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Role of c-Src inhibitor in the regulation of hepatocarcinoma cell migration

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It has been discovered that hepatocellular carcinoma (HCC) has high ability of migration and angiogenesis. This study aimed to explore the mechanism of HCC cell migration and angiogenesis. BEL-7402 cell line was used as HCC cell model for investigating the regulation of cell migration upon c-Src inhibitors (PP2 and SU6656) treatment. Western blot was used for detecting the expression of MT1-MMP and VEGF-C. The activity of MMP2 and MMP9 was monitored with gelatin zymography assay. BEL-7402 cell migration and invasion was detected by wound healing assay and Transwell. Immunoprecipitation was used for detecting the interaction among c-Src, pro-MT1-MMP, Furin and VEGF-C. Our results have show that the expression of MT1-MMP and VEGF-C were inhibited by PP2 and SU6656, in accordance with c-Src activity. Zymography assay demonstrated that the activity of MMP2 and MMP9 decreased upon PP2 or SU6656 treatment. The invasion and migration of BEL-7402 were inhibited. We also found that c-Src interacted with Furin *in vivo*. The interaction between Furin and its substrates pro-MT1-MMP、pro-VEGF-C decreased upon c-Src inhibitors treatment. These findings indicate that the activity of c-Src inhibition associated with cell invasion and migration decreased by down-regulating the interaction between Furin and its substrates (pro-MT1-MMP, pro-VEGF-C).

Key words: Hepatocellular carcinoma (HCC), Furin, c-Src inhibitor, MT1-MMP, VEGF-C, cell migration.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the leading cause of cancer- related death in the world, which has a high potential ability of metastasis in tumor progression (GLOBOCAN, 2008). However, the underlying mechanisms of HCC initiation, progression and metastasis are still not fully understood (Tang et al., 2010). Cell migration related protein MT1-MMP, MMP2, VEGF must be cleaved by protein convertase, followed by maturation and activation.

Furin is the best-characterized representative of the mammalian subtilisin-like family of proprotein convertase. It is synthesized as inactive proenzyme and rapidly matured by autocatalytic cleavage between the prodomain and the catalytic domain in endoplasmic reticulum (ER) (Vey et al., 1994). Following this initial cleavage, the propeptide-Furin complex leaves the ER and enters the

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trans-golgi network TGN) for its second cleavage (Anderson et al., 1997). Hence, Furin becomes active to process substrate molecules in multiple compartments in the TGN/endosomal system (Molloy et al., 1994). Many protein precursors such as matrix metalloproteases, hormones, growth factors, serum proteins, receptors, and adhesion molecules have identified the Furin substrates (Fujisawa et al., 2004; Louagie et al., 2008; Yana and Weiss, 2000). MT1-MMP proenzyme cleavage by Furin is considered to be a principal event in the activation of this substrate and it may be important in HCC cell migration and invasion (Dangi-Garimella et al., 2011).

Furin activation plays a vital role in tumor process (Lopez de Cicco et al., 2005). Furin inhibitor α1-PDX has been used to block Furin activity and to prevent cancer metastasis in biochemical, cellular and animal studies (Molloy and Thomas, 2001). As the most closely related members of the Src family of nonreceptor tyrosine kinases, it up-regulates c-Src correlates with a variety of human tumors, including cancer of the HCC (Hilbig, 2008; Ischenko et al., 2007). c-Src and Furin have been found to be upregulated in human cancer, but the ubiquitous c-Src participating in the interaction between Furin and substrates still remains unknown.

Stawowy et al. (2002) have demonstrated that Furin-like proprotein convertase PC5 was strongly upregulated by platelet derived growth factor-BB (PDGF-BB) through PI3-kinase/p70s6-kinase pathway (Philipp et al., 2002). A similar mechanism may also apply to the convertase Furin. We then investigate whether Furin is regulated by PDGF-BB through c-Src kinase and how Furin activity is controlled to mediate the processing of its substrate MT1-MMP and VEGF-C.

In this study, we detected the protein level and the interaction between Furin and its substrates stimulated with PDGF-BB 30 min after pretreatment with c-Src inhibitor PP2 or SU6656 in HCC cells. In this short article, we will attempt to understand the new pathogenesis that activation of Furin is c-Src dependent in HCC cells and has a promising strategy against HCC metastasis.

MATERIALS AND METHODS

Cell culture and experimental reagents

HCC cell lines BEL-7402 were cultured in RPMI 1640 *invitrogen* supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in a 5% CO_2 humidified atmosphere at 37℃. In some experiments, cells were cultured in serum-free medium wherever indicated. PDGF-BB (20 ng/ml), research and development (R&D, USA) or c-Src inhibitors PP2 or SU6656 (10 μM) were added to the medium whenever necessary as indicated in the figure legend.

Primary antibodies against pSrcY416, Furin, MT1-MMP, VEGF-C and β-actin were purchased from Santa Cruz Biotechnology Santa Cruz, USA. Gelatin Zymography kit Millipore (USA), 4-amino-5-4-chlorophenyl)-7-t-butyl) pyrazolo (3,4-d) pyrimidine PP2) and PDGF-BB were purchased from Enzo Life Sciences International, USA; SU6656 Sigma (USA).

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer of 50 mM Tris with pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, 5 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaF, and 1 mM Na_3VO_4 containing protease inhibitor cocktail for 30 minutes at 4℃. All cell lysates (16,000 g) were centrifuged at 4°C for 30 min. The protein concentration was determined with the pierce bicinchoninic acid (BCA) method (USA). Aliquots of cell lysates were fractionated by electrophoresis in sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE): 8% for the analysis of Furin and c-Src, 10% for the analysis of MT1-MMP and VEGF-C. Total proteins were electroblotted to polyvinylidene fluoride (PVDF) membrane using a wet transblot system Bio-Rad, Hercules (CA). Blots were then blocked for 1 h at room temperature with 10% bovine serum albumin (BSA) or 5% nonfat dry milk. Membranes were incubated overnight at 4°C with antibodies against $pSrc^{Y416}$, Furin, MT1-MMP, VEGF-C and β-actin (1:1000). After subsequent washing, the membranes were incubated for 1 h with horseradish peroxidase conjugate of goat anti-rabbit or anti-mouse second antibody, diluted in 1:5,000 phosphate buffered saline with Tween (PBST). After washing, the membrane was processed using Super Signal West Pico chemiluminescent substrate Pierce (USA), followed by exposure to Fujifilm LAS3000 Imager Fuji (Japan). Densitometric analysis was performed with Image J densitometer using the software Excel.

Co-immunoprecipitation

BEL-7402 cells (10,000 g) were washed twice with ice-cold PBS, lysed in 1 ml of RIPA buffer for 30 min on ice, clarified by centrifugation at 4°C, and then the supernatant was subjected to immunoblot or immunoprecipitation. Each cell lysate (500 μg) was incubated with 2 μg appropriate antibody anti-c-Src or anti-Furin overnight at 4°C. 50 μl of protein G was added and mixed at 4°C for 2 h with gentle agitation. The pellet was washed three times with RIPA buffer, boiled with 50 μl 2×loading buffer Tris with pH 6.8, 0.1% SDS, 10% glycerol, 0.025% Bromophenol blue, 20 mM 1,4-dithiothreitol (DTT| for 5 min prior to gel loading. Proteins were detected by Western blot with anti-Furin, c-Src, MT1-MMP and VEGF-C. Some experiments substituted the secondary antibody with Clean-Blot IP Detection Reagent for clear IP/Western blot results.

Gelatin zymography

Levels of the active and latent forms of MMP-2, MMP-9 were analyzed by gelatin zymography as described in kit. BEl-7402 cells were washed with ice-cold PBS and lysed with RIPA buffer for 30 min on ice. Mixtures (12,000 g) were centrifuged at 4°C for 20 min. The supernatant was aliquoted and protein content was determined using BCA Protein Assay Reagent Pierce. After electrophoresis and washing, the gel was incubated at 37°C for 24 h, stained with Coomassie brilliant blue R250 and destained.

Wound healing assay

BEL-7402 cells were plated into 24-well plates and grown to confluence. The monolayer was artificially wounded using the tip of a sterile 200 μl pipette. Cell debris was removed by washing with PBS. The cells were then incubated with c-Src inhibitors (10 μM PP2 or SU6656) at the appropriate time. The cells migrated into the wounded areas were photographed. Wound closure was photographed at the indicated times in the same spot with an

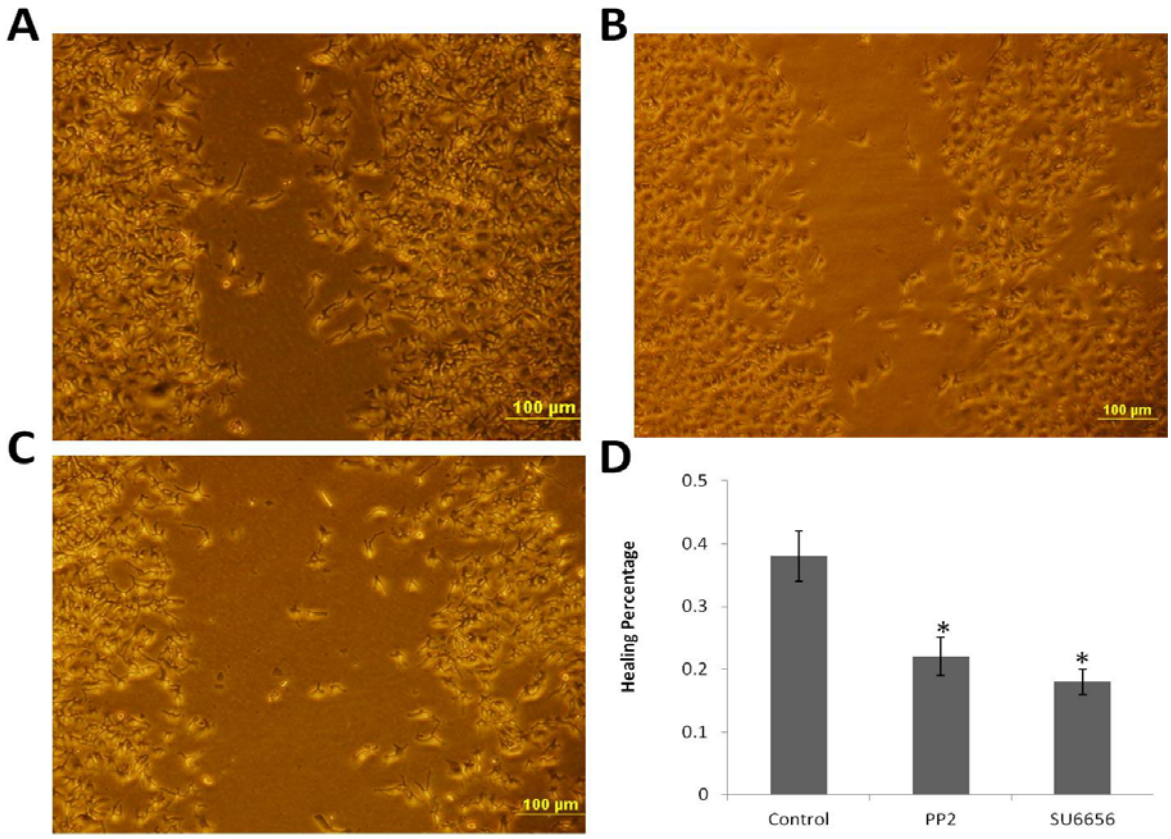

Figure 1. Effect of c-Src inhibitor on the BEL-7402 migration. Confluent 90% BEL-7402 were wounded by 200 μl sterile pipette and then treated with c-Src inhibitors for 48 h. The cells migrated into the wounded areas were photographed. Wound closure was photographed at the indicated times at the same spot with an inverted microscope equipped with a digital camera. The extent of healing was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area. **A.** Control group; **B.** PP2 treatment for 48 h; **C.** SU6656 treatment for 48 h; **D.** The graph represents the mean±S.E. of at least three independent experiments.

inverted microscope equipped with a digital camera. The extent of healing was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area.

Transwell invasion assay

Matrigel Invasion Chambers were hydrated for 4 h before starting the invasion assay. Log-phase cells (4×10^4) were plated in 200 μl completed RPMI 1640 containing 10% FBS in the upper chamber of the transwell, and the lower chamber was filled with 500 μl completed RPMI 1640 containing 10% FBS. After incubation for 2 h, the cells were treated with PP2 or SU6656 as previously described for 24 h. The cells were allowed to migrate for 10 h at 37°C and 5% CO_2, followed by carrying out the invasion assay. The cells were fixed for 15 min at room temperature by replacing the culture medium in the bottom and top of the chamber with 4% formaldehyde dissolved in PBS. The cells that remained on the bottom of the chamber were stained with 0.1% crystal violet; the migrated clones were photographed under an optical microscope. The cell number was counted at 12 different areas. Data were averaged from three parallel experiments, which were normalized to that of the control.

Statistical analysis

Western blots were quantified by measuring the relative density of protein bands recognized by a particular antibody using Image J software NIH (USA). The results were expressed as mean ± standard deviation SD). Statistical analysis was done with Student's t-test for comparison of two groups; differences with $P < 0.05$ were considered statistically significant.

RESULTS

Effects of c-Src inhibitors on the invasion and migration of BEL-7402 cells

In order to detect whether c-Src activity regulated the invasion and migration of BEL-7402 cells, we did wound healing assay and Transwell assay. As expected, c-Src inhibitors have obvious roles in modulation of BEL-7402 cells invasion and migration. The ability of invasion and migration of the cells treated with PP2 or SU6656 de-creased significantly compared with the control (Figure 1).

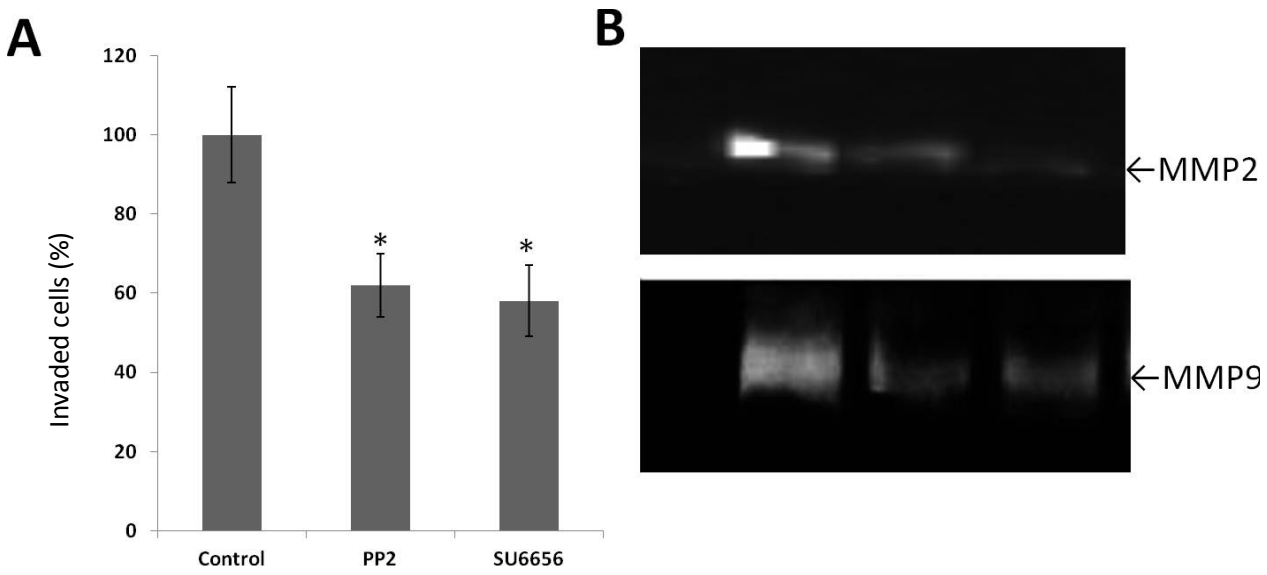

Figure 2. Effect of c-Src inhibitor on the ability of BEL-7402 cell invation through down-regulation of the activity of MMP2 and MMP9 in BEL-7402 cells. BEL-7402 cells treated with 10 μmol/L PP2 or SU6656 for 48 h, cell invaded ability was detected by Transwell assay. **A.** The invade cells were counted for analysis. The results shown are representative of at least three independent experiments. At the same time, the activity of MMP2 and MMP9 were detected with Zamograph assay **(B).**

Effects of c-Src inhibitors on the expression of Furin and its substrates in BEL-7402 cells

BEL-7402 cells were treated with 10 μM PP2 or SU6656 for 24 h; the expression of pc-SrcY416, MT1-MMP, VEGF-C was detected by immunoblot. Upon inhibition with PP2 or SU6656, a quantitative decrease in the pSrcY416 band intensities was observed (Figure 2A). Especially, the level of MT1-MMP, VEGF-C was also massively decreased, whereas Furin showed no obvious variation (Figure 2A). It seems that down-regulation expression of Furin substrates is in accordance with c-Src activity.

Direct binding of c-Src to Furin *in vivo*

It is not clear whether binding between c-Src and Furin may exist and how Furin interacts with its substrates in BEL-7402 cells. We then did co-immunoprecipitation of c-Src and Furin to see if c-Src is directly associated with Furin in BEL-7402 cells. As shown in Figure 3, we found that endogenous significant amounts of c-Src and Furin were specifically immunoprecipitated with the counterpart antibody for Furin and c-Src in BEL-7402 cells, respectively. The results suggest that endogenous c-Src may physically associate with Furin *in vivo*.

c-Src activity as a requirement for efficient association between Furin and its substrates

To explore the possible mechanism of the modulation of Furin interaction with its substrates, we also analyzed the maturation of MT1-MMP or VEGF-C by Furin through co-ip experiments. Serum free BEL-7402 cells were treated as previously described. They were stimulated with 20 ng/ml PDGF-BB for 30 min or pretreated with 10 μM PP2 or SU6656; and followed by stimulation with PDGF-BB. The whole cell lysates were immunoprecipitated with anti-Furin antibody, and then detected with anti-MT1-MMP or VEGF-C antibody. Our results show that MT1-MMP can be found only in PDGF-BB stimulation group, and there was almost no band detection in SU6656 treatment group. Similar results have been found in VEGF-C detection (Figure 4). This seems that c-Src activity is necessary for efficient interaction between Furin and its substrates.

DISCUSSION

Furin has a crucial role in the progress of tumor (Cheng, 1997; Bassi et al., 2001). It may constitute a marker for tumor progression and could contribute to the prediction of the outcome of this disease (Page et al., 2007). It is a Ca^{2+}-dependent cellular endoprotease that activates a large number of precursor proteins in secretory pathway compartments (Thomas, 2002). Inhibition of Furin activity decreases substrate activation, proliferation rate and invasive potential of cancer cells, suggesting that it is a potentially useful target for therapeutics (Bassi et al., 2003).

Our present study first found that c-Src activity may directly regulate the HCC cell invasion and migration by modulating the maturation of MT1-MMP/VEGF-C. In our

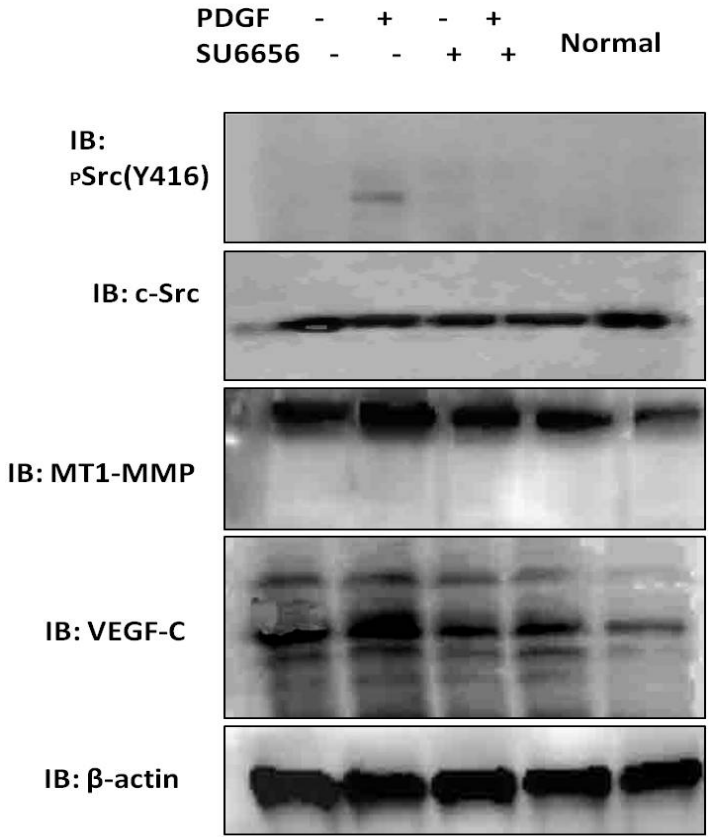

Figure 3. Expression of MT1-MMP and VEGF-C which were inhibited by c-Src inhibitor in BEL-7402 cells. BEL-7402 cells were made to undergo serum starvation for overnight, followed by incubation with 20 ng/mL PDGF-BB for 30 min. Some groups were pre-treated with SU6656 for 30 min then treated with PDGF-BB stimulation. Expression of p-c-Src, MT1-MMP and VEGF-C were detected with western blot. Protein loading control was monitored with β-actin blot.

study, we first found that the ability of BEL-7402 cells invasion and migration decreased upon PP2/SU6656 treatment. To explore the mechanism, we then detected the effects of c-Src inhibitors on the protein expression of MT1-MMP or VEGF-C in BEL-7402 cells. Results have shown that MT1-MMP or VEGF-C decreased significantly in accordance with c-Src activity, but expression of Furin had no obvious variation. The results indicate that regulation of MT1-MMP or VEGF-C does not depend on the down-regulation of Furin, another mechanism may exist.

We then hypothesized that c-Src may directly interact with Furin *in vivo* in BEL-7402 cells. Co-IP assay demonstrated that c-Src binds with Furin *in vivo*. This interaction may have a potential role in regulation of Furin activity in maturation of its substrates. So it is necessary to detect the effects of c-Src on the interaction between Furin and its substrates. MT1-MMP and VEGF-C have played a vital role in regulation of cancer cell invasion and migration. Upregulation of MT1-MMP can effectively cause elevated invasiveness in human cancer cell (Tolde et al., 2010; Seiki, 2003; Zucker et al., 2003). To be active, the zymogen of MT1-MMP or VEGF must be cleaved to the pro-peptide by protein convertase Furin.

Our results show that activation of c-Src with PDGF-BB was found to enhance the formation of the complex between Furin and pro-MT1-MMP, but SU6656 treatment resulted in the reversion of the interaction. That is, c-Src activity is required for efficient association between Furin and its substrate pro-MT1-MMP. Similar results were seen in interaction between Furin and VEGF-C.

The platelet derived growth factor, PDGF-BB recognize both PDGFR-α and PDGFR-β subunits (Hart et al., 1988). PDGFR activation occurs through dimerization and phosphorylation of tyrosine residues in its intracellular domain, thus leading to activation of intracellular signaling, particularly of c-Src (Choudhury et al., 2006). We also found that PDGF-BB, through the activation of c-Src, regulates its own converting enzyme, Furin, creating a unique regulation loop of potential importance in a variety of cell fate and functions (Blanchette et al., 2001).

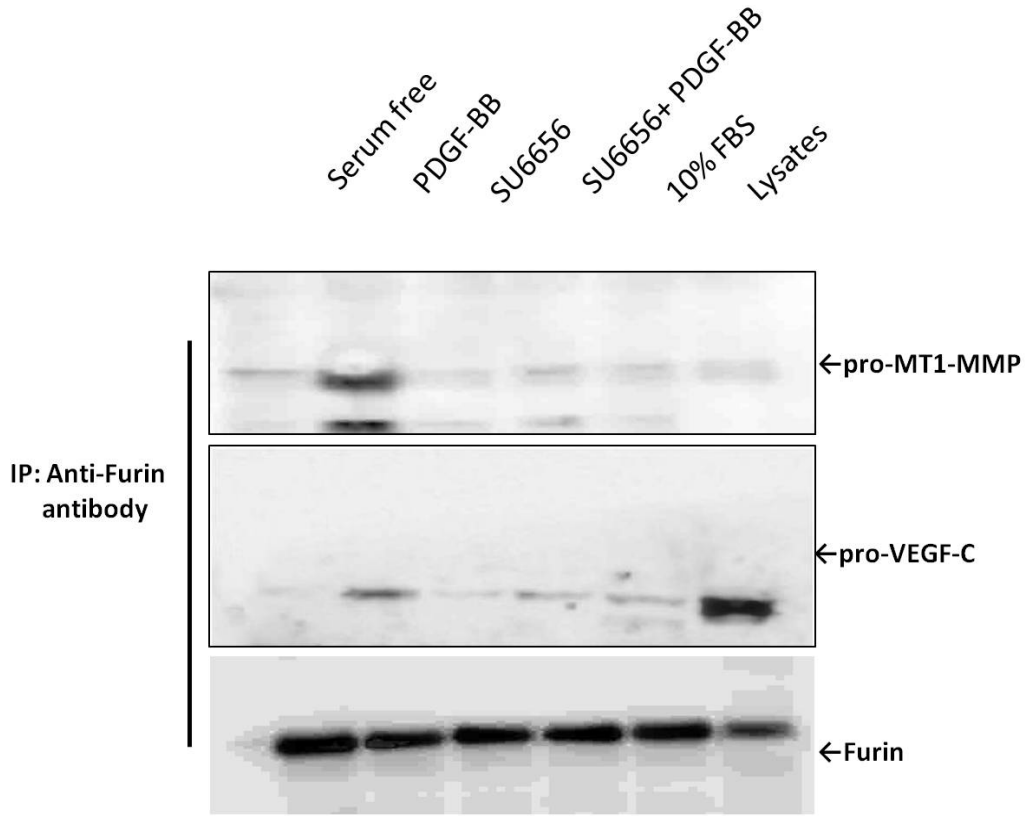

Figure 4. Modulation of fusin interaction with its substrates by PDGF-BB and c-Src inhibitor in BEL-7402 cells. BEL-7402 cells were made to undergo serum starvation for overnight, then treated with PDGF-BB and SU6656 as previous description. Whole cell protein was collected and 2 μg anti-Furin antibody was used for immunoprecipitated for overnight. The immunoprecipitates were used for detection of the expression of pre-MT1-MMP or pre-VEGF-C by specifical antibody

In conclusion, we examined the role of c-Src in the process of Furin breaking down its substrates. The potential inhibitor to block Furin and subsequent processing activity are more attractive therapeutic agents for HCC cancer.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENT

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