Curcumin stimulates proliferation, stemness acting signals and migration of 3T3-L1 preadipocytes

JEONG-HWAN KIM[1], SHIN-HYUNG PARK[5], SOO-WAN NAM[1,2,4], HYUN-JOO KWON[1,3,4], BYUNG-WOO KIM[1,3,4], WUN-JAE KIM[7] and YUNG HYUN CHOI[1,4,6]

Departments of [1]Biomaterial Control, [2]Biotechnology and Bioengineering, [3]Life Science and Biotechnology, [4]Blue-Bio Industry RIC, Dong-Eui University, Busan 614-714; Departments of [5]Pathology, [6]Biochemistry, Research Institute of Oriental Medicine, Dong-Eui University College of Oriental Medicine, Busan 614-052; [7]Department of Urology and Personalized Tumor Engineering Research Center, College of Medicine, Chungbuk National University, Cheongju 361-763, Republic of Korea

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Abstract. In the present study, the potential of curcumin to stimulate proliferation, stemness acting signals and migration of 3T3-L1 preadipocytes and the associated molecular mechanisms were investigated. Low concentrations of curcumin stimulated cell proliferation, whereas high concentrations were cytotoxic to 3T3-L1 cells. In particular, application of 0.02 μM of curcumin for 24 h resulted in significantly increased cell proliferation and was determined to be the optimal treatment for this study. In a colony-forming cell assay, cells treated with 0.02 μM of curcumin showed an approximately 1.5-fold increase in colony formation. Curcumin treatment up-regulated the proliferation-related marker proteins coupled with increased cell growth, telomerase activity and over-expression of stemness acting signals, which was associated with activation of the phosphoinositide 3-kinase (PI3K) pathway. In addition, curcumin significantly inactivated p38 mitogen-activated protein kinases (MAPK) and stress-activated protein kinase/c-Jun N-terminal kinases (SARK/JNK), coupled with inhibition of p53 and p21 tumor suppressor gene products. In addition, curcumin significantly increased cell migration through activation of migration-associated transcription factors. Therefore, these results clearly show that activation of cell proliferation by curcumin is associated with improved stem cell potency in 3T3-L1 preadipocytes.

Introduction

Curcumin (diferuloylmethane) is a natural polyphenol derived from the plant *Curcuma longa*, which is used in some cultures for the treatment of diseases associated with oxidative stress and inflammation (1). Due to its ability to scavenge free radicals and inhibit inflammation, curcumin has been investigated for cancer chemoprevention and tumor growth suppression (2-6). Previous results have indicated that this compound has been recognized as a promising anticancer drug due to its efficient induction of proliferation arrest and cell death, including apoptosis and necrosis, through diminishing reactive oxygen species (ROS) generation in a variety of tumor cells (7-12). Furthermore, curcumin has also been reported to prevent tumor-induced T cell apoptosis (13). Also, overwhelming *in vitro* evidence and completed clinical trials suggest that curcumin may prove useful for chemoprevention of human colon cancer (14). Some reports have also suggested the possibility that curcumin can reduce oxidative damage and cognitive deficits associated with aging (15-17). In addition, studies of animal models have suggested that curcumin may be beneficial in neurodegenerative conditions, such as Alzheimer's disease (AD) (18-20) and focal cerebral ischemia (21). Furthermore, curcumin treatment can protect hippocampal neurons against excitotoxic and traumatic injury (22,23). Therefore, curcumin has attracted commercial interest as a useful medicinal reagent.

A stem cell is a special type of cell with a unique capacity to renew itself, to give rise to specialized cell types, and to migrate, proliferate and survive (24). Although most cells of the body, such as heart cells or skin cells, are committed to the conduct of specific functions, a stem cell is uncommitted and remains uncommitted, until it receives a signal to develop into a specialized cell. Its proliferative capacity combined with the ability to obtain active self renewal activity and to become specialized makes stem cells unique (24-27). To orchestrate self-renewal via an increase in cell proliferation, multiple signaling networks are activated by various intra-cellular and extracellular factors.

In the present study, we hypothesized that curcumin as an extracellular factor can improve stem cell potency via stimulated proliferation, stemness acting signals, and migration of 3T3-L1 preadipocytes. To estimate this hypothesis, we examined

Correspondence to: Dr Yung Hyun Choi, Department of Biochemistry, Research Institute of Oriental Medicine, Dong-Eui University College of Oriental Medicine, Busan 614-052, Republic of Korea
E-mail: choiyh@deu.ac.kr

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the ability of curcumin to induce improvement of self-renewal via an increase in cell growth, telomerase activity, and migration potential after overexpression of stemness acting signals in 3T3-L1 preadipocytes.

Materials and methods

Cell culture and curcumin treatment. Mouse 3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD) were maintained at 37˚C in humidified 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated bovine calf serum (BCS) and 1% penicillin-streptomycin (28). Curcumin (Sigma Co., St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and stored at -20˚C. For curcumin treatment, 3T3-L1 cells were seeded and stabilized in 5% BCS containing DMEM for 24 h. The cells were then treated with curcumin at various concentrations in serum-free DMEM for different time points. Following treatments, the optimum concentration of curcumin was selected on the basis of cell viability studies using the trypan blue exclusion assay.

Trypan blue exclusion assay. 3T3-L1 cells were seeded in 6-well plastic culture dishes at a density of $7x10^4$ cells/well in DMEM containing 5% BCS and cultured for 24 h. The medium was then replaced with serum-free DMEM with the indicated curcumin concentrations for different time points. After treatment, cells were collected and viable cells, determined by the uptake of 0.4% trypan blue, were counted using a hemocytometer. Triplicate wells were used in all cell viability assays and each experiment was repeated at least three times.

Colony-forming cell (CFU) assay. 3T3-L1 cells were seeded in 10-cm cell culture dishes at a density of $3.5x10^3$ and stabilized in 5% BCS containing DMEM for 24 h. Following stimulation with curcumin (0.02 μM) for another 24 h in serum-free DMEM, the medium was replaced with complete medium containing 10% BCS. After 15 days, cells were fixed with 4% paraformaldehyde (PFA) and stained with 0.1% toluidine blue in 1% PFA. The proliferation efficiency was determined by counting the visual colonies (29).

Telomerase activity assay. 3T3-L1 preadipocytes were seeded in 10-cm dishes at a density of $5x10^5$ cells/dish and stabilized in 5% BCS containing DMEM for 24 h. The cells were treated with curcumin (0.02 μM) in serum-free DMEM for 24 h and collected. Telomerase activity was then measured using a telomerase-polymerase chain reaction (PCR) ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Cell migration assay. To evaluate the migration activity of curcumin-treated 3T3-L1 preadipocytes, cells were seeded in 10-cm cell culture dishes at a density of $5x10^5$ and incubated in 5% BCS containing DMEM for 24 h at 37˚C in a CO_2 incubator. The cells were then treated with curcumin (0.02 μM) for another 24 h. The cultured cells were transferred into Costar Transwell membranes (8-μm pore size; Corning Costar, Acton, MA) and placed in 6-well plates. Below the membrane, curcumin containing DMEM with 2% BCS was added to each well. In the upper chamber, cells were incubated for 2 h at 37˚C in 2% BCS containing DMEM, and the plate was incubated overnight at 37˚C in a CO_2 incubator. Cells on the lower surface were air-dried, counterstained with Harris hematoxylin for 20 min and then washed. The stained inserts were placed on object slides, and the number of cells on the lower surfaces was assessed at x200 using an inverted bright-field microscope. Ten x20 fields/insert were counted. Migration was expressed as the count of cells/field of spontaneous migration toward the cell bottom. To obtain clearer evidence regarding the role of curcumin in 3T3-L1 cell migration, we conducted a simple cell-scraped wound model assay. Cells were seeded in 10-cm cell culture dishes at a density of $5x10^5$ and incubated in 5% BCS containing DMEM. After 24 h of stabilization, cells were scraped in a straight line across the dish and washed with medium three times. The cells were then treated with curcumin (0.02 μM) for 24 h in serum-free DMEM. Cells that migrated onto the wounded region were photographed under the microscope at x40 magnification.

RNA extraction and reverse transcription-PCR. 3T3-L1 preadipocytes were treated with curcumin, as in the previous paragraph and harvested. Total-RNA was prepared according to the manufacturer's instructions of the RNeasy kit (Qiagen, La Jolla, CA) and primed with random hexamers by the AMV reverse transcriptase (Amersham Corp., Arlington Heights, IL) for synthesis of complementary DNA. PCR was then carried out in a Mastercycler (Eppendorf, Hamburg, Germany) using 20 pM of specific primers. The conditions for the PCR reactions were 1 cycle at 94˚C for 3 min; 35 cycles at 94˚C for 45 sec, 56.5˚C for 45 sec and 72˚C for 1 min; and 1 cycle at 72˚C for 10 min. Amplified products obtained by PCR were mixed with 6X agarose gel loading dye (Bioneer, Seoul, Korea) and electrophoretically separated on 1% agarose gel containing ethidium bromide (EtBr) (30).

Protein extraction and Western blot analysis. 3T3-L1 cells treated with curcumin were collected, lysed with lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μM Tris-HCl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin and 2 μg/ml aprotinin), and the protein concentrations were quantified using a Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. For Western blot analysis, equal amounts of protein were loaded on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by electroblotting. The blots were probed with the specific antibodies and incubated overnight at 4˚C. After 1 h of incubation with the diluted enzyme-linked secondary antibodies, the blots were visualized by enhanced chemiluminescence (ECL) according to the manufacturer's procedure (Amersham). Primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Cell Signaling Technology (Boston, MA), and both anti-rabbit and anti-mouse immunoglobulins were purchased from Amersham.

Statistical analysis. All data are presented as the mean ± SD from three or more independent experiments. Statistical significance of the differences between groups was calculated using the Student's two-tailed t-test.

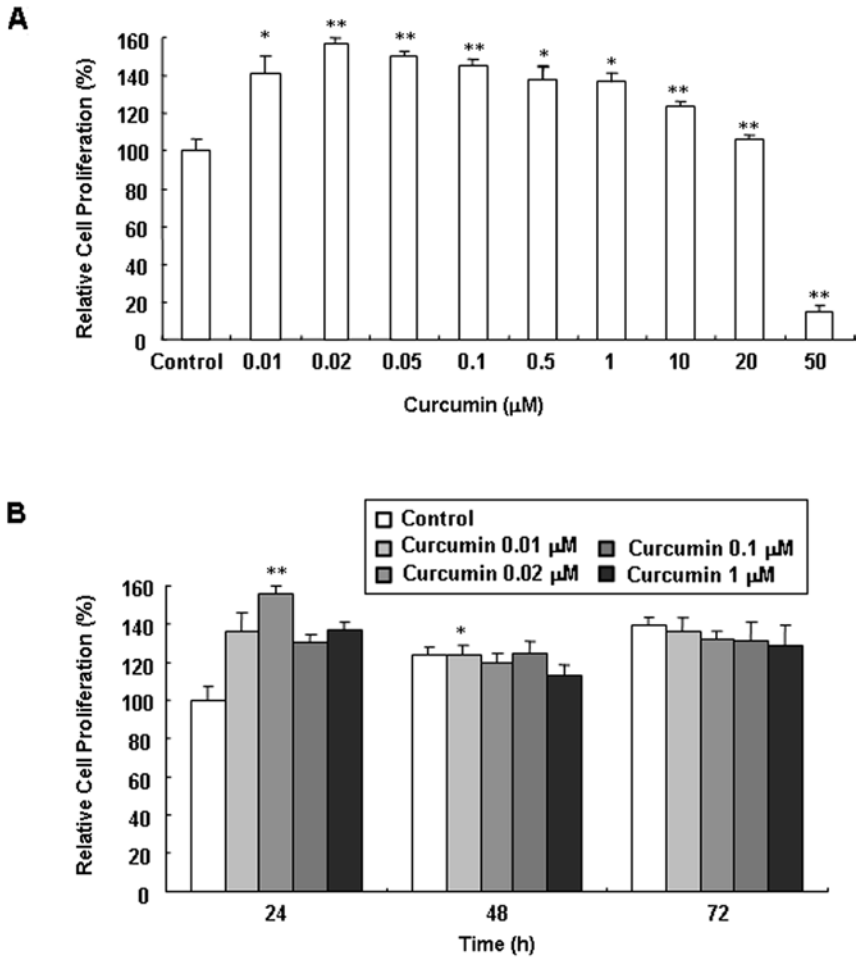

Figure 1. Effects of curcumin on the cell proliferation in 3T3-L1 preadipocytes. Proliferation of 3T3-L1 preadipocytes treated with various concentrations of curcumin was evaluated by trypan blue exclusion assay. Cells were seeded in 6-well plastic culture dishes at a density of $7x10^4$ cells/well in DMEM containing 5% BCS and cultured for 24 h. The medium was then replaced with serum-free DMEM with the indicated curcumin concentrations (A) for 24 h or (B) the indicated times. After treatment, the cells were collected and the viable cells were counted using a hemocytometer.

Results

Effects of curcumin on the cell growth and proliferation in 3T3-L1 preadipocytes. To examine the potential of curcumin to induce cell growth, proliferation of 3T3-L1 preadipocytes treated with various concentrations of curcumin for 24 h was evaluated by the trypan blue exclusion assay. After 24 h of curcumin treatment, low concentrations (0.01, 0.02, 0.1 and 1 μM) of curcumin increased cell proliferation, whereas high concentrations (≥50 μM) caused a decrease in cell proliferation. Among the doses tested, 0.02 μM of curcumin was the most effective in stimulating cell proliferation (Fig. 1A). The proliferative effects of low doses of curcumin, evaluated during a 72-h treatment period, revealed that 0.02 μM of curcumin administered for 24 h was the most effective in stimulating cell proliferation (Fig. 1B). Active proliferation of curcumin-treated cells was clearly visible by observation of the increase of cell density under a phase-contrast microscope (Fig. 2A). Therefore, application of 0.02 μM of curcumin for 24 h was determined to be the optimal treatment for this study. A clonogenic assay was also performed to confirm the proliferation efficiency of curcumin-treated 3T3-L1 preadipocytes. In the CFU assay, cells treated with 0.02 μM of curcumin showed an approximately 1.5-fold increase in colony formation, compared with control cells (Fig. 2B).

Curcumin enhances telomerase activity of 3T3-L1 preadipocytes. In order to determine whether or not the increased proliferation efficiency of 3T3-L1 preadipocytes by curcumin is associated with activation of telomerase, telomerase activity and the transcriptional levels of its regulatory factors were measured after 24 h of 0.02 μM of curcumin treatment. Cells treated with 0.02 μM of curcumin showed an increase in positive telomerase enzyme activity of ~1.6-fold (Fig. 3). This result is generally consistent with overexpression of telomerase-associated transcription factors, including telomerase RNA subunits (TR), telomerase reverse transcriptase (TERT) and a telomerase-associated protein (TEP1) after curcumin treatment (Fig. 3).

Curcumin induces stemness and proliferation-related gene expression in 3T3-L1 preadipocytes. Several proliferation-related proteins and transcription factors were assessed for identification of possible activated signaling molecules involved in active cell proliferation occurring after curcumin treatment. The data indicate that curcumin induces significant

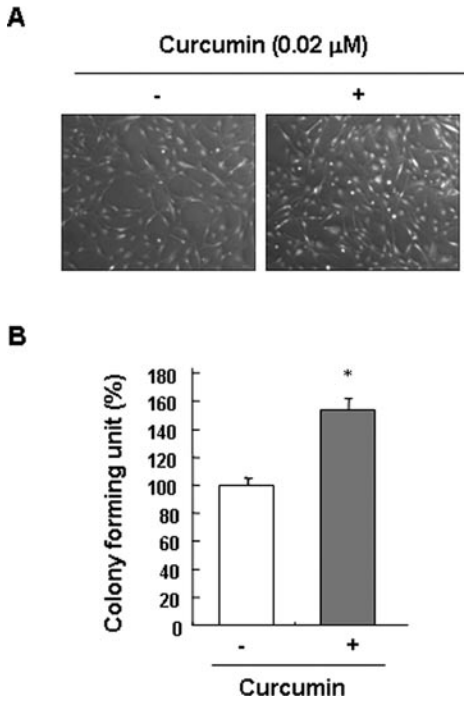

Figure 2. Effects of curcumin on cell morphology and colony formation in 3T3-L1 preadipocytes. (A) For the morphological observation, the cells were incubated with 0.02 μM of curcumin for 24 h and shown in phase-contrast images with untreated control cells (original magnification, x50). (B) For the CFU assay, the cells were treated with 0.02 μM of curcumin for 24 h in serum-free DMEM; the medium was replaced with complete medium containing 10% BCS, as described in Materials and methods. After 15 days, the cells were fixed with 4% PFA and stained with 0.1% toluidine blue in 1% PFA. The proliferation efficiency was then determined by counting the visual colonies. Each bar represents the mean ± SD of three independent experiments. Data were analyzed using analysis of variance with the Fisher's test or t-test; *P<0.05.

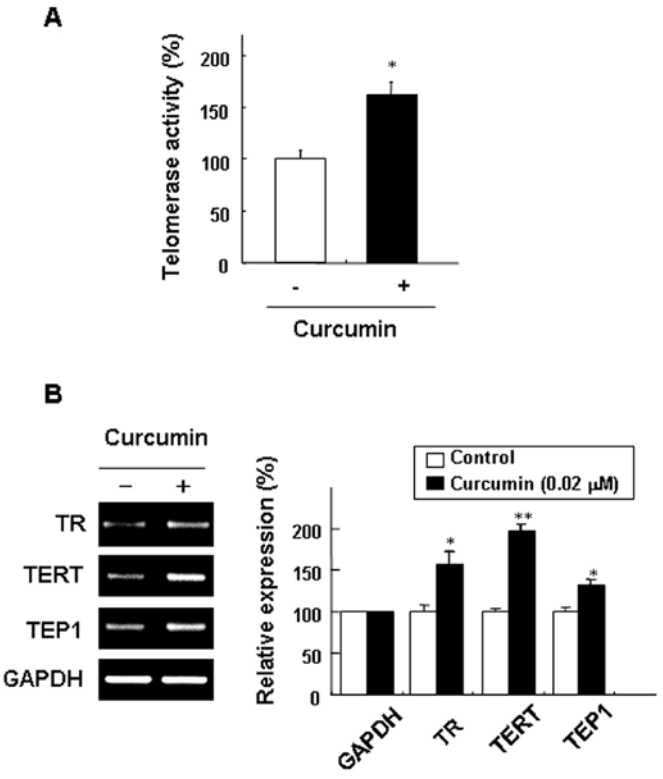

Figure 3. Increase of telomerase activity and its regulatory factors by curcumin in 3T3-L1 preadipocytes. (A) Following a 24-h period of incubation with 0.02 μM of curcumin, telomerase activity of 3T3-L1 cells was measured using a telomerase-PCR ELISA kit. (B) The total-RNA was isolated from cells grown under the same conditions as A and reverse-transcribed. The resulting cDNAs were subjected to PCR with the indicated primers and the reaction products were subjected to electrophoresis on a 1% agarose gel and visualized by EtBr staining. GAPDH was used as the internal control. mRNA levels of TR, TERT and TEP1 in curcumin-treated cells are expressed as a percentage of the GAPDH mRNA levels. Data were analyzed using analysis of variance with the Fisher's test or t-test; *P<0.05; **P<0.01.

activation of phosphoinositide 3-kinase (PI3K) and its downstream mediators, phosphorylated-mitogen-activated protein kinases (MAPK)/extracellular signal-regulated protein kinase (ERK) (p-MEK), p-ERK and p-AKT in 3T3-L1 preadipocytes (Fig. 4), whereas it directly attenuated the levels of apoptosis-related proteins, p38 MAPK and p-stress-activated protein kinase/c-Jun N-terminal kinases (p-SAPK/JNK) (Fig. 4). These results clearly show that curcumin-induced 3T3-L1 cell proliferation via activation of the PI3K and MEK signaling pathways with direct inhibition of p38 and p-SAPK/JNK. In addition, curcumin exerted prominent effects on the overexpression of proliferation-related transcription factors, including REX1, SP1, cyclin-dependent kinase1 (CDK1) and CDK2. Curcumin also induced the overexpression of the c-Myc protein and the down-regulation of the tumor suppressor, p53, and the CDK inhibitor, p21. In addition, the curcumin-induced activation of p-ERK and p-AKT in 3T3-L1 cells was associated with the subsequent induction of expression of stemness transcription factors, including OCT4, SOX2, KLF4 and c-Myc (Fig. 5). These results indicate that curcumin can induce active proliferation, stemness acting signals, along with enhanced telomerase activity in 3T3-L1 preadipocytes.

Curcumin induces cell migration of 3T3-L1 preadipocytes. In vitro Transwell plate and scratch-inducing cell migration assays were conducted in order to assess the migration efficiency of curcumin-treated 3T3-L1 preadipocytes. The curcumin treatment significantly increased the migration efficiency of 3T3-L1 preadipocytes in a time-dependent manner, compared with control cells (Fig. 6A). In order to obtain clearer evidence of the active migration in curcumin-treated cells, a simple cell-scraped wound model assay was used, in which curcumin-treated cells and untreated control cells were scraped from one side of a marked reference line (simulating a wound) and were then allowed to migrate on their own. Cells that migrated across the marked reference line were photographed under a phase-contrast microscope. The curcumin treatment actively induced migration across the reference line, compared with the untreated control cells, thereby indicating an almost 2.5-fold increase in the migration of curcumin-treated cells over the untreated control cells (Fig. 6B). These results are consistent with the overexpression of migration-associated transcription factors, including matrix metalloproteinase-1 (MMP1), MMP2, MMP3, of the stromal cell-derived factor (SDF)1 and the vascular endothelial growth factor (VEGF) coupled with the down-regulation of tissue inhibitors of MMP1 (TIMP1), TIMP2 and thrombospondin-1 (TSP-1) following curcumin treatment (Fig. 6C).

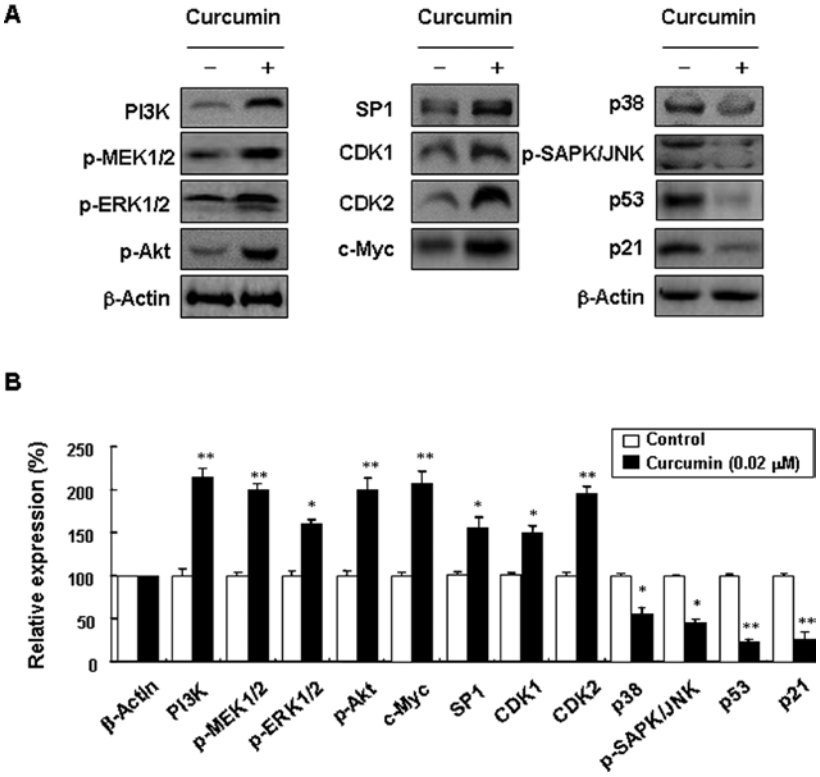

Figure 4. Effects of curcumin on the activity of PI3K and its downstream mediators, p-MEK, p-ERK and p-AKT, and proliferation-related transcription factors, including SP1, CDK1 and CDK2 in 3T3-L1 preadipocytes. (A) 3T3-L1 cells were treated with 0.02 μM of curcumin for 24 h, lysed, and equal amounts (50 μg) of cell lysate were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Western blotting was then performed using the indicated antibodies and an ECL detection system. β-actin was used as an internal control. (B) Relative protein levels in curcumin-treated cells are expressed as a percentage of the levels of β-actin protein. Data were analyzed using analysis of variance with the Fisher's test or t-test; *P<0.05; **P<0.01.

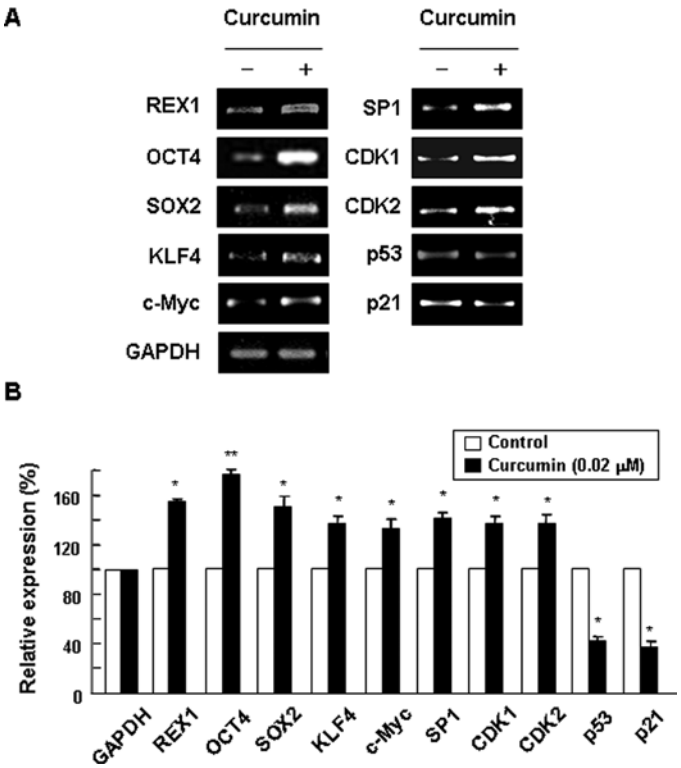

Figure 5. Effects of curcumin on the transcriptional levels of proliferation-related functional genes (REX1, SP1, CDK1 and CDK2), stemness genes (OCT4, SOX2, KLF4 and c-Myc), and p53 and p21 tumor suppressor genes. (A) After incubation with curcumin under the same conditions described in Fig. 4 legend, total-RNAs were isolated and reverse-transcribed. Resulting cDNAs were then subjected to PCR with the indicated primers and the reaction products were subjected to electrophoresis on a 1% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control. (B) mRNA levels are expressed as a percentage of the levels of GAPDH mRNA. Data were analyzed using analysis of variance with the Fisher's test or t-test;*P<0.05; **P<0.01.

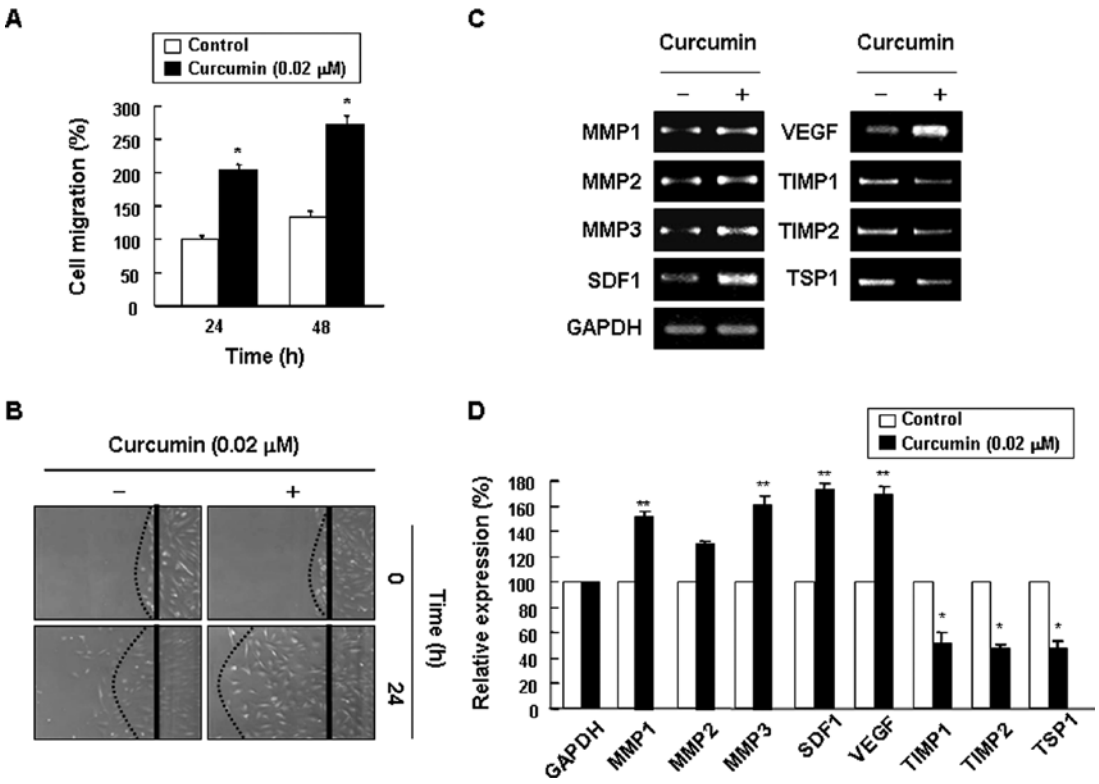

Figure 6. Increase of cell migration by curcumin and its effects on the levels of migration-associated transcription factors in 3T3-L1 preadipocytes. (A) As described in Materials and methods, an *in vitro* Transwell plate assay was conducted using Costar Transwell membranes in order assess the migration efficiency of curcumin-treated 3T3-L1 cells. (B) A simple cell-scraped wound model assay was performed in order to obtain clearer evidence of the active migration in curcumin-treated cells. Cells that migrated across the marked reference line were photographed under a phase-contrast microscope at x40 magnification. (C) 3T3-L1 cells were treated with 0.02 μM of curcumin for 24 h and total-RNAs were isolated, and reverse-transcribed. Resulting cDNAs were then subjected to PCR with the indicated primers and the reaction products were subjected to electrophoresis in a 1% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control. (D) mRNA levels are expressed as a percentage of the levels of GAPDH mRNA. Data were analyzed using analysis of variance with the Fisher's test or t-test; $^{*}P<0.05$; $^{**}P<0.01$.

Discussion

Curcumin is a major component of a dietary spice derived from the roots of *C. longa*. Previous reports have documented antioxidant and anti-inflammatory effects of curcumin in cultured tumor cell lines as well as normal cells (15-17). Also, treatment of cell cultures and adult rodents with curcumin can protect neurons from being damaged and killed in models relevant to the pathogenesis of AD, Parkinson's disease, and stroke (18,19,21,22). However, there is no evidence of the molecular mechanisms involved in the curcumin-induced improvement of stem cell potency in 3T3-L1 preadipocytes. In this study, we hypothesized that curcumin can improve stem cell potency through an increase in the proliferation potential of 3T3-L1 preadipocytes. To estimate this hypothesis, this study explored the ability of curcumin to increase the proliferation of 3T3-L1 cells and the involved signaling mechanisms.

We first examined the question of whether curcumin enhanced the proliferative capacity of 3T3-L1 preadipocytes. Low doses of curcumin stimulated cell proliferation, whereas high doses were cytotoxic in 3T3-L1 cells. In particular, 0.02 μM of curcumin administered for 24 h was the most effective in stimulating cell proliferation. The CFU assay also showed that curcumin can actively stimulate proliferation of 3T3-L1 cells, which was associated with increased telomerase enzyme activity coupled with overexpression of telomerase-associated transcription factors, including TR, TERT and TEP1 (31-33).

To characterize the signaling pathways involved in the curcumin-induced stem cell potency, the effects of curcumin on the levels of various cell proliferation, stemness and migration-related gene products were investigated. As indicated in Fig. 4, curcumin treatment significantly increased activation of PI3K and its downstream mediators, p-MEK, p-ERK and p-AKT in 3T3-L1 cells, which was connected with the subsequent induction in the expression of proliferation-related transcription factors (REX1, SP1, CDK1 and CDK2) and stemness genes (OCT4, SOX2, KLF4 and c-Myc). However, the levels of p38 and p-SAPK/JNK coupled with the inhibition of p53 and p21 tumor suppressor gene products were markedly down-regulated in curcumin-treated 3T3-L1 cells. In particular, REX1 overexpression after curcumin treatment is consistent with our recent reports in which enhancement of REX1 expression causes an increase in the proliferation efficiency of cells (34,35). We next investigated the effects of curcumin on the migration of 3T3-L1 cells in order to show that increased cell proliferation by curcumin was related to the migration efficiency. As shown in Fig. 6, curcumin significantly-induced cell migration via activation of migration-associated transcription factors, including MMP1, MMP2, MMP3, SDF1 and VEGF. However, the levels of TIMP1, TIMP2 and TSP1 were markedly

inhibited by curcumin treatment in 3T3-L1 fibroblasts. These data clearly show that curcumin can induce proliferation as well as migration of 3T3-L1 cells through activation of the PI3K and MEK signaling pathways with direct inhibition of p38 and p-SAPK/JNK, and modulation of cell migration regulatory gene products.

In conclusion, our findings suggest that curcumin can improve stem cell potency via increased proliferation, stemness acting signals, and migration of 3T3-L1 fibroblasts. The association between curcumin-induced cell proliferation and stem cell potency is still unclear, and there is still the question of whether curcumin can induce cell migration through other pathways; however, the results provide novel information on possible mechanisms for a novel biological function of curcumin.

Acknowledgements

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