# The role of serum response factor in hepatocellular carcinoma: An association with matrix metalloproteinase

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**Abstract.** Serum response factor (SRF) regulates transcription of immediate early genes and triggers proliferation, migration and differentiation in several types of cells. Matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases, play a crucial role in tumor invasion and metastasis. However, expression of SRF and its association with MMPs in hepatocellular carcinoma (HCC) have not been elucidated. We examined the expression levels of SRF, MMP-2 and MMP-9 in HCC tissues using Western blot assay. We also examined the effect of SRF on MMP expression and enzyme activity in HCC by transfection of SRF cDNA in HLE cells. Protein expression of SRF, MMP-2 and MMP-9 showed a significant increase in HCC tissues, compared with those of corresponding non-tumor tissues. High SRF expressing HCC tissues showed higher levels of expression of MMP-2 and MMP-9, compared with low SRF expressing HCC tissues. In addition, overexpression of SRF in HLE cells led to increased levels of expression of mRNA and protein, as well as increased enzyme activity of MMP-2 and MMP-9. Overexpression of SRF also led to significantly enhanced migration of HLE cells. These results suggest that overexpression of SRF in HCC may play an important role in tumor cell migration and invasion through upregulation of MMP-2 and MMP-9.

## Introduction

Hepatocellular carcinoma (HCC) is the sixth most common solid malignant tumor worldwide, with an incidence of 626,000 cases and 598,000 deaths annually, making it the third most common cause of cancer deaths throughout the world (1,2). In recent years, considerable advances have been made in diagnosis and treatment of HCC. However, HCC is still associated with a high rate of mortality, and the prognosis of this tumor is poor, even with treatment that is considered potentially curative. Morbidity and mortality of patients with HCC is predominantly the result of tumor invasion and metastasis (3).

Serum response factor (SRF) is a transcription factor of the MADS box family, which is composed of 508 amino acids containing three major domains: a serum response element (SRE) DNA binding domain, a transactivation domain, and several phosphorylation sites (4-8). Serum response factor (SRF) is a transcription factor involved in regulation of many genes, including immediate early genes, such as *c-Fos, Jun,* and *Egr,* and tissue-specific genes involved in cellular activities, such as proliferation, migration, differentiation, angiogenesis, and apoptosis (4-11). Recent studies have strongly implicated a role for SRF in tumor progression, including HCC (12,13).

Matrix metalloproteinases (MMPs) are a family of structurally related zinc-dependent endopeptidases, which are collectively capable of degrading essentially all components of extracellular matrix (ECM), including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan (14,15). They play an important role in various physiological processes, including tissue remodeling, organ development, angiogenesis, inflammatory processes, vascular and autoimmune disorders, and in cancer (14-17). Among the MMPs, activity of MMP-2 and -9 has been associated with progression of HCC and some studies have demonstrated an association of overexpression of MMP-2 or -9 with invasion and metastasis of HCC (18,19). However, SRF expression and its association with MMPs in HCC have not been elucidated. We examined the expression and role of SRF in HCC, specifically in association with MMPs expression.

In the present study, we examined a) expression levels of SRF, MMP-2, and -9 in HCC tissues, b) the relationship between expression of SRF and MMP-2, -9 in HCC tissues, c) the effect of SRF in expression and enzyme activities of MMP-2 and -9 using SRF-overexpressed HCC cells, and d) the role of SRF in migration of HCC cells.

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## Materials and methods

*Cell culture*. The human HCC cell line, HLE, was purchased from the Health Science Research Resources Bank (Osaka, Japan). HLE cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin and streptomycin (100 U/ml) and 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA), and the cells were grown at 37°C in a humidified 5% $CO_2$ incubator.

*Stable transfection of SRF complementary DNA*. A previously described method was used in performance of stable trans-fection (13). Briefly, cells were rinsed with serum-free medium and without antibiotics, and were transfected with pcDNA 3.1-SRF 1 $\mu$g/lipofectin 8-10 $\mu$l or pcDNA3.1 1 $\mu$g/lipofectin 8-10 $\mu$l (mock control), followed by incubation at 37°C in a 5% $CO_2$ incubator for 12 h. The medium was then replaced by DMEM with 10% FBS and without antibiotics. After 18 h, cells in each group were removed for detection of transient expression of SRF by Western blot methods, whereas others were continuously cultured for stable expression of SRF. Cells were subcultured with DMEM with 10% FBS with G418 (600-800 $\mu$g/ml). After 2 weeks of culture in the presence of G418, positive clones were selected and transferred into 24-well plates.

*RNA extraction and RT-PCR analysis*. Total RNA was extracted using TRI Reagent (Molecular research center, Inc., Cincinnati, OH) according to the manufacturer's protocol. The concentration of total RNA can be determined by spectrophoto-metry. Reverse transcription was carried out using reverse transcriptase (Roche Applied Science, Mannheim, Germany), which involved preparation of a master-mixture. The master-mixture was comprised of 5X colorless Go Taq Flexi buffer, 25 mM $MgCl_2$, 10 mM dNTPs, and Go Taq polymerase (Promega, Madison, WI), each of both forward and reverse primers and extracted RNA in a 25 $\mu$l reaction mixture. Conditions for PCR of MMP-2 were: 95°C for 2 min, 58°C for 50 sec, 72°C for 30 sec, and a final extension at 72°C for 5 min for 35 cycles; and MMP-9 conditions were: 95°C for 2 min, 62°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 5 min for 35 cycles. In this study, primers used were: MMP-2: 5'-CGGCCGCAGTGACGGAA-3' and 5'-AAGAA CTTCCGTCTGTCCCAGGATG-3'; MMP-9: 5'-GACGCA GACATCGTCATCCAGTTT-3' and 5'-TTTGGAAACGCAG ATGGCGCGGC-3'; β-actin: 5'-CGCGAGAAGATGACCCA GATC-3' and 5'-TCGTGATGGACTCCGGTGA-3'.

*Protein extraction and Western blot analysis*. HCC tissues and cells were homogenized and extracted with PRO-PREP Protein Extraction Solution (iNtRON Biotechnology Inc., Korea). Lysates were incubated on ice for 10 min, and centri-fuged at 14,000 rpm for 10 min at 4°C. Protein concentration was determined according to the Bradford method (Bio-Rad, Richmond, CA). Proteins were separated by electrophoresis on an 8% SDS-polyacrylamide gel, and the separated proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane using a semidry transfer method (Bio-Rad). The membrane was then blocked with 5% nonfat dry milk in Tris-buffered saline (TBS)-0.1% Tween-20 (15 mM NaCl,

100 mM Tris-HCl, pH 7.5) for 1 h to reduce nonspecific binding. The membrane was incubated with anti-SRF (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MMP-2 (R&D Systems, Minneapolis, MN), and anti-MMP-9 (Thermo Fisher Scientific, Scotts Valley, CA) overnight at 4°C. The membrane was incubated for 1 h at room temperature with corresponding secondary antibodies, and the immune complexes were visual-ized using the ECL detection system (Amersham Biosciences, Buckinghamshire, UK) and exposed to a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan). Equal loading of proteins in each lane was confirmed by probing the membrane with mouse anti-β-actin (Sigma, St. Louis, MO) or rabbit anti-GAPDH (Sigma).

*Measurement of MMP-2 and -9 enzyme activity*. Assessment of MMP-2 and -9 activities of conditioned media was performed using the colorimetric Biotrak MMP-2, -9 activity assay (GE Healthcare Biosciences, Piscataway, NJ), according to the manufacturer's instructions. The assay uses the pro-form of a detection enzyme that can be activated by captured active MMPs into an active detection enzyme, through a single proteolytic event. Standard and samples were incubated in microplate wells pre-coated with anti-MMP-2 or anti-MMP-9 antibody. Optical densities were quantified using a microtiter plate reader (Bio-Rad) at a wavelength of 405 nm, referenced to 650 nm. Three samples were used for each experimental condition. Experiments were performed in triplicate and mean values were calculated.

*Wound-healing assay*. HLE cells transfected with either the SRF expression plasmid or the control vector plasmid were cultured in 0.2% gelatin-coated 6-well plates containing DMEM medium until confluence. Confluent cells were scraped with a sterile pipette tip and washed with serum-free DMEM medium to remove cellular debris. Cells were then starved in serum-free DMEM medium for 12 h. After 12 h, cells were treated with 10% fetal bovine serum for 24 h. Migration of cells into the wound area was allowed at 37°C in a 5% $CO_2$ incubator for 24 h. The wound area was photographed with a Nikon digital camera connected to the microscope at 0, 6, 12, and 24 h. All experiments were performed in triplicate.

*Statistical analysis*. Data are expressed as means ± SD for three or more independent experiments. Difference of expres-sion of SRF, MMP-2, and -9 between HCC and non-malignant hepatocytes was tested by the Student's t-tests. $P < 0.05$ were considered to indicate statistical significance.

## Results

*Expression of SRF, MMP-2, and -9 in hepatocellular carcinoma tissues*. We evaluated expression of SRF, MMP-2, and -9 in 16 cases of HCC tissues and corresponding non-tumor hepatic tissue. Representative SRF, MMP-2, -9 expressions are shown in Fig. 1A. In HCC samples, SRF, MMP-2 and -9, were significantly increased by 2-, 1.5-, and 2.5-fold, respectively, when compared with those of the corresponding non-tumor tissues (Fig. 1A).

*Relationship between SRF expression and expression of MMP-2 and -9 in hepatocellular carcinoma tissues*. ImageJ software was used for measurement of SRF protein band
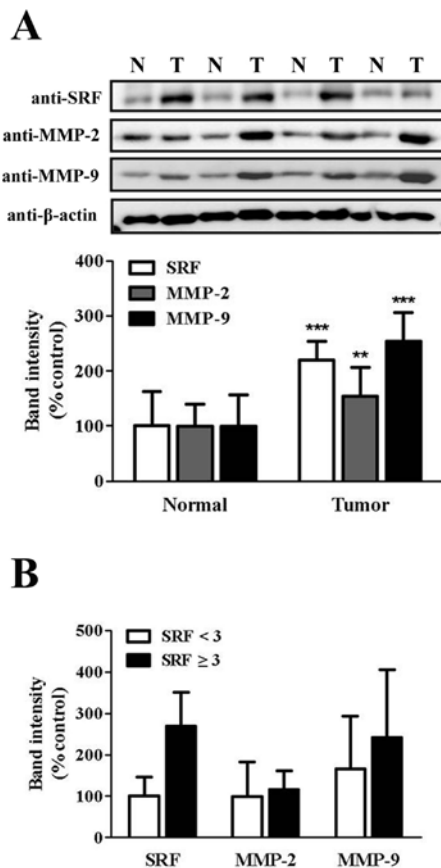
Figure 1. Protein levels of SRF, MMP-2, and -9 in hepatocellular carcinoma (HCC) tissues. (A) Expression levels of SRF, MMP-2, and -9 in HCC tissues was significantly up-regulated when compared with those of corresponding non-tumor tissues ([**]P<0.01; [***]P<0.001). (B) The high SRF expression group (SRF intensity ≥3-fold as compared to that of non-tumor tissues) showed increased expression of MMP-2 and -9 when compared with those of the low SRF expression group (SRF <3-fold as compared to that of non-tumor tissues).



Figure 2. Enhanced expression of MMP-2 and -9 mRNA in HLE cells trans-fected with the SRF gene (three selected clones; C-2, C-7 and C-7-1).

intensities. Then, by adoption of cut-off values according to SRF band intensities 3-fold level, compared with that of non-tumor tissues, we divided results according to the high SRF expression group (SRF intensity ≥3-fold) and the low SRF expression group (SRF <3-fold). According to the results, the high SRF expression group (7 cases) showed higher expression of MMP-2 and -9 than the low SRF expression group (9 cases) (Fig. 1B).

*Overexpression of SRF in HLE cells induced MMP-2 and -9 gene expression.* Based on the above results, we attempted to determine whether SRF increases MMPs production in SRF-transfected HLE cells. Using RT-PCR, we first analyzed mRNA levels of MMP-2 and -9 in SRF-transfected HLE cells. According to the results, expression of MMP-2, -9 mRNA showed a marked increase in SRF-transfected HLE cells, when compared with normal control cells (Fig. 2). Next, we analyzed the protein levels of MMP-2, -9 in SRF-transfected HLE cells by Western blotting. Overexpression of SRF in HLE cells resulted in markedly increased expression of MMP-2 and -9 (including pro-, latent-, active form), when compared with those of the control. Representative SRF, MMP-2, and -9 protein expressions are shown in Fig. 3. In SRF-transfected
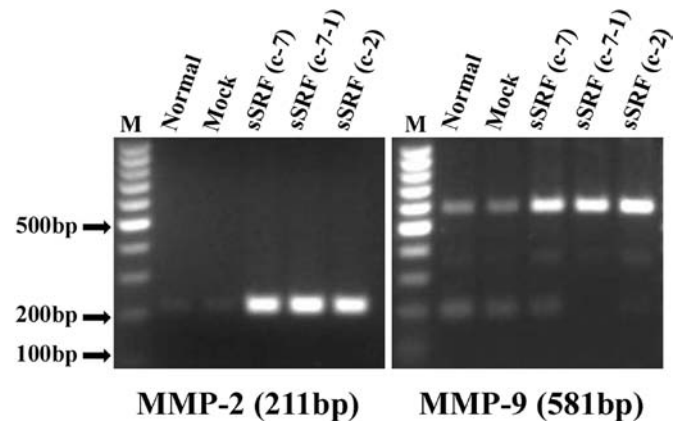
HLE cells, SRF, the active form of MMP-2 and -9 was increased by 3.8-, 2.3-, and 2.2-fold, respectively (Fig. 3), when compared with those of control cells.

*Enzyme activity of MMP-2 and -9 in HLE cells transfected with the SRF gene.* Overexpression of SRF led to a significant increase in MMP-2, -9 enzyme activities at 0.1, 1, 5, and 10% serum after serum starvation, compared with normal controls, respectively (Fig. 4). This result indicated that transfection of SRF cDNA in HCC cell induces activation of MMP-2 and -9. Taken together, these results indicated that SRF could induce an increase in expression of MMP-2 and -9 and also induce activation of these enzymes in HCC cells.

*Overexpression of SRF stimulated cell migration.* Incubation of SRF overexpressing HLE cells with 1, 5, and 10% serum resulted in significant dose-dependent increases in cell migration, respectively (Fig. 5A). Overexpression of SRF in HLE cells led to significantly increased cell migration by 1.5- and 3.1-fold, when compared with those of the control at 12 and 24 h, respectively (Fig. 5B).

**Discussion**

Incidence of hepatocellular carcinoma (HCC) is increasing in many countries and is becoming one of the most common lethal tumors worldwide (1,2). Although significant progress has been made in early detection, the overall outcome for patients with advanced or metastatic HCC remains poor (3). The morbidity and mortality experienced by HCC patients is due mainly to invasion and metastasis of the primary tumor. MMPs are up-regulated in almost every type of human cancer, including HCC, and their expression is associated with cancer cell invasion and metastasis (16-19). Recent studies have implicated expression of SRF in carcinogenesis and cancer progression in several cancers (12,13,20-24). However, the relationship between expression of SRF and MMPs has not been examined, forming the basis for this study.

　　This study demonstrated the following for the first time: a) expression of SRF, MMP-2, and -9 protein was significantly elevated in HCC tissues when compared with corresponding non-tumor tissues, b) expression of SRF showed correlation with
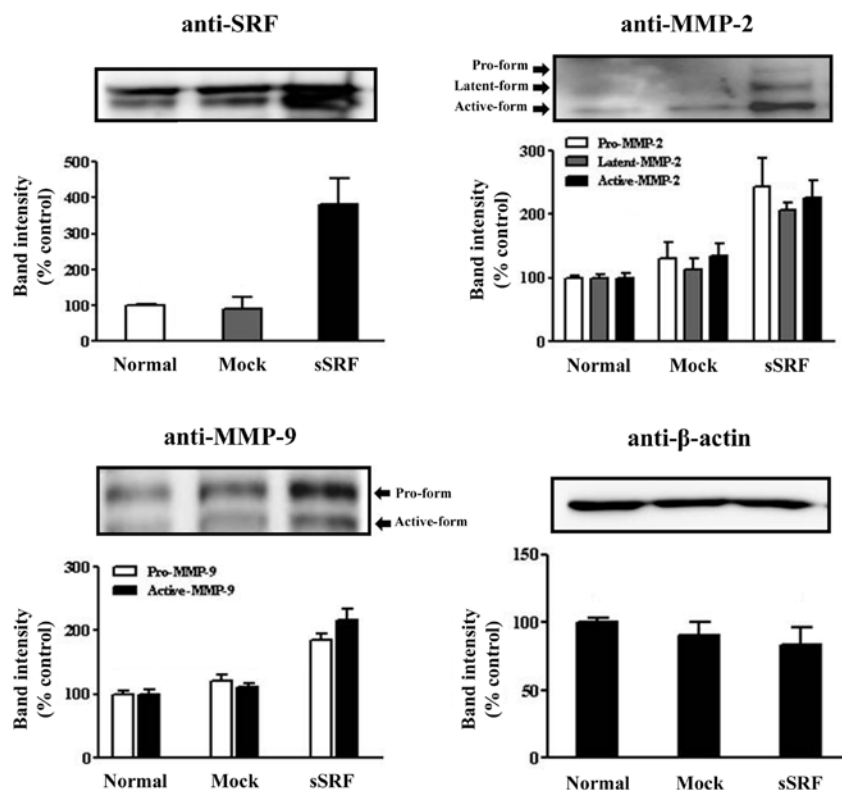
Figure 3. Enhanced expression of SRF, MMP-2, and -9 in SRF-transfected HLE cells. In SRF-transfected HLE cells, SRF, active form of MMP-2 and -9 were increased by 3.8-, 2.3-, and 2.2-fold, respectively.

MMP-2 and -9 expression in HCC tissues, c) overexpression of SRF led to increased expression levels of mRNA and protein of MMP-2 and -9 in HLE cells, d) overexpression of SRF also induced an increase in MMP-2, -9 enzyme activities and expression levels of the active form of MMP-2 and -9. In addition, overexpression of SRF resulted in enhanced HLE cell motility. These findings clearly indicate the important role of SRF in HCC cell migration through up-regulation of MMP-2 and -9 in HCC.

Accumulating evidence has suggested that SRF plays multiple roles in carcinogenesis and metastasis in various cancers (12,13,20-24). In this study, we found that expression of SRF is up-regulated in HCC tissues when compared with that of non-tumor tissues. We also found that overexpression of SRF resulted in enhanced HLE cell motility. We have previously demonstrated that expression of SRF in meta-static colorectal carcinoma cells is significantly higher than that of primary tumors (20). We have shown that expression of SRF correlates with tumor cell de-differentiation (23) in HCC and forced overexpression of SRF in HCC cells results in significantly increased cell growth and proliferation (13). Farra *et al* recently determined that SRF depletion by a siRNA affects expansion of highly and poorly differentiated HCC cell lines (22). Expression of SRF was associated with cell proliferation and poor patient survival in cholangiocarcinoma (24). Overexpression of SRF led to significantly enhanced cell migration and invasiveness of HCC cells through altered expression of mesenchymal markers and proteins involved in the Wnt/β-catenin pathway. In addition, inhibition of SRF expression in poorly differentiated HCC cells by SRF anti-sense cDNA resulted in significantly decreased migration

and invasion (13). Taken together, our data suggest that SRF expression may act as a tumor promoter and appears to be necessary for tumor progression in various cancers, including HCC.

MMPs are a major group of proteolytic enzymes that regulate cell-matrix composition (14-17). Considerable evidence exists on the role of MMPs in normal and pathologic conditions, including embryogenesis, inflammation, and disorders of the immune system, cardiovascular disease, and cancer. MMP-2 and MMP-9 degrade the extracellular matrix components of the basement membrane, removing tissue boundaries and facilitating cancer cell motility (14-17). MMP-2 and -9 have been implicated in migration and invasion of HCC cancer cells (18,19). In this study, we found that overexpression of SRF increased production of mRNA of MMP-2 and -9 in HLE cells. Furthermore, overexpression of SRF also resulted in increased MMP-2, -9 enzyme activities and in the expression levels of the active form of MMP-2 and -9. This is in agreement with a previous study demonstrating that increased expression of SRF in pulmonary lymphangioleiomyomatosis (LAM) and overexpression of SRF up-regulates expression of MMP-2 and -14 in mouse lung fibroblasts (25). Zhe *et al* have shown that overexpression of SRF in human lung fibroblasts up-regulates urokinase-type plasminogen activator and finally increases MMP activities (26). They have concluded that SRF plays an important role in regulation of the plasminogen/MMP system, which allows degradation of extracellular matrix by LAM cells, consequently promoting destruction of lung parenchyma and expansion of lesions (26).

The mechanism whereby SRF up-regulates MMP-2 and -9 is currently unknown. SRF is a transcription factor, which binds
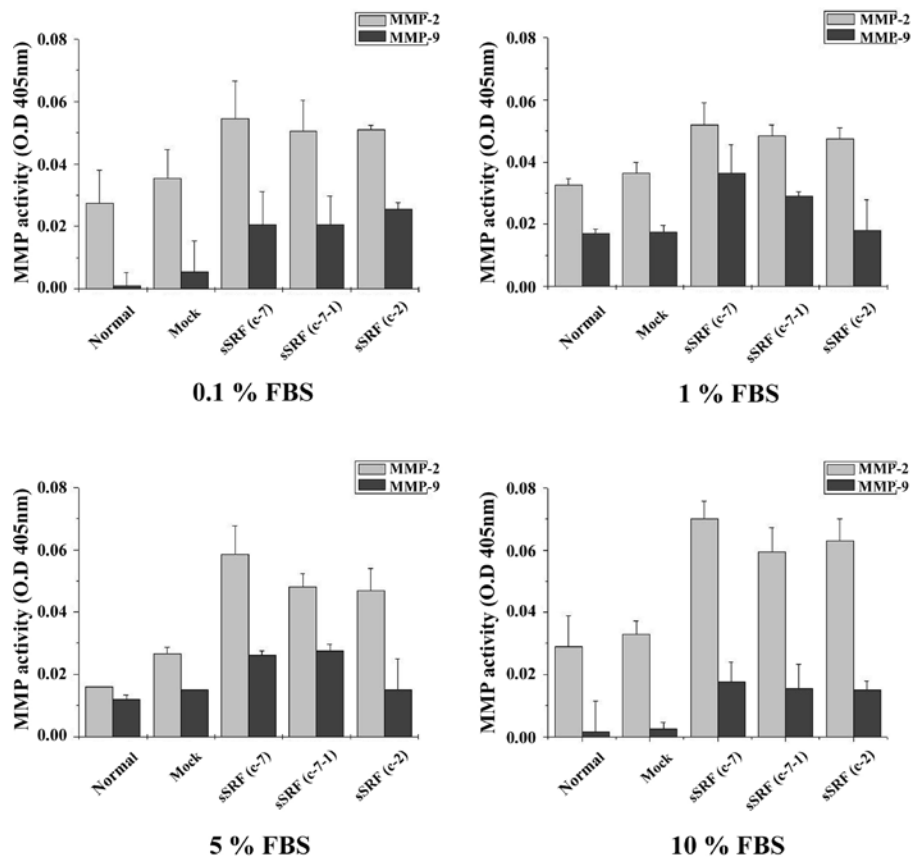
Figure 4. Enzyme activity of MMP-2 and -9 in SRF-transfected HLE cells. Overexpression of SRF in HLE cells led to increased levels of enzyme activity of MMP-2 and -9 at 0.1, 1, 5, and 10% serum when compared with those of normal controls, respectively.
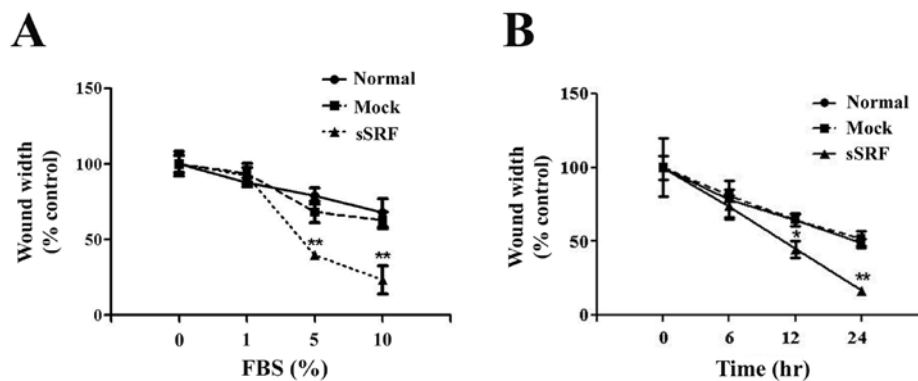


Figure 5. (A) Overexpression of SRF in HLE cells effectively increased cell migration by 2- and 3-fold when compared with those of the control at a concentration range of 5 and 10% serum for 24 h, respectively (**P<0.01). (B) Overexpression of SRF in HLE cells resulted in significantly increased cell migration by 1.5- and 3.1-fold when compared with those of the control at 12 and 24 h, respectively (*P<0.05; **P<0.01). The experiment was independently repeated three times.

to an SRE associated with immediate early genes, such as *c-Fos, FosB, Jun,* and *Egr-1* and *-2*, and controls cellular activities by regulation of these immediate early genes (8-10). Most MMPs respond to stimuli at the transcription level and are downstream targets of immediate-early genes, such as *c-Fos* and *Jun*, which are induced within minutes of cell stimulation and in the absence of new protein synthesis (16,27). In papillary carcinoma of the thyroid, expression of SRF was up-regulated and it enhanced expression of the SRF target gene, *c-Fos*, which was associated with tumor progression and metastasis (21). Activator protein-1

(AP-1) complexes are heterodimers of proteins of two proto-oncogene (*Fos* and *Jun* gene) is found at ~70 bp in the promoter region of inducible MMP gene and is implicated in induction of MMPs (16,28). Therefore, it is possible that SRF could control MMP expression by regulating expression of immediate early genes, including Fos and Jun genes. Although it is not clear whether overexpression of SRF itself or regulation of one or more of its target genes is responsible for activation of MMPs, the above findings indicate a critical role of SRF in modulation of the MMPs pathway in HCC.

In conclusion, our study indicates that expression of SRF is closely related to regulation of the MMP system and that it may be one of the mechanisms for promotion of migration and invasion in human HCC cells.

## Acknowledgements

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