

An Anticancer Effect of Curcumin Mediated by Down-regulating PRL-3 Expression on Highly Metastatic Melanoma Cells

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Running Title: Curcumin Suppresses Expression of PRL-3

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Number of Document Statistics:

Text pages: 32

Tables: None

Figures: 5

References: 26

Abstract: 150 words

Introduction: 430 words

Discussion: 973 words

Abbreviations:

Act D, Actinomycin D; AP-1, activator protein 1; CHX, Cycloheximide; CSK, C-terminal Src kinase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, Electrophoretic mobility shift assay; FBS, fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinases; MEF, murine embryonic fibroblast; NF-kB, nuclear factor-kB; NP40, Polyoxyethylene(10)nonyl phenyl ether; PBS, phosphate-buffered saline; PCR, Polymerase Chain Reaction; PRL-3, Phosphatase of regenerating liver-3; siRNA, small interfering RNA; Tyr, tyrosine.

Abstract

Phosphatase of regenerating liver-3 (PRL-3) has been suggested as a potential target for anti-cancer drugs based on its involvement in tumor metastasis.

However, little is known about small molecule inhibitor against PRL-3. In this study, we report that curcumin, the component of the spice turmeric, shows its anti-tumor effect by selectively down-regulating the expression of PRL-3 but not its family members PRL-1 and -2 in a p53-independent way. Curcumin inhibited the phosphorylation of Src and stat3 partly through PRL-3 down-regulation. Cells with PRL-3 stably knocked down show less sensitivity of curcumin treatment, which reveals that PRL-3 is the very upstream target of curcumin. Curcumin treatment also remarkably prevented B16BL6 from invading the draining lymph nodes in the spontaneous metastatic tumor model, which is likely of relevance to PRL-3 down-regulation. Our results reveal a novel capacity of curcumin to down-regulate oncogene PRL-3, raising its possibility in therapeutic regimen against malignant tumor.

Introduction

The phosphatase of regenerating liver (PRL) as a tyrosine phosphatase family includes 3 members PRL-1, PRL-2 and PRL-3. In 2001, PRL-3 was first reported to be over-expressed in metastatic lesions derived from colorectal cancers, but expressed at lower levels in primary tumors and normal colorectal epithelium (Saha et al., 2001). The elevated PRL-3 expression was then found in other highly metastatic cancers such as gastric carcinomas (Miskad et al., 2004), Hodgkin's lymphoma (Schwering et al., 2003), melanomas (Wu et al., 2004), breast (Parker et al., 2005) and ovarian tumors (Zeng et al., 2003), suggesting that PRL-3 may be a molecular marker for metastatic tumor cells. Indeed, several *in vitro* and *in vivo* studies support a causal link between PRL-3 and tumor metastasis. Over-expressing PRL-3 promotes motility and invasion of both tumor cell lines and normal cell lines (Wu et al., 2004; Zeng et al., 2003), whereas knocking down endogenous PRL-3 with small interfering RNA attenuates cancerous cell motility and metastatic tumor formation (Qian et al., 2007). Treatment with monoclonal antibody of PRL-3 massively inhibited the tumor growth *in vivo* (Guo et al., 2008; Li et al., 2005). Therefore, PRL-3 is considered as a tractable target for anti-cancer drugs and regulating its expression and function may become a new strategy to prevent or treat tumor metastasis. However, there is no report on the natural small molecule compounds that can regulate PRL-3 expression.

Curcumin is a polyphenol derived from dietary spice turmeric. It possesses wide-ranging anti-inflammatory and anti-cancer properties (Sharma et al., 2005). The abilities of curcumin to induce apoptosis of cancer cells, as well as to inhibit angiogenesis and cell adhesion contribute to its chemotherapeutic potential in the treatment of cancer. Several phase I and phase II clinical trials indicate that curcumin is quite safe and may exhibit therapeutic efficacy in patients with progressive advanced cancers (Dhillon et al., 2008). Although inhibition of several cell signaling pathways involving Akt (Woo et al., 2003), NF- κ B (Aggarwal S et al.,2006), AP-1 (Balasubramanian et al., 2007) or JNK (Chen et al., 1998) have been implicated in the biological effects of curcumin, its direct molecular target and mechanism of inhibition in tumor metastasis remain to be well clarified.

In the present study, we showed a novel activity of curcumin as the first reported specifically down-regulating the PRL-3 expression, which contributes to the *in vivo* anti-metastatic effect of curcumin. Such activity of curcumin was further demonstrated to be at the transcriptional level without affecting the stability of either PRL-3 mRNA or protein, and to result in the inhibition of the phosphorylation of Src and stat3.

MATERIALS AND METHODS

Reagents

Curcumin (>98%) was purchased from Shanghai R&D Centre for Standardization of Traditional Chinese Medicine (Shanghai, China) and the stock solution was prepared with dimethyl sulfoxide (DMSO). Cycloheximide and Actinomycin D was purchased from Sigma (St Louis, MO), and dissolved at 5 mg/ml in PBS and cycloheximide dimethyl sulfoxide, respectively.

Animals

C57BL/6J mice (6 to 8 weeks old) were obtained from the Shanghai Laboratory Animal Center (Shanghai). Throughout the experiments, mice were maintained with free access to pellet food and water in plastic cages at 21 ± 2 °C and kept on a 12-h light-dark cycle. Animal welfare and experimental procedures were performed strictly in accordance with the “Principles of laboratory animal care” (NIH publication No.86-23, revised 1985) and the related ethical regulations of China (Ministry of Science and technology, 2006). All efforts were made to minimize the animals’ suffering and to reduce the number of animals used.

Cell culture

Mouse melanoma cells B16 and B16BL6, mouse breast carcinoma cells EMT-6, human prostate cancer cells PC3 and human breast carcinoma cells MCF-7 were purchased from the American Type Culture Collection (Rockville,

MD). MEFs were generated from E13.5 embryos. All the cells used were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

RT-PCR and real-time PCR

RNA samples were treated by DNase and subjected to semiquantitative RT-PCR. First-strand cDNAs were generated by reverse transcription using oligo (dT). The cDNAs were amplified by PCR for 28 cycles (94 °C for 30 seconds, 59°C for 30 seconds, and 72 °C for 30 seconds) using TaqDNA polymerase (Promega Corp., Shanghai). The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The Gel Imaging and Documentation DigiDoc-It System (version 1.1.23; UVP, Inc., Unpland, CA) was used to scan the gels and the intensity of the bands was assessed using Labworks Imaging and Analysis Software (UVP, Inc.). Quantitative PCR was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green I dye (Biotium, Inc.), and threshold cycle numbers were obtained using ABI Prism 7000 SDS software version 1.0. Conditions for amplification were one cycle of 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 59°C for 35 second, and 72°C for 45 seconds. The primer sequences used in this

study were as follows: PRL-1 forward, 5'-CAACCAATGCGACCTTAA-3';
PRL-1 reverse, 5'-CAATGGCATCAGGCACCC-3'; PRL-2
forward, 5'-ATTTGCCATAATGAACCG-3'; PRL-2
reverse, 5'-ACAGGAGCCCTTCCCAAT-3'; PRL-3
forward, 5'-CTTCCTCATCACCCACAACC-3'; PRL-3
reverse, 5'-TACATGACGCAGCATCTGG-3'; GAPDH forward,
5'-CATGGCCTTCCGTGTTCTTA-3'; GAPDH
reverse, 5'-GCGGCACGTCAGATCCA-3'. The resultant PCR products were
472 bp (PRL-1), 339 bp (PRL-2), 468 bp (PRL-3) and 191 bp (GAPDH).

Cell proliferation assay

Cells (3×10^3 /well) were prepared in 96-well plates in serum-containing medium. After 24 hours, various concentrations of curcumin were added into the plates. The cells were cultured for 48 hours and then MTT (Sigma) solution (40 μ l per well, 2 mg/ml in PBS) was added. The cells were incubated for 4 hours at 37°C. After discarding the supernatant, 200 μ l of DMSO were added and the absorbance of the color substrate was measured with an enzyme-linked immunosorbent assay reader (Sunrise Remote/Touch Screen; TECAN, Austria) at 540 nm. After subtraction of background of bovine serum albumin-coated wells, the percentage of the remaining cells was calculated according to the OD value.

Cell adhesion assay

The cell adhesion assay was performed as before (Wu et al., 2004). In brief, 96-well, flat-bottom culture plates were coated with 50 μ l of fibronectin (10 μ g/ml) (Calbiochem, San Diego, CA) in PBS overnight at 4°C, respectively. Plates were then blocked with 0.2% bovine serum albumin for 2 hours at room temperature followed by washing three times with DMEM. The cells were harvested with trypsin/EDTA, washed with ice cold PBS for twice, and re-suspended in DMEM medium. Cells (2×10^4 /well) were added to each well in triplicate and incubated for 30 minutes at 37 °C. Plates were then washed three times with DMEM medium to remove unbounded cells. Cells remaining attached to the plates were quantified with MTT assay as described above.

Luciferase assay

Luciferase assays were performed with the Dual Luciferase System (Promega). A fragment of 1986 bp nucleotides of 5' untranscriptional region of PRL-3 promoter was amplified with the primers as follows: PRL-3 promoter sense: 5'-CGCGCTAGCGGGCAAATCTCCAGTCATG-3'; anti-sense: 5'-CGCAAGCTTCAACAGGCACTCAGTCAAGC-3', which was subcloned into the luciferase reporter plasmid PGL-3 (Promega). With the β -galactosidase reporter as an internal control, cells were co-transfected for 12 hours with the full-length of mouse PRL-3 promoter vectors. The cells were then exposed to curcumin for 12 hours. The luciferase activity of the PRL-3 promoter reporters

was determined and normalized to that for the β -galactosidase reporter.

Electrophoretic mobility shift assay

Nuclear proteins were extracted from B16BL6 melanoma cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Rockford, IL,) with a protease inhibitor cocktail (Sigma). An oligonucleotide probe containing the p53-binding motif of the PRL-3 promoter (5'-GGGTAGACTCAGGCATGCTGTGGGCATGCCCCTTTTGGCC -3') was prepared as Shashwati Basak et al. did (Basak et al., 2008) and then labeled with biotin by Invitrogen (Shanghai). Detection of p53-oligonucleotide complex was performed using a LightShift chemiluminescent EMSA kit (Pierce). Briefly, nuclear protein (10 μ g) was incubated with 20 fmol of biotin-labeled oligonucleotide for 20 minutes at room temperature in binding buffer consisting of 10 mM Tris at pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 50 ng of poly(dA-dT), and 0.05% NP-40. The specificity of the p53 DNA binding was determined in competition reactions in which a 50-fold molar excess (1 pmol) and 100-fold molar excess (2 pmol) of unlabeled oligonucleotide were added to the binding reaction respectively. Products of binding reactions were resolved by electrophoresis on a 6% polyacrylamide gel using 0.5 x Tris-borate EDTA buffer (Invitrogen). P53-oligonucleotide complex was electroblotted to a nylon membrane (Invitrogen). After incubation in blocking buffer for 15 minutes at room temperature, the membrane was

incubated with streptavidin-HRP conjugate for 30 minutes at room temperature. The membrane was incubated with chemiluminescent substrate for 5 minutes, and allowed to expose radiographic film.

Stably interfering PRL-3 in B16BL6 melanoma cells

We have followed previously described methods (Qian et al., 2007) to generate the pRNA-U6.1/Neo PRL-3 siRNA (5'-GCUCACCUACCUGGAGAAGUA-3'), and pRNA-U6.1/Neo luciferase siRNA (5'-GCUUACGCUGAGUACUUCGAU-3') was used as the monitor and a negative control. B16BL6 melanoma cells were cultured to 60% confluence in a 35-mm plate and transfected with luciferase siRNA or PRL-3 siRNA using the Lipofectamin 2000 (Life Technologies Inc.). After transfection (48 hours), cells were passaged to a 100-mm dish and geneticin (G418 sulfate; Sigma) was added to final concentration of 1 mg/ml. Resistant cells were allowed to grow for 10 days. Individual G418-resistant colonies were picked and screened for monoclonality in 96-well plates by limited dilution. P1 and P9 were clone 1 and clone 9 of B16BL6 cells stably transfected with PRL-3 siRNA. L10 and L13 were clone 10 and clone 13 of B16BL6 cells stably transfected with luciferase siRNA.

Western blot

The Western blot was performed as described (Qian et al., 2007). The cells

were collected and lysed (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 5 mM EDTA, 0.1 mM PMSF, 0.15 U/ml aprotinin, 1 µg/ml pepstatin, and 10% glycerol). Anti-phosphorylation of Src (Tyr416 or Tyr527), anti-Src, anti-stat3, anti-phosphorylation of stat3 (Tyr705), anti-cyclin D1, anti-cyclin D3 (Cell Signaling Technology, Inc.), anti-tubulin, anti- p53, anti-gapdh (Santa Cruz, CA) and anti-PRL-3 antibodies (ProSci Inc, CA) were used for Western Blot.

Cell migration assay

Cell migration assay was performed using 8.0 µm pore size Transwell inserts (Costar Corp., Cambridge, MA). In brief, the under surface of the membrane was coated with fibronectin, laminin (10 µg/ml , Sigma) in phosphate- buffered saline, pH 7.4, for 2 hours at 37 °C. The membrane was washed in PBS to remove excess ligand, and the lower chamber was filled with 0.6 ml of DMEM with 10% FBS. Cells were serum-starved overnight (0.5% FBS), harvested with trypsin/ EDTA, and washed twice with serum-free DMEM. Cells were resuspended in the medium (DMEM with 0.5% FBS) and 1×10^5 cells in 0.1 ml were added to the upper chamber. After 6 hours at 37 °C, the cells on the upper surface of the membrane were removed using cotton tips. The migrant cells attached to the lower surface were fixed in 10% formalin at room temperature for 30 minutes, and stained for 20 minutes with a solution containing 1% crystal violet and 2% ethanol in 100 mM borate buffer (pH 9.0).

The number of migrated cells on the lower surface of the membrane was counted under a microscope in five fields.

***In vivo* metastasis assay**

The B16BL6 tumor cells metastasis model was performed as reported previously (Qian et al., 2007). 12 days after injection, the mice were distributed into three groups with six mice each according to tumor size. Curcumin, dissolved in olive oil, was given by intraperitoneal injection at dose of 50 and 100 mg/kg every two days, respectively. Tumor volumes were measured every 2 days from day 12 to 26 and calculated by the following formula: $0.5236 \times L1 \times (L2)^2$, where L1 is the long axis and L2 is the short axis of the tumor. At the end of experiment, mice were killed and the right footpads and draining popliteal lymph nodes were resected and photos were taken.

Statistical analysis

Data are expressed as mean \pm SEM. Student's *t* test was used to evaluate the difference between two groups. Kaplan-Meier method was used to evaluate the survival test. *P* < 0.05 was considered to be significant.

Results

Curcumin selectively down-regulates PRL-3 expression in multiple tumor cell lines.

Several mouse and human cell lines were treated with various concentrations of curcumin and tested for PRL-3 mRNA level. As shown in Figure 1A, curcumin decreased PRL-3 mRNA level in mouse melanoma B16BL6, B16, EMT-6, human breast cancer MCF-7 and human prostate cancer PC3 cells. Then, B16BL6 cells with high PRL-3 expression were chosen for further use and a time-dependent inhibition of PRL-3 level was confirmed in curcumin-treated cells (Fig. 1B). Interestingly, the expressions of PRL-1 and PRL-2 which have been identified homologous to PRL-3 were hardly affected by curcumin (Fig. 1C).

Curcumin inhibits PRL-3 expression at the transcriptional level in a p53-independent way.

The stability of PRL-3 mRNA in B16BL6 cells was examined after treated with or without 20 μ M curcumin in the presence of 5 μ g/ml Actinomycin D. Curcumin had no impact on degradation of PRL-3 mRNA (Fig. 2A). There was also no appreciable difference in PRL-3 protein stability between B16BL6 cells exposed to curcumin and those exposed to the solvent, DMSO (Fig. 2B). In order to evaluate whether the inhibitory effect of curcumin is at the transcriptional level, B16BL6 cells were transiently transfected with the PRL-3 promoter luciferase reporter plasmid pGL-3, in which a fragment of 5' untranscriptional region of PRL-3 promoter was subcloned. Compared with the untreated control, curcumin decreased the PRL-3 transcriptional activity in a

dose-dependent manner (Fig. 2C). Next, the effect of curcumin was examined on p53, an important transcription factor associated with PRL-3 expression (Basak et al., 2008). After B16BL6 cells were exposed to various concentrations of curcumin, no reduction in p53 protein amount was observed (Fig. 2D).

The anticancer effects of curcumin are related to PRL-3.

The expression of PRL-3 verified in mouse melanoma cell lines. The highly metastatic B16BL6 cells express PRL-3 mRNA approximately 3 folds more than the lowly metastatic B16 cells, which is consistent with our previous result (Wu et al., 2004), and so does the protein level (Fig. 3A). In order to detect whether PRL-3 is a key target of curcumin, we generated B16BL6 cells with PRL-3 stably knocked down (P1 and P9), in which the PRL-3 level was about 10% of those cells transfected with the control siRNA (L10 and L13). Comparably, curcumin strongly inhibited adhesion (Fig. 3B) and migration (Fig. 3C) of cells with high PRL-3 expression, while the cells with low PRL-3 expression, both endogenous and stably knocked down, showed less sensitive to the treatment of curcumin. Similar results were obtained in the inhibitory effect of curcumin on cell proliferation (Fig. 3D).

Curcumin blocks the Src/stat3 pathway through down-regulating PRL-3 expression.

Curcumin is able to inhibit the phosphorylation of stat3 (Bharti et al., 2003;

Blasius et al., 2006). In addition, PRL-3 can induce Src activation which initiates a number of signal pathways cumulating in the phosphorylation of ERK1/2, stat3 and p130^{Cas} (Liang et al., 2007). To evaluate the involvement of PRL-3 in the inhibitory effect on stat3 phosphorylation by curcumin, B16BL6 cells were treated with 10, 20 or 40 μ M of curcumin for 24 hours followed by immunoblotting. Curcumin reduced PRL-3, phosphorylated Src (Tyr416) and phosphorylated stat3 (Tyr705) at the protein level, whereas phosphorylated Src (Tyr527), total Src and stat3 were not affected (Fig. 4A). After B16BL6 cells were transfected with various amount of PRL-3 expressing plasmids, over-expression of PRL-3 reversed the inhibition of the phosphorylation of Src (Tyr416) and stat3 (Tyr705) by curcumin in a dose-dependent manner (Fig. 4B). As a direct transcript target of stat3, cyclin D1 but not cyclin D3 was also reversed (Fig.4B). In order to exclude the possibility caused by the nonspecific effect of over-expression system, L13 and P1 cells were treated with curcumin for 24 hours followed by immunoblotting. As shown in Figure 4C, tyr-416 phosphorylation of Src kinase was reduced by about 73% and 28%, and tyr-705 phosphorylation of stat3 was reduced by about 53% and 14% in L13 and P1 cells, respectively.

Curcumin inhibits the tumor growth and spontaneous metastasis of B16BL6 cells *in vivo*.

C57BL/6J mice were injected subcutaneously with B16BL6 cells into right

footpads, and treated with 50 and 100 mg/kg of curcumin by intraperitoneal injection every two days. Compared with olive oil, curcumin significantly inhibited the tumor growth in a dose-dependent manner (Fig. 5A). After 26 days of tumor cell inoculation, the tumor tissues were removed. As shown in Figure 5B, the mRNA and protein levels of PRL-3 were significantly inhibited in the tumor by curcumin treatment. Moreover, curcumin dose-dependently reduced the metastatic potential of the tumor cells (Fig. 5C). To investigate whether curcumin treatment influences lymphatic metastasis, the popliteal lymph nodes were collected and photographed. The control group showed 100% visible metastasis. In contrast, lower ratios of metastasis were found in curcumin-treated mice with approximately 50% in 50 mg/kg group, and 17% in 100 mg/kg group, respectively (Fig. 5D). Histologic analysis revealed that all lymph nodes from the control group contained metastases. But curcumin-treated mice had developed lymph node metastases at lower frequency (Fig. 5D).

Discussion

It is a well accepted that PRL-3 is a metastasis-associated gene. In the initial study, we observed that curcumin inhibited cell proliferation and adhesion of mouse melanoma B16BL6 in which high invasive and metastatic activity is closely correlated with its high level of PRL-3 expression. These findings implicate that curcumin might show anticancer effects at least partially by regulating PRL-3. Indeed, curcumin decreased PRL-3 mRNA of B16B16 in a dose- and time- dependent manner and the inhibitory effect occur at the transcriptional level. The cells with PRL-3 stably knocked down were less susceptible to curcumin inhibition. PRL-3 expression in other cell lines was also inhibited by curcumin, suggesting this mechanism is not unique to B16B16 highly expressing PRL-3. Interestingly, curcumin had no effect on the expression of PRL-1 and PRL-2, which share a high degree (>75%) of amino acid sequence identity. It is likely that curcumin down-regulates PRL-3 transcription through a pathway different from PRL-1 or PRL-2. The 5' non-coding regions of mouse PRLs are much more divergent and the expression pattern of the PRLs differs among tissues, which supports possibility of differential transcription regulation (Zeng et al., 1998).

The exact mode of PRL-3 transcription regulation is unclear. Although recent study has demonstrated that PRL-3 is a p53 target gene and induces G1

cell-cycle arrest in a p53-dependent manner in primary cells (Basak S et al., 2008), we found that curcumin had no effect on p53 despite the PRL-3 down-regulation. Upon exposure to curcumin, no changes were detected in either p53 protein level or p53 binding to the corresponding site in the promoter of PRL-3 (Supplemental Fig. 1A). Moreover, curcumin still decreased PRL-3 even in p53-deficient MEFs (Supplemental Fig. 1B). Curcumin has ever been reported to accelerate the p53 accumulation in some tumor cell lines, such as MCF-7 (Tathagata et al., 2002; Aggarwal BB et al., 2007). In our test, PRL-3 down-regulation in MCF-7 cells seemed to be less sensitive to curcumin treatment than in B16BL6 cells. That is, the PRL-3 level of MCF-7 cells showed no significant change after exposure to 20 μ M curcumin for 12 hours (Fig. 1A), which may be caused by the accumulation of wild type of p53. However, such accumulation of p53 did not up-regulate PRL-3, which can be explained by the reason that the target transcription factor of curcumin could be more active than p53 in the transcription regulation of PRL-3. Taken together, these results suggest that there are other transcription factors, rather than p53, involving in regulating PRL-3 transcription in tumor cells and they are targeted by curcumin. Consistently, some tumor cells were not arrested by up-regulated PRL-3 in p53-dependent manner like primary cells (Ryan et al., 2001). On the other hand, the p53 tumor suppressor plays a critical role in protecting organisms from developing cancer (Liang et al., 2007). Degrading wild-type p53 might lead to accumulation of DNA-damaged cells by inhibiting

their p53-induced apoptosis (Ryan et al., 2001; Vousden et al., 2002).

Therefore, it seems to be unreasonable to down-regulate PRL-3 expression by targeting the degradation of p53 in tumor cells. Further study is in progress to elucidate the mechanism of PRL-3 transcription regulation by using curcumin as a tool.

Considerable studies suggest that curcumin shows wide-ranging anti-inflammatory and anti-cancer properties, and is able to affect multiple targets (Preetha et al., 2008). In this study, we demonstrated that PRL-3 is not only a normal target of curcumin, but a trigger one. Elevated PRL-3 will lead to Src activation through down-regulating the synthesis of C-terminal Src kinase (CSK) protein, which in turn leads to tyrosine phosphorylation of a number of proteins in HEK293 cells (Tathagata et al., 2002). Surprisingly, in highly metastatic melanoma cells, we did find decreases in tyrosine phosphorylation in PRL-3 stably knocked down cell lines (P1 and P9), compared to L1 and L13 cells, which stably express luciferase siRNA. However, not like that in HEK293 cells, we haven't noticed significant change of the tyr-527 but the tyr-416 phosphorylation of Src (Supplemental Fig. 2). This result indicates that there might be other relationship between PRL-3 and Src activation that independent of CSK. As an important substrate of Src, stat3 can be also elevated by PRL-3, and the Src/stat3 pathway has been demonstrated to be implicated in tumor metastasis, including proliferation, invasion and motility (James et al., 2002) As

shown in Figure 3C, the inhibition of the activity of Src and stat3 made by curcumin was about 45% and 39 % through down-regulating PRL-3 expression, respectively. Cells with PRL-3 stably knocked down by siRNA showed less susceptible to the anticancer effect of curcumin. These findings suggest that PRL-3 is the very upstream target of curcumin. In this study, it is the first to reveal the relationship between the inhibition effect of curcumin on stat3 phosphorylation and PRL-3, and provide a possible mechanism by which curcumin inhibits the metastasis of different cancers.

In vivo study showed that curcumin dose-dependently inhibited the tumor growth and prevented B16BL6 cells in primary tumor from invading the draining lymph nodes. As expected, the PRL-3 expression in the tumor tissues was remarkably decreased by curcumin. These results are similar to those in our previous study using PRL-3 siRNA (Qian et al., 2007). Due to high level of PRL-3 mRNA in heart, it is considered that therapeutic targeting PRL-3 might exhibit cardiotoxicity (Stephens et al., 2005). However, we detected no changes either of PRL-3 mRNA level in the cardiac muscle tissues or of myocardial function in the mice treated with curcumin. Moreover, we did not detect visible protein of PRL-3 in the heart and muscle of adult mice like the tumor cell lines, which indicates that the role of PRL-3 protein synthesis system is unique in normal tissues and it also supports that targeting the expression of endogenous PRL-3 by curcumin is safe and feasible as a novel

therapy of cancer.

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Acknowledgements

This study was supported in part by Natural Science Foundation of China (No.30730107), Science Fund for creative Research Groups (No.30821006)

and Natural Science Foundation of Jiangsu Province (BK2008022)

Legends for Figures:

Figure 1.

Curcumin selectively inhibited PRL-3 expression in multiple cell lines.

A. Different cell lines were grown in 6-well plates and were treated with different concentrations of curcumin for 12 hours. Total RNA was prepared for analyses of PRL-3 expression by RT-PCR for three times, and the electrophoresis presented is the representative one. GAPDH was used as an invariant control.

B. B16BL6 cells were treated with 20 μ M curcumin for different hours. At the indicated time, the mRNA level of PRL-3 was measured by real-time PCR. and calculated by using GAPDH as an invariant control. Data are mean \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs cells cultured without curcumin.

C. B16BL6 cells were treated with various concentrations of curcumin for 12 hours. The mRNA level of PRL1, PRL-2 and PRL-3 were measured by real-time PCR and calculated by using GAPDH as an invariant control. Data are mean \pm SEM of three independent experiments.

Figure 2.

Curcumin inhibited PRL-3 expression in a p53-independent pathway.

A. B16BL6 cells were treated with 20 μ M curcumin in the presence of 5 μ g/ml Actinomycin D for different hours and total RNA was extracted. The mRNA level of PRL-3 was measured by real-time PCR and calculated by using GAPDH as an invariant control. Data are mean \pm SEM of three independent experiments.

B. B16BL6 cells were treated with 20 μ M curcumin in the presence of the protein synthesis inhibitor, cycloheximide (CHX, 5 μ g/ml). At the indicated time, cell lysates were collected and the protein level of PRL-3 and tubulin were determined by immunoblotting for at least 3 times and representative data were shown. The bands were semi-quantified by gray scale scanning.

C. B16BL6 cells were transfected with the PRL-3 promoter luciferase reporter plasmid. After transfected for 12 hours, cells were incubated with different concentrations of curcumin for additional 12 hours. Luciferase activity was expressed as relative units after β -galactosidase normalization. Data are mean \pm SEM of three independent experiments. ** $P < 0.01$, vs cells transfected without curcumin.

D. B16BL6 cells were treated with various concentrations of curcumin for 12 hours, p53 and GAPDH proteins were detected by immunoblotting.

Figure 3.

The anticancer effects of curcumin are related to PRL-3.

P1 and P9 cells were B16BL6 cells with PRL-3 stably knocked down by PRL-3 specific siRNA. L10 and L13 cells were those transfected with luciferase siRNA

as control.

A. The mRNA levels of PRL-3 were measured by real-time PCR, and the protein levels were detected by immunoblotting. GAPDH was used as an invariant control.

B. B16, B16BL6, L10, L13, P1 and P9 cells were incubated with various concentrations of curcumin for 24 hours. The adhesion ability to fibronectin of cells was measured by cell adhesion assay. Data are mean \pm SEM of three independent experiments, and each experiment includes triplicate sets. * $p < 0.05$, ** $p < 0.01$ vs B16BL6 cells treated without curcumin.

C. B16, B16BL6, L10, L13, P1 and P9 cells were incubated with various concentrations of curcumin for 24 hours followed by starvation for 8 hours. The migration ability of cells was measured by cell migration assay. Data are mean \pm SEM of three independent experiments, and each experiment includes triplicate sets. * $p < 0.05$, ** $p < 0.01$ vs B16BL6 cells treated without curcumin.

D. B16, B16BL6, L13 and P1 cells (3000/well) were seeded in 96-well plates and treated with various concentrations of curcumin for 48 hours, The inhibition rate was measured by cell counting.

Figure 4.

Curcumin induced inhibition on src/stat3 pathway through down-regulating PRL-3 expression.

A. B16BL6 cells were treated with various concentrations of curcumin for 24 hours. Whole cell extracts were prepared for western blotting analysis. The

protein levels of PRL-3, total Src, phosphorylated Src (tyr-416), phosphorylated Src (tyr-527), total stat3 and phosphorylated stat3 (tyr-705) were determined for at least 3 times and representative data were shown. Tubulin was used as an internal control.

B. B16BL6 cells were seeded in 6-well plates for 12 hours, then they were transfected with different amount of myc-PRL-3 expressing plasmid for 12 hours followed by incubated with 20 μ M curcumin for an additional 24 hours. The proteins mentioned above as well as cyclin D1 and cyclin D3 were examined by immunoblotting.

C. P1 and L13 cells were incubated with 20 μ M curcumin for 24 hours. Protein analysis was performed as described in Fig. 4A. Representative data were shown. The bands were semi-quantified by gray scale scanning. Data are mean \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs cells cultured without curcumin.

Figure 5.

Curcumin significantly inhibited the tumor growth and spontaneous metastasis of B16BL6 cells in mice.

B16BL6 cells (20 μ l, 2.5×10^6 cells/ml) were injected subcutaneously into the right footpads of C57BL/6J mice. 12 days after injection, the mice were distributed into four groups according to tumor size. Then, they were treated with 50 and 100 mg/kg of curcumin dissolved in olive oil by intraperitoneal injection every two days for total 14 days.

A. Time course of tumor growth and tumor volumes. Data are mean \pm SEM of six mice in each group. * $p < 0.05$, ** $p < 0.01$ vs. control.

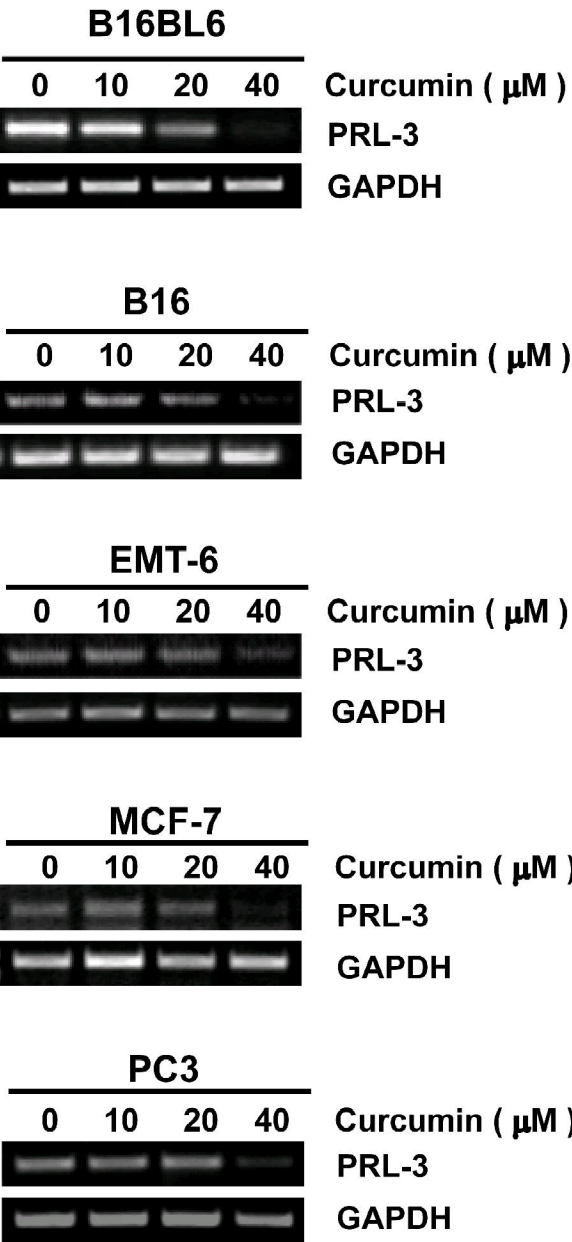
B. After 26 days, tumor tissues were removed. The mRNA of PRL-3 was detected by real-time PCR and the protein level of PRL-3 was determined by immunoblotting. GAPDH was used as an internal control.

C. Tumor cells were separated and the migration ability was measured by transwell assay. Data are mean \pm SEM of three independent experiments, and each experiment includes triplicate sets. * $p < 0.05$, ** $p < 0.01$ vs separated tumor cells from olive oil group.

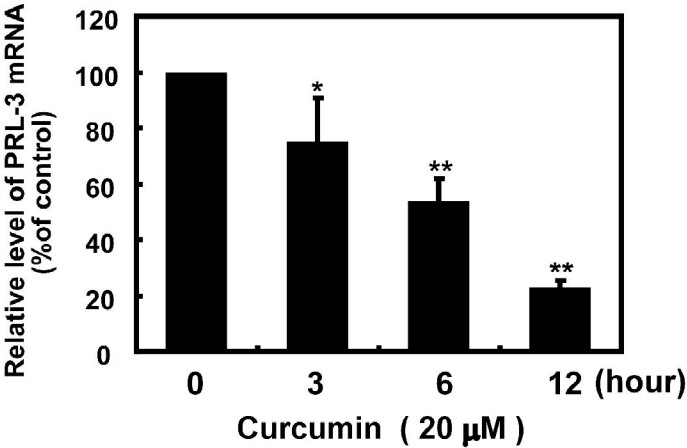
D. Draining popliteal lymph nodes from each group were photographed (upper). And lymph node sections were stained with H&E (lower). Arrows = tumors in the lymph nodes. Original magnification $\times 100$ or $\times 200$.

Figure 1

A



B



C

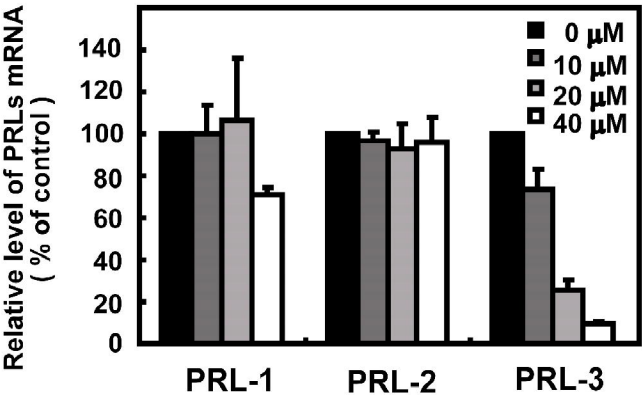


Figure 2

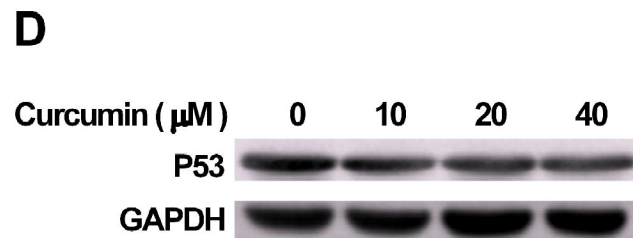
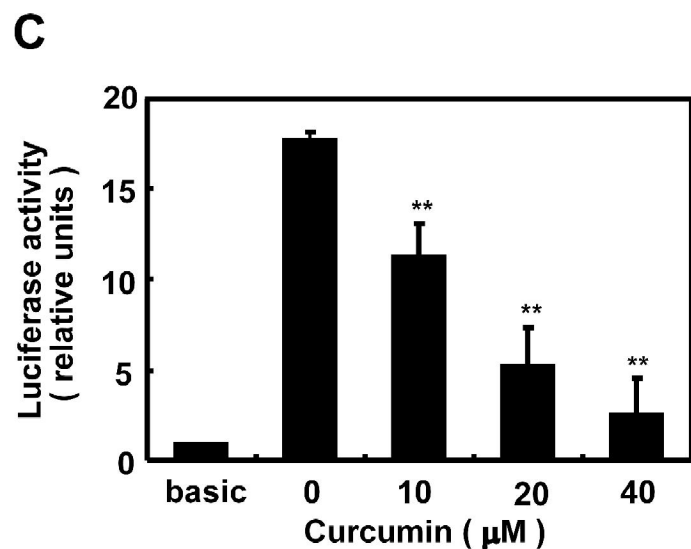
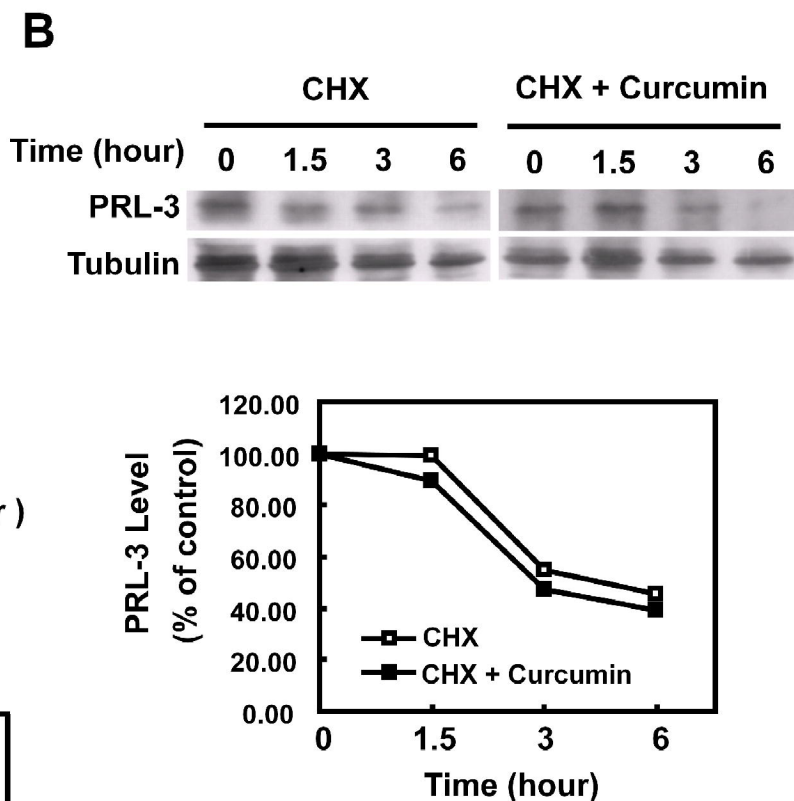
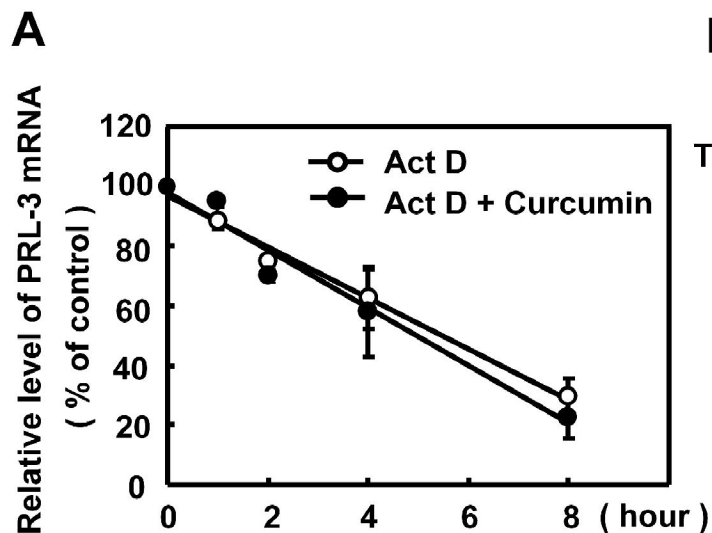


Figure 3

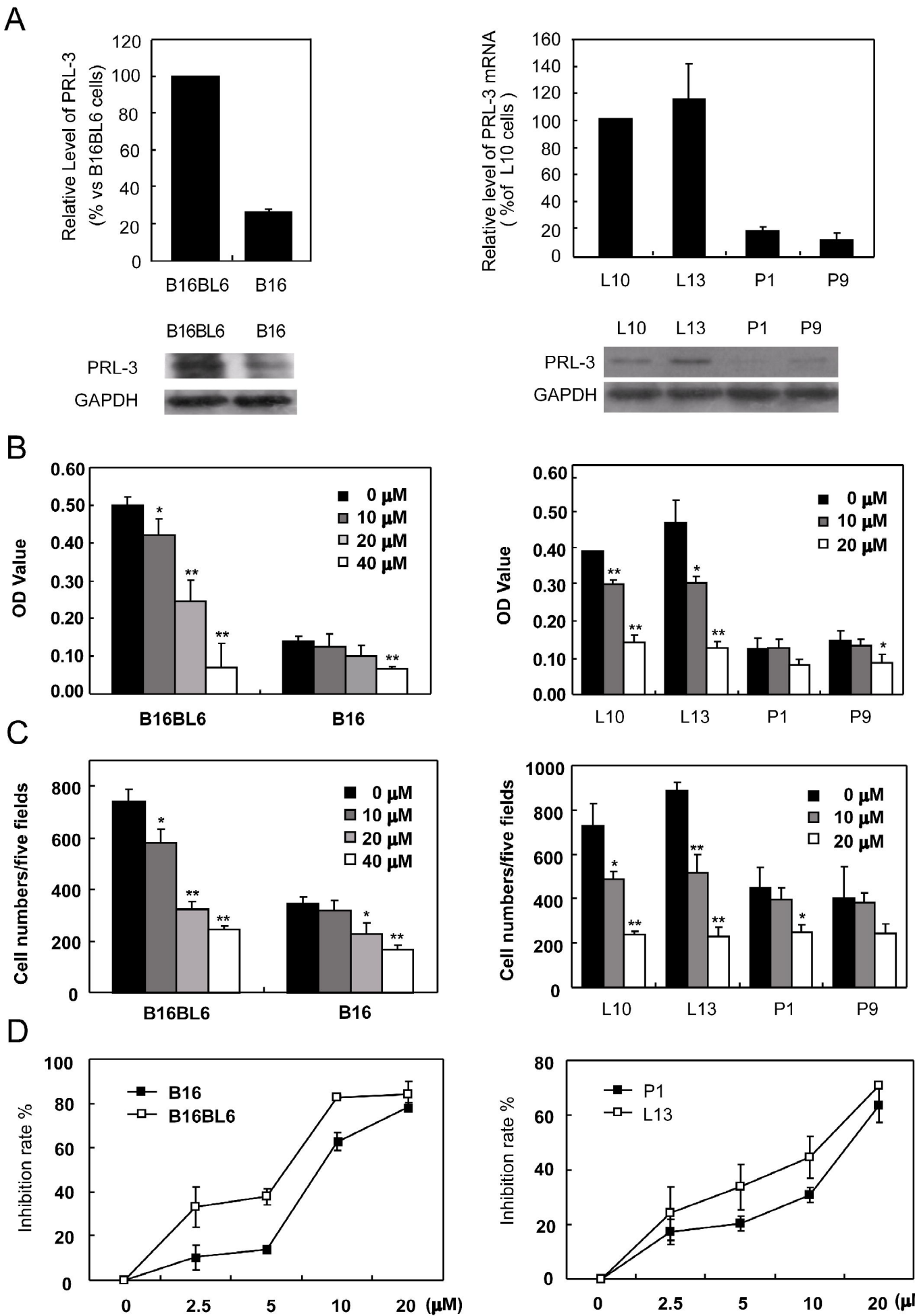
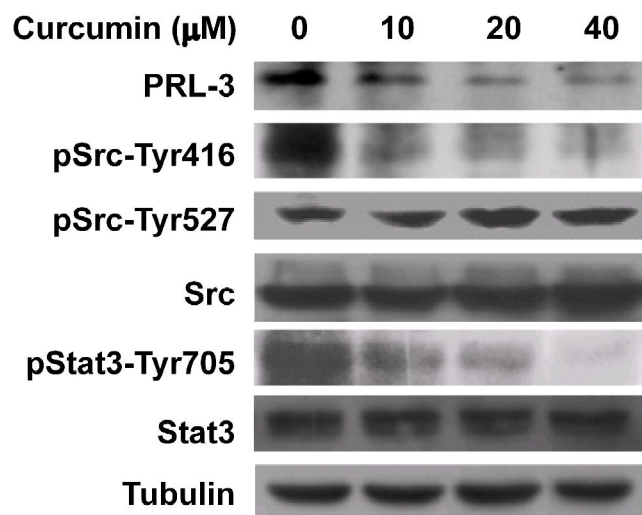
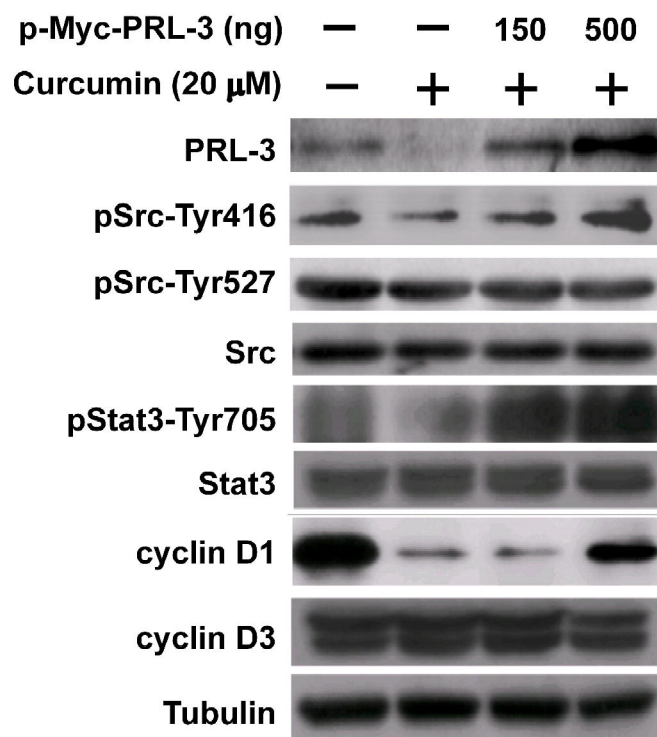


Figure 4

A



B



C

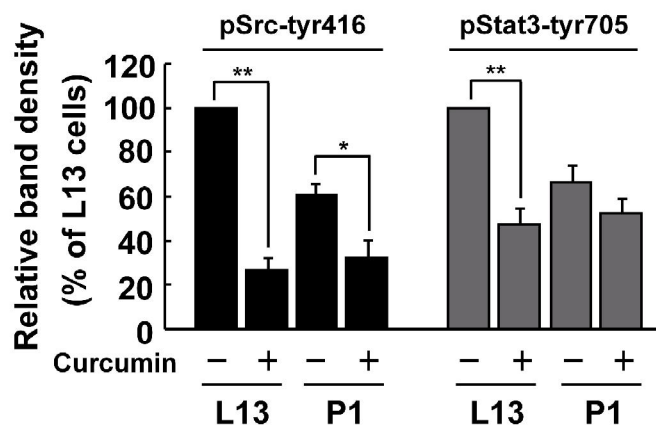
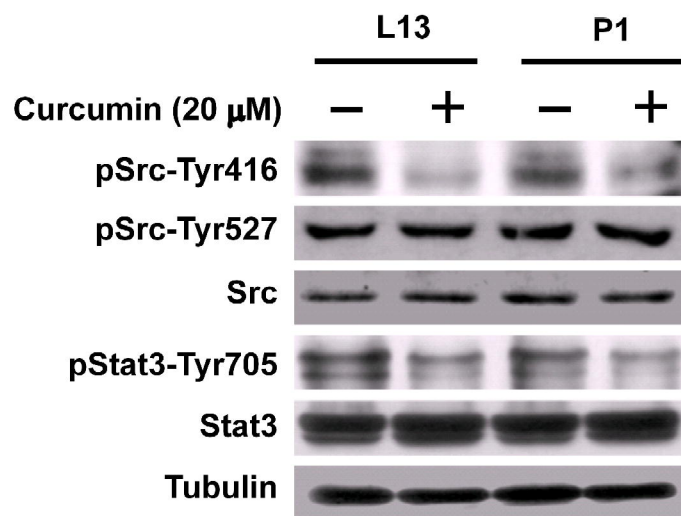
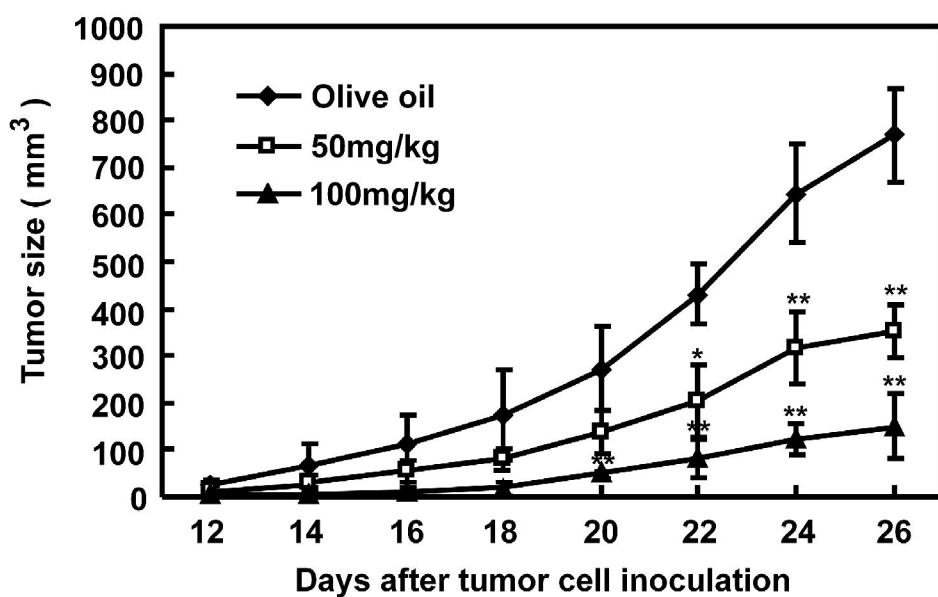
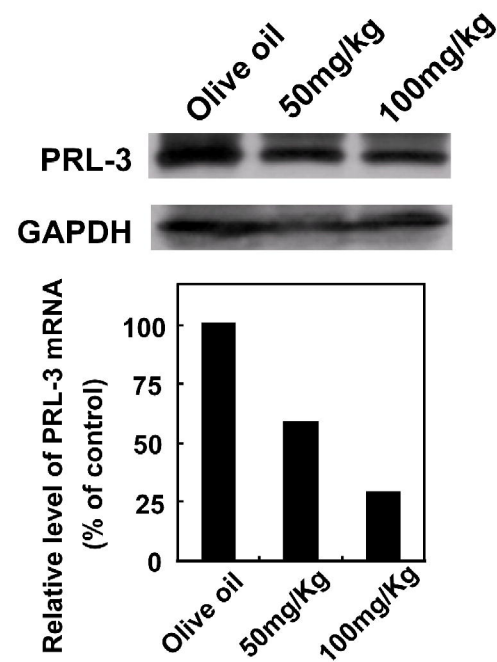


Figure 5

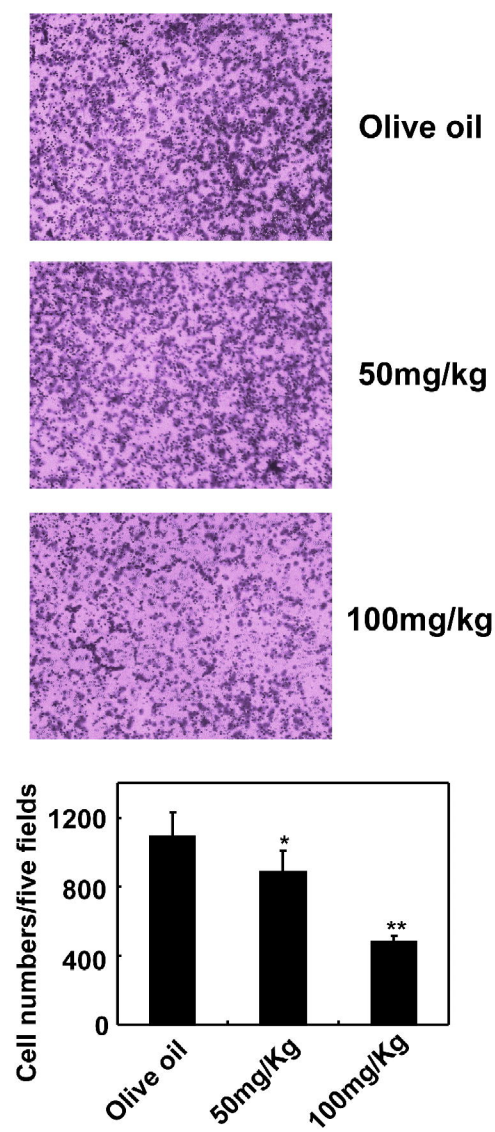
A



B



C



D

