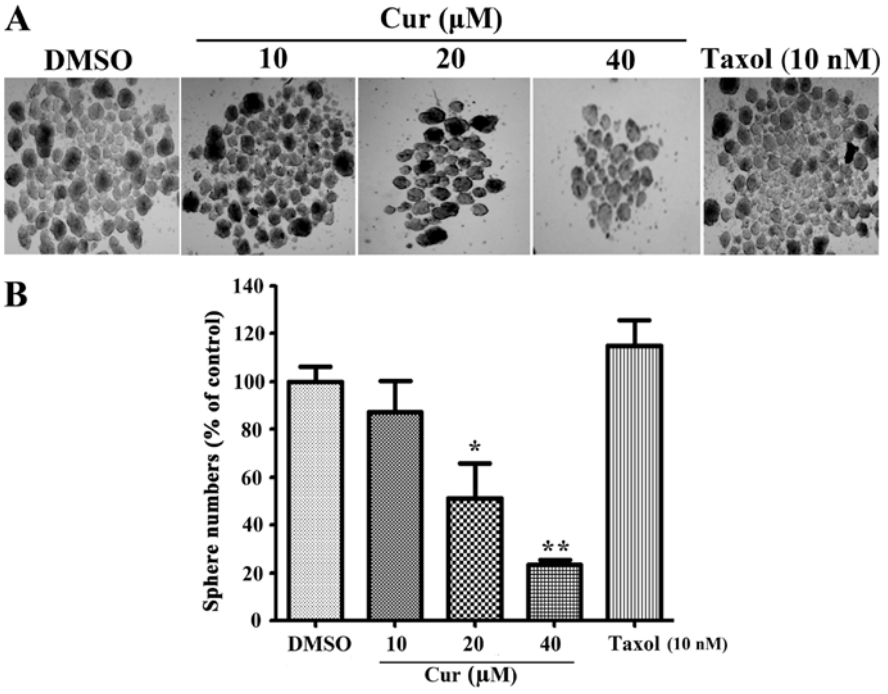

Figure 2. Curcumin impairs tumor sphere formation of NCI-H460 lung cancer cells. (A and B) NCI-H460 cells were plated into ultra low-attachment 24-well plates at a density of 1,000/well and cultured in tumor sphere medium containing different concentrations of curcumin and Taxol for 5 days. Tumor spheres were photographed (A) and tumor sphere numbers were counted using ImageJ (B). The data are presented as the mean ± standard deviation with three independent experiments. *P<0.05, **P<0.01, compared with the control group.

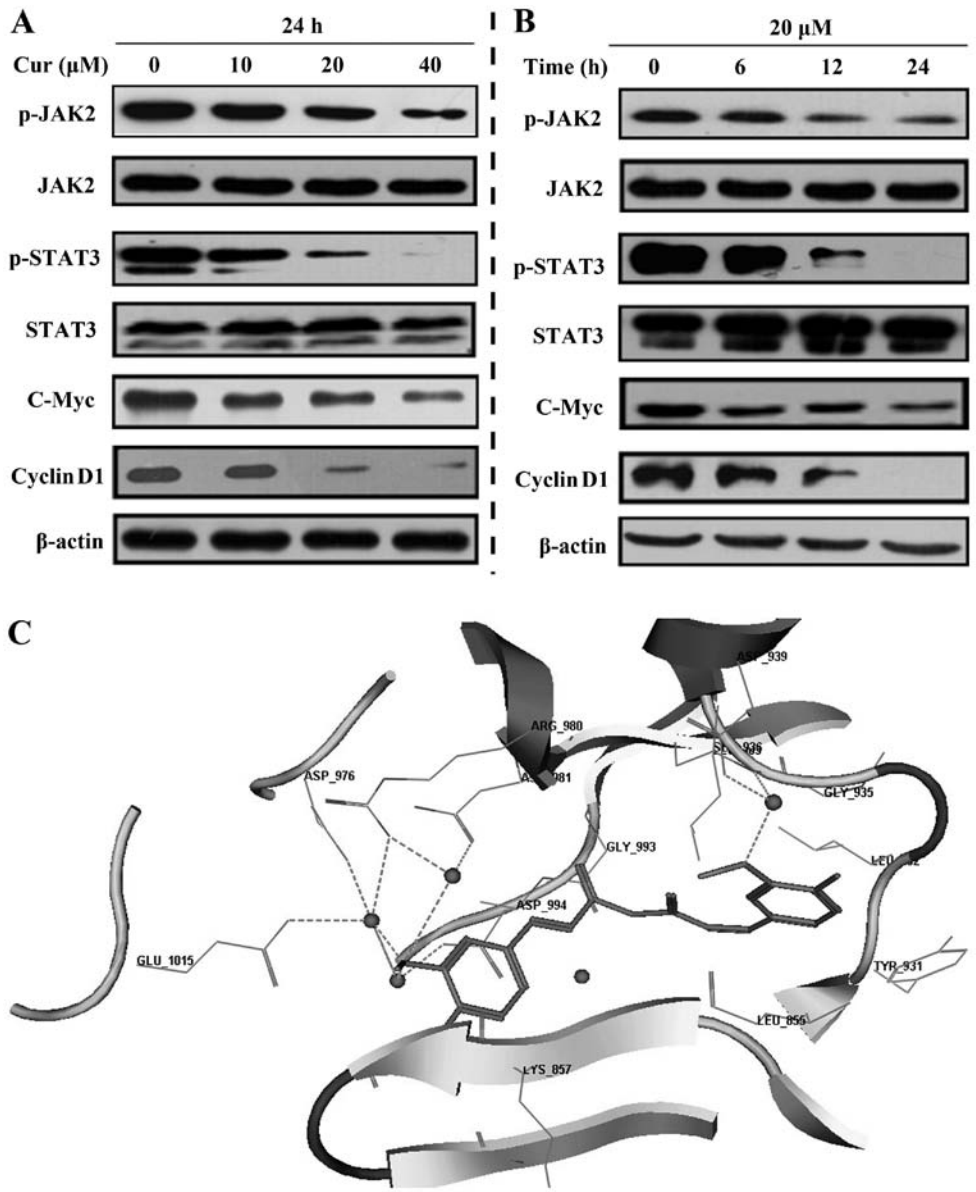

Figure 3. Curcumin inhibits JAK2/STAT3 signaling pathway activity. (A and B) Tumor spheres of NCI-H460 cells were treated with different doses of curcumin for 24 h (A) or 20 μm curcumin for indicated times (B). Then western blotting assay was conducted using indicated antibodies. (C) Plausible binding model of curcumin to JAK2 is presented.

long and narrow hydrophobic pocket formed by Leu_855, Leu_983, Gly_858, Asp_994 and Gly_935 of JAK2 (Fig. 3C). The methoxyl group of curcumin formed hydrogen bonds with the side chains of Asp_976, Asp_994, Glu_1015, Arg_980 and Asn_981 of JAK2 through water molecules (Fig. 3A). These results inferred that curcumin attenuated the JAK2/STAT3 pathway.

The role of STAT3 in curcumin-induced tumor sphere suppression. To further clarify JAK2/STAT3 activity in curcumin-induced tumor sphere suppression, we first evaluated STAT3 activity in NCI-H460 tumor sphere formation. STAT3 overexpressing and knockdown assays were carried out using plasmids and siRNA. We found when overexpressing STAT3, not only the number but the size of tumor spheres were increased (Fig. 4A). Conversely, when STAT3 was knoced down, both the number and size of tumor spheres were decreased (Fig. 4B). These results indicate that active stat3 promotes tumor sphere formation of NCI-H460 lung cancer cells. Next, by stat3 overexpression and curcumin co-treatment, stat3 overexpression can restore curcumin-induced tumor sphere inhibition (Fig. 4C). Further, stat3 knockdown and curcumin treatment can synergistically inhibit tumor spheres of NCI-H460 lung cancer cells (Fig. 4D). Taken together, these results inferred curcumin could reduce tumor spheres via inhibiting the JAK2/STAT3 signaling pathway.

Curcumin inhibits tumor spheres growth of NCI-H460 lung cancer cells in vivo. We next assessed whether curcumin could inhibit stem-like tumor growth *in vivo*. NCI-H460 derived tumor spheres were digested into single cells and subcutaneously inoculated into nude mice in the right flank area (100 cells for each mouse). When the tumors reached 50 mm^3, the mice were randomized into 3 groups (n=5 for each group). The mice were administered curumin (40 mg/kg) or Taxol (5 mg/kg) every 2 days with intraperitoneal injection

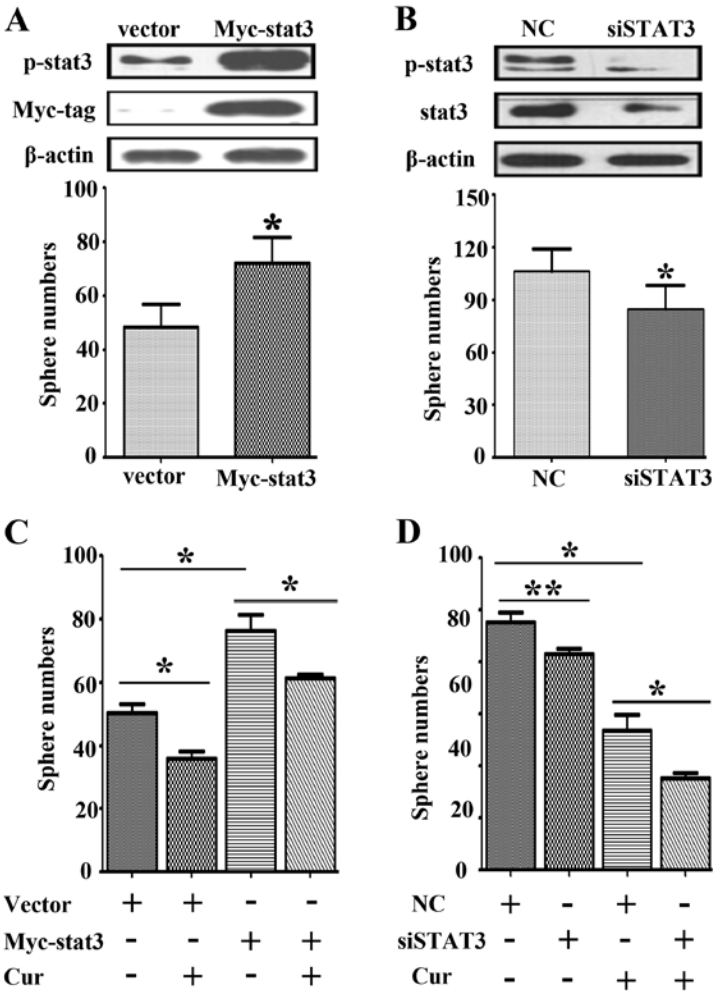

Figure 4. The role of STAT3 in curcumin induced NCI-H460 tumor sphere inhibition. (A and B) NCI-H460 cells of stat3 overexpression (A) or stat3 interference by siRNA (B) were seeded into ultra low-attachment 24-well plates at a density of 1,000/well. Five days later, tumor sphere numbers were counted and western blotting assay was conducted to detect p-STAT3 and STAT3 expression. (C and D) NCI-H460 cells of stat3 overexpression (C) or stat3 interference by siRNA (D) were treated with curcumin (20 μM) for five days in tumor sphere medium and the number of tumor spheres was counted. The data are presented as the mean ± standard deviation with three independent experiments. *P<0.05, compared with the control group.

(i.p). Tumor growth and mice body weight were monitored every other day for 15 days. Intriguingly, we found that curcumin significantly inhibited tumor growth compared with vehicle control or Taxol (P<0.01) (Fig. 5A and B). Moreover, body weight loss was not observed in curcumin-treated groups compared to vehicle and Taxol groups (Fig. 5C). Proteins were extracted from tumor samples and western blot assay was performed. We found that in curcumin treatment group, the expression of p-JAK2 and p-STAT3 were downregulated as compared with the samples separated from vehicle or Taxol control mice (Fig. 5D). These results demonstrate that curcumin suppressed tumor sphere growth *in vivo*.

Discussion

Tumor recurrence and drug resistance are the primary causes of poor survival rates in patients with advanced cancer. Since the isolation of CSCs in 1997, substantial research has demonstrated CSCs are highly related to tumor recurrence and drug resistance (12-14). Thus, eliminating CSCs may be a feasible approach for cancer therapy (40). Previous research showed that tumor spheres enrich cells with CSC-like property and are capable of forming tumors *in vivo* (41,42). Unlike the main population of cancer cells, CSC-like cells can form tumor spheres in non-adherent conditions and serum-free medium (43). Tumor sphere-forming assay has its own advantage, as it does not rely on specific markers to identify CSCs, which could vary greatly from one cell line to another (44). Curcumin has been reported to be an effective drug by preventing emergence of chemoresistance and eliminating CSCs in breast, glioblastoma, pancreatic and colon cancer (29-33). To date, the effects and molecular mechanisms of curcumin on lung CSCs still remain unclear. In the present study, we performed tumor sphere assays to determine the effects of curcumin on lung CSCs and found curcumin impaired the ability of tumor spheres in NCI-H460 lung cancer cells (Fig. 2A and B). Moreover, in the *in vivo* nude mouse model, 100 cancer stem-like cells derived from NCI-H460 tumor spheres were injected into each mouse and tumors formed. Curcumin strongly repressed tumor growth compared to vehicle or Taxol treatment groups (Fig. 5A and B). These results indicated that curcumin suppressed tumor sphere growth of NCI-H460 lung cancer cells *in vitro* and *in vivo*.

Attempts to eliminate CSCs by targeting relevant signaling pathways are being carried out in several preclinical studies. In

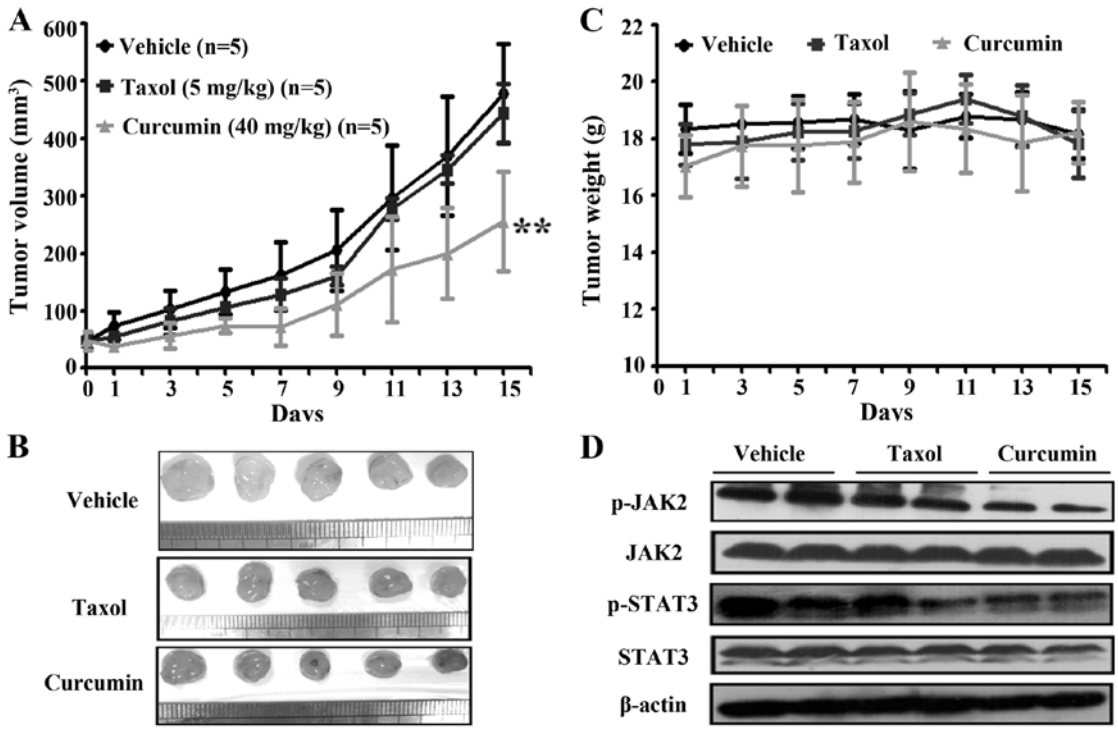

Figure 5. Curcumin inhibits tumor sphere derived tumor growth in xenograft mouse model. (A) Tumor volume was measured every two days and calculated according to the formula: $V = 0.5 \times L \times W^2$. (B) Images of xenograft tumors obtained from mice. (C) Body weight of mice was measured every two days. (D) Western blot analysis of lysates from tumor samples using indicated antibodies. The data are presented as the mean ± standard deviation. $^{**}P<0.01$, compared with the control group.

lung CSCs, interference with Wnt/β-catenin signaling by RNAi markedly decreased cancer cell proliferation, clone formation and drug resistance (45). PTEN, Hedgehog, JAK-STAT, Notch and PI3K/AKT pathways also offer latent intervention targets against CSCs (46,47). In the present study, by molecule docking we found curcumin interacted with the residues of JAK2 by forming hydrogen bonds though water molecules (Fig. 3C). We assumed that curcumin could inhibit JAK2 activity and repress the JAK2/STAT3 signaling pathway. Western blotting results confirmed our hypothesis. Under curcumin treatment, the activity of JAK2/STAT3 pathway was inhibited *in vitro* and *in vivo* (Figs. 3A and B, and 5D). Further, to elucidate the role of STAT3 in tumor sphere formation of NCI-H460 lung cancer cells, we conducted an overexpressing and RNAi knockdown assay. By transfection with STAT3 overexpression plasmid or STAT3 siRNA, we found STAT3 activation promotes tumor sphere formation, while STAT3 inhibition suppresses this effect (Fig. 4A and B). Furthermore, by stat3 overexpression and curcumin co-treatment, stat3 overexpression restored curcumin-induced tumor sphere inhibition, and stat3 knockdown with curcumin could synergistically inhibit tumor sphere formatioin (Fig. 4C and D). Taken together, our results infer that curcumin repressed tumor spheres of NCI-H460 lung cancer cells by inhibiting the JAK2/STAT3 signaling pathway.

In conclusion, we have documented the antitumor sphere formation effects of curcumin *in vitro* and *in vivo*. Our findings highlighted the fact that curcumin could inhibit lung cancer cell proliferation, colony formation and tumor spheres. The underlying mechanisms of curcumin-induced tumor spheres suppression are mainly due to the inhibition of the JAK2/STAT3 signaling pathway. The present study implies that curcumin may be a potential drug in lung CSC elimination and cancer therapy.

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