

Curcumin blocks migration and invasion of mouse-rat hybrid retina ganglion cells (N18) through the inhibition of MMP-2, -9, FAK, Rho A and Rock-1 gene expression

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Abstract. Cancer metastasis involves multiple processes which may complicate clinical management and even lead to death. Matrix metalloproteinases (MMPs) play an important role in cancer cell invasion, metastasis and angiogenesis, depending on whether agents can inhibit MMPs which could lead to inhibition of the migration and invasion of cancer cells. Curcumin, the active constituent of the dietary spice turmeric, has potential for the prevention and therapy of cancer. However, there is no study to address the effects of curcumin on migration and invasion of mouse-rat hybrid retina ganglion cells (N18). This is the first study to explore the anti-migration and -invasion of curcumin in mouse-rat hybrid retina ganglion cells (N18) *in vitro*. Curcumin exerted a dose- and time-dependent inhibitory effect on the invasion and migration of N18 cells *in vitro*. Results from Western blotting showed that curcumin inhibited the protein levels of PKC, FAK, NF- κ B p65 and Rho A leading to the inhibition of ERK1/2, MKK7, COX-2 and ROCK1, respectively, finally causing the inhibition of MMP-2 and -9 for the inhibition of migration and invasion of N18 cells. Moreover, this action was involved in the inhibition of gene expression of MMP-2 and -7, FAK, ROCK1 and Rho A. Overall, the above data show that the anticancer effect of curcumin also exists for the

inhibition of migration and invasion in N18 cells, and that curcumin may be a powerful candidate for developing preventive agents for cancer metastasis.

Introduction

Many types of neoplasms possess higher levels of activated matrix metalloproteinases (MMPs) that have been demonstrated in more invasive and/or metastatic tumors (1). The interactions between cells and the extracellular matrix (ECM) could enhance cell migration, invasion and ECM degradation. Metastasis of cancer is involved in the degradation of ECM and the overexpression of MMPs and u-PA (2,3). Many epidemiological and laboratory studies have shown that dietary agents play an important role in reducing cancer risk (4-6). Moreover, substantial research suggests that numerous naturally occurring compounds can act as antioxidants, cancer preventative and therapeutic agents (7-9). Many prescription drugs in clinical used for cancer patients are derived from natural plant species (10,11).

Curcumin (diferuloylmethane) is an active component from the spice turmeric (*Curcuma longa*). Curcumin has been reported to have chemopreventive and therapeutic properties against many tumors *in vitro* and *in vivo* models (12,13). Curcumin inhibited cell proliferation and inflammation and caused cell cycle arrest and apoptosis in many types of cancer cells (14-16). In particular it can suppress invasion, angiogenesis, and metastasis of cancer cells (14). It is well known that the biological activities of curcumin involve Akt, activator protein-1, cyclooxygenase-2 lipooxygenase, nuclear factor- κ B, I κ B α kinase, mitogen-activated protein kinases (MAPK) tumor necrosis factor, chemokines, and cell cycle machinery and Akt/mTOR signaling (15,17-24). Our previous studies have shown that curcumin induced cell cycle arrest and apoptosis (25) and DNA damage and

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inhibited DNA repair associated gene expression (26) in mouse-rat hybrid retina ganglion cells (N18). However, the effects of curcumin on migration and invasion of mouse-rat hybrid retina ganglion cells (N18) and the mechanisms underlying these effects have not been studied.

In the present study, we examined whether curcumin inhibits migration and invasion of N18 cells. We demonstrated that curcumin potently inhibits the migration and invasion of mouse-rat hybrid retina ganglion cells (N18), an effect which may be mediated through inhibition of MMP-2 and MMP-7 signaling.

Materials and methods

Materials and chemicals. Curcumin, dimethyl sulfoxide (DMSO), pyruvate, penicillin G, trypan blue, triton X-100 and streptomycin were purchased from Sigma Chemical (St. Louis, MO, USA). Anti-MMP-2, anti-MMP-9, anti-ERK, anti-c-jun-p, anti-FAK, anti-GRB2, anti-JNK, anti-MEKK3, anti-MKK7, anti-PI3K, anti-PKC, anti-Ras, anti-VEGF and anti-Rho A were purchased from Santa Cruz Biotechnology. Materials and chemicals for electrophoresis were obtained from Bio-Rad.

Cell culture. Mouse-Rat Hybrid retina ganglion cell line (N18: derived from retina ganglion cells hybrid with lymphoma cells) was purchased from the Japanese Collection of Research Bioresources Bank. N18 cells are placed onto the 75 cm² flasks with DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin) and 2 mM L-glutamine and were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C as described previously (10,26).

Transwell migration assay. N18 cells were serum deprived in DMEM medium supplemented with 1% charcoal-stripped FBS and maintained for 24 h. The lower side of the Transwell filter was pre-coated with 10 µg Type IV collagen. Then from each well, DMEM containing 1% charcoal-stripped FBS was added. The filter in 6.5-mm Transwell was inserted in 24-well plates. Then ~2.5x10⁴ cells/filter of N18 cells were placed on the filter and cells were treated with 0, 7.5 and 15 µM curcumin for 24 and 48 h. Migrated cells were stained with hematoxylin and eosin (H&E) and then were examined and photographed under a microscope (11,26).

Transwell invasion assay. The same protocols were performed as described in the migration assay except that cells were placed on a matrigel-coated Transwell filter (Matrigel invasion chamber, BD Biosciences) and then examined and photographed under a microscope (11,26).

Western blot analysis. N18 cells (1x10⁶/well) in DMEM medium were plated onto 6-well plates and then were treated with 15 µM curcumin for 0, 6, 12, 24 and 48 h. After treatment in each time point, cells from each well were harvested and isolated by centrifugation in 1,500 rpm for 5 min. The isolated cells from each treatment were lysed individually and the total protein levels were quantified. To determine the proteins levels associated with invasion and

Table I. The DNA sequence was evaluated using the Primer Express software.

Primer name	Primer sequence
Rat MMP2-F	CTTGCTGGTGGCCACATTC
Rat MMP2-R	CTCATTCCTGCGAAGAACAC
Rat MMP7-F	GATGGGCCAGGAAACACTCTAG
Rat MMP7-R	CCTCACCATCCGTCCAGTACTC
Rat MMP9-F-2	CGCTCATGTACCCCATGTATCA
Rat MMP9-R-2	TCAGGTTTAGAGCCACGACCAT
Rat FAK-F	AGGGAGGACGGGAGTTTTCA
Rat FAK-R	GGCGAGGTGGTTTCTTTGG
Rat Rho-F	TCATGGTCTTCGGAGGATTCA
Rat Rho-R	GTGGCAAAGAAGCCCTCAAG
Rat ROCK1-F	ATTCATTCCCTACCCTCTACCACTTTC
Rat ROCK1-R	TGTGGGACTTAACATGGCATCT
Rat GAPDH-F	GGTGGACCTCATGGCCTACA
Rat GAPDH-R	CAGCAACTGAGGGCCTCTCT

Each assay was conducted at least twice to ensure reproducibility.

migration (Ras, GRB2, MEKK3, FAK, MKK7, JNK, ERK, MMP-9, MMP-2, Rho A, PI3K, PKC, VEGF and c-jun-p) in N18 cells, all samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (11,26,27).

Real-time PCR of MMP-2, MMP-7, FAK, Rho A and ROCK1 in N18 mouse-rat hybrid retina ganglion cells. N18 cells (1x10⁶/wells) in DMEM medium were plated onto 6-well plates and then were treated with 15 µM curcumin for 24 and 48 h. Cells were isolated from each treatment and the total RNA was extracted using the Qiagen RNeasy mini kit as described previously (27-29). All RNA samples were reverse-transcribed individually for 30 min at 42°C with high capacity cDNA Reverse transcription kit according to the standard protocol of the supplier (Applied Biosystems). The quantitative PCR from each sample was performed by the condition: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C, 1 min at 60°C using 1 µl of the cDNA reverse-transcribed as described above, 2X SYBR Green PCR master mix (Applied Biosystems) and 200 nM of forward and reverse primers as shown in Table I. Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicates and expression fold-changes were derived using the comparative C_T method (27,29,30).

Statistical analysis. The differences between the curcumin-treated and control group were analyzed by Student's t-test.

Results

Curcumin inhibits the migration of N18 cells in vitro. Representative pictures are shown in Fig. 1A and the percentage of inhibition is shown in Fig. 1B, and both results

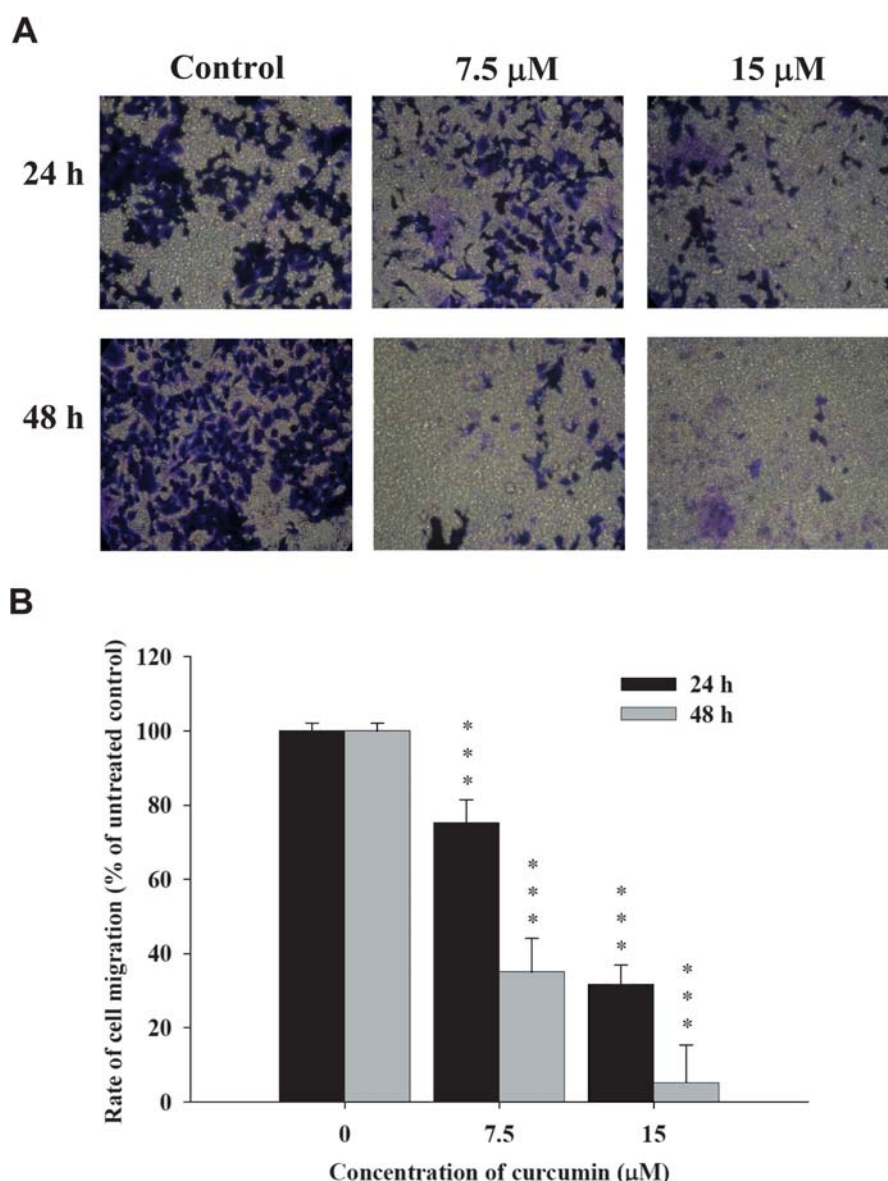


Figure 1. Curcumin inhibited the migration of N18 cells *in vitro*. The filter in 6.5-mm Transwell was inserted in 24-well plates. Then $\sim 2.5 \times 10^4$ cells/filter of N18 cells were placed on the filter and cells were treated with 0, 7.5 and 15 μM curcumin for 24 and 48 h. Migrated cells were stained with hematoxylin and eosin (H&E) and then were examined and photographed under microscope (x200). Quantification of cells in the lower chamber was performed by counting cells at x200 (B). *** $P < 0.001$, significant difference between curcumin-treated groups and the control.

indicated that curcumin inhibited N18 cell migration. The higher dose of curcumin and the longer (48 h) incubation time period of N18 cells lead to greater inhibition of cell migration, and the percentage of inhibition ratio is 62-94%. These effects were dose- and time-dependent.

Curcumin inhibits the invasion of N18 cells in vitro. The inhibition of curcumin on the N18 cells invasion was examined and results are shown in Fig. 2A and B. Fig. 2A shows that N18 cells invaded from the upper to the lower chamber in majority of cells when compared to the control group, but the penetration of the matrigel-coated filter by N18 cells was inhibited in the presence of curcumin. This inhibitory effect was higher at a curcumin concentration of 15 μM than 7.5 μM . The quantification of cells in the lower chamber when compared to the upper chamber group from Fig. 2B indicated that curcumin significantly inhibited N18 cell

invasion, the inhibition range was 80-84% at 24 h and these effects were dose- and time-dependent.

Curcumin inhibits the levels of mRNA expression in N18 cells. To confirm that curcumin inhibited the migration and invasion of N18 cells, and ultimately resulted in the alterations of the expression of these genes at the mRNA level, which are associated with protein levels, we conducted a series of real-time PCR analyses for examining the levels of MMP-2, MMP-7, FAK, Rho A and ROCK1 and the results are shown in Fig. 3 which indicated that expression levels of MMP-2, MMP-7, FAK, Rho A and ROCK1 mRNA were decreased. These effects were time-dependent.

Curcumin inhibits levels of GRB2, Ras, PKC, MKK7, FAK, Rho A, ROCK1, MMP-2, MMP-9, iNOS, NF- κB p65, COX-2, JNK1/2 and ERK1/2 in N18 cells. Levels of invasion- and

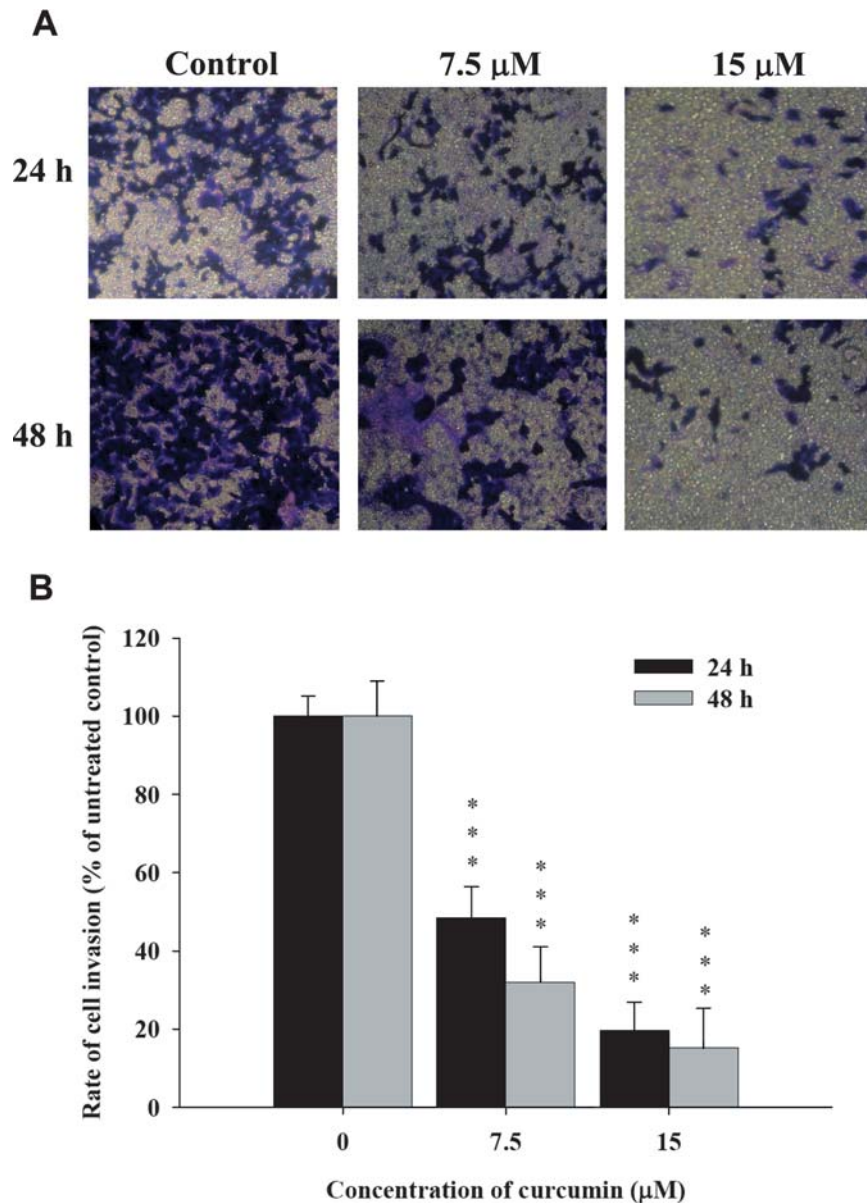


Figure 2. Curcumin inhibited the invasion of N18 cells *in vitro*. N18 cells (10^4 cells/0.4 ml DMEM medium) were placed and cells that penetrated through the matrigel to the lower surface of the filter were stained with crystal violet and were photographed under a light microscope at x200 (A). Quantification of cells in the lower chamber was performed by counting cells at x200 (B). *** $P < 0.001$, significant difference between curcumin-treated groups and the control.

migration-associated proteins in N18 cells after treatment with 7.5 and 15 μ M of curcumin were determined and quantitated by Western blotting. Results presented in Fig. 4 indicate that the levels of GRB2, Ras, PKC, MKK7, FAK, Rho A, ROCK1, MMP-2, MMP-9, iNOS, NF- κ B p65, COX-2, JNK1/2 and ERK1/2 were lower than the corresponding control group. MMP-2 and -9 play an important role in cell invasion and migration and these effects may lead to the inhibition of invasion and migration of N18 cells.

Discussion

Many studies have shown that curcumin exhibits anticancer activity and is associated with the molecular mechanism. It is also being observed for cell cycle arrest and induction of apoptosis. Our previous studies also showed that curcumin decreased the percentage of viable N18 cells through G2/M

phase arrest and apoptosis and those effects were dose- and time-dependent. We also found that curcumin induced DNA damage and inhibited DNA repair gene expression in N18 cells. However, actions of curcumin on migration and invasion of N18 cells and the possible signaling pathways have not been addressed. Herein, we found that curcumin suppressed the migration and invasion of N18 cells through the EEK3, FAK, Rho A and MKK7 pathway before leading to the inhibitions of MMP-2 and MMP-9. Other studies demonstrated that curcumin inhibited the invasion and metastasis in human lung adenocarcinoma (CL1-5) cells through activation of the tumor suppressor DnaJ-like heat shock protein 40 (HLJ1) through activation of the JNK/JunD pathway (23). It was reported that cell migration involves several growth factors for stimulating downstream signaling pathways to form cytoskeletal reorganization and stimulation of motility machinery of the cell (31), and they also provide multiple

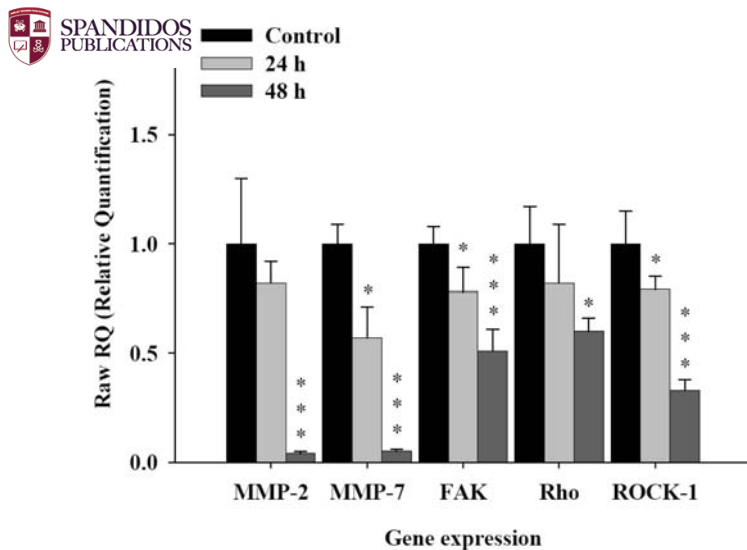


Figure 3. Curcumin inhibited the mRNA expression of migration and invasion in N18 cells. Cells were treated with 15 μ M curcumin for 0, 24 and 48 h and then the total RNA was prepared. The evaluation of the gene expression was carried out by real-time PCR for examining the mRNA expression levels of MM-2, MMP-7, FAK, Rho A and ROCK1. Significantly different between curcumin and control treated groups (* P <0.05; ** P <0.01; *** P <0.001).

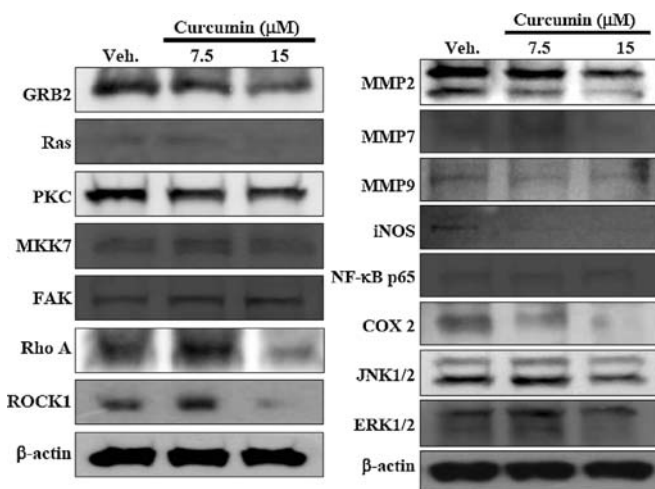


Figure 4. Curcumin inhibited the levels of associated proteins in migration and invasion of N18 cells. The N18 cells (1×10^6 cells/well) were treated with curcumin at 7.5 and 15 μ M for 48 h before total protein was prepared and determined from each sample, as described in Materials and methods. The levels of GRB2, Ras, PKC, MKK7, FAK, Rho A, ROCK1, MMP-2, MMP-7, MMP-9, iNOS, NF- κ B p65, COX-2, JNK1/2 and ERK1/2 expressions were estimated by Western blotting, as described in Materials and methods.

targets for the development of therapeutic agents to inhibit cancer invasion and metastasis (32,33).

It is well known that cancer invasion is due to the cancer cells responding and migrating towards gradients of stimuli and that it requires proteolysis of basement membrane (BM) and extracellular matrix (ECM) proteins to create a path for migration. Matrix metalloproteinases (MMPs) are a multigene family of zinc-dependent endopeptidases responsible for the proteolysis of BM and ECM proteins (34), and the expression

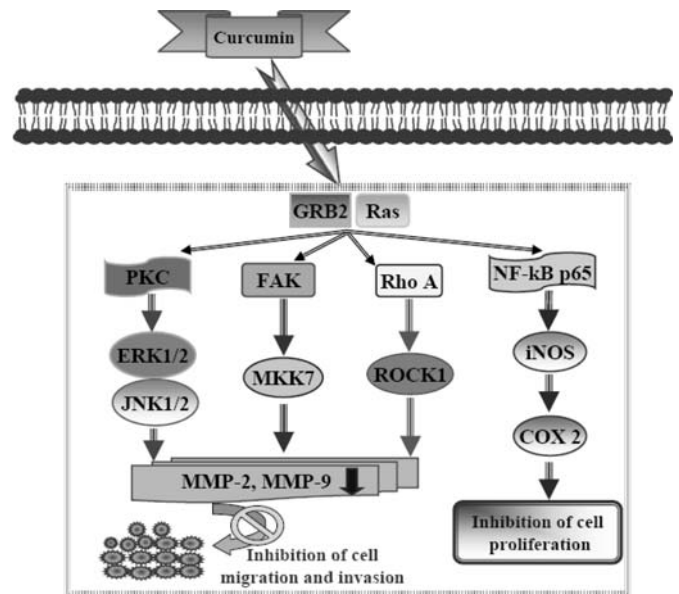


Figure 5. Molecular signaling pathways for curcumin-inhibited invasion and migration in mouse-rat hybrid ganglion cells.

level of MMPs appears to correlate with the invasiveness of cancer cells (35). Among MMPs, MMP-2 and MMP-9 play the most important role for basement membrane type IV collagen degradation (23,31,32). The MMP-2 expression had been shown to be associated with tumor invasion, angiogenesis, metastasis and recurrence (36). In the present study, we found that curcumin inhibited the gene expression of MMP-2 and MMP-7 in N18 cells. It was reported that agents affecting MMPs may go through different mechanisms including direct inhibition of the enzymatic activity of MMPs, interference in the activation of pro-MMPs and reduced expression of MMP genes and protein levels (36). Thus, it seems quite likely that curcumin could inhibit or delay cancer invasion and migration in N18 cells via modulation of MMP-2 and -9.

Herein, our results already showed that curcumin inhibited the migration (Fig. 1A and B) and invasion (Fig. 2A and B) in N18 cells and those effects are dose- and time-dependent. We also showed that curcumin inhibited the expression of mRNA of MMP-2 and -7, Rho A, Rho kinases- ROCK-1 (ROCK-1) and focal adhesion kinase (FAK) in N18 cells (Fig. 4). ROCK-1 mediates some of the downstream signaling of Rho A (37) and is involved in regulation of cell adhesion and migration and therefore ROCK-1 could have potential therapeutic value in lung cancer (38).

Cell migration involves assembly and disassembly of focal adhesions. Furthermore, FAK is activated mainly in focal adhesions and it was reported that FAK plays an important role in cell-ECM interactions that affect cell migration, proliferation and survival (39,40). It is not known what concentrations of curcumin are reached *in vivo* under the conditions used in the present experiments. This will require further study. Therefore, the exact molecular signaling pathway for curcumin inhibiting the migration and invasion in N18 cells still needs further investigation.

Collectively, our data is the first finding to show that curcumin inhibited migration and invasion of human N18

cells through MEKK, ERK and NF- κ B signaling pathways resulting in the inhibition of MMP-2 and -9 as summarized in Fig. 5. Curcumin was recognized to be a therapeutic agent for inhibiting metastasis and invasion.

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