Hepatoma-derived growth factor stimulates podosome rosettes formation in NIH/3T3 cells through the activation of phosphatidylinositol 3-kinase/Akt pathway

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ABSTRACT

Hepatoma-derived growth factor (HDGF) stimulates the migration, invasion and metastasis in several types of cancer cells. However, the mechanism underlying HDGF-stimulated migration remains unclear. In this study, we investigated the influence of HDGF on cytoskeleton remodeling and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in non-transformed NIH/3T3 cells. Exogenous HDGF promoted the migration and the formation of dorsal ruffles and podosome rosettes. Besides, HDGF supply increased the PI3K expression and Akt phosphorylation in dose- and time-dependent manners. Application of LY294002, a PI3K inhibitor, attenuated the HDGF-induced migration, dorsal ruffles and podosome rosettes formation. Consistently, the HDGF-overexpressing NIH/3T3 transfectants exhibited significantly increased motility and elevated PI3K/Akt activities, which were repressed by LY294002 or adenovirus-mediated overexpression of endogenous PI3K antagonist, PTEN. In summary, HDGF elicits the activation of PI3K/Akt signaling cascade, thereby promoting cytoskeleton remodeling to stimulate cellular migration.

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1. Introduction

Cell migration is a crucial and intricate process that is involved in physiological and pathological processes such as angiogenesis and metastasis [1,2]. Class I PI3K enzymes involve in intracellular signal transduction and mediate numerous cellular processes, such as cell proliferation and migration. Phosphatidylinositol- 3,4,5-tris-phosphate (PIP3), a lipid second messenger generated by the PI3K, triggers various cellular signaling including the activation of Akt signaling. The PI3K/Akt signaling recruits guanine nucleotide exchange factors (GEFs) to activate Rho GTPases, such as Rho, Rac and Cdc42 [3,4], which subsequently elicit the cytoskeletal rear-rangements and cellular adhesion, thereby stimulating cell migration [5]. The alteration of PI3K signaling frequently leads to the dysfunction of growth arrest and contributes to tumorigenesis and metastasis [6]. The tumor suppressor phosphatase and tensin homolog (PTEN), a cellular antagonist of PI3K/Akt signaling, dephosphorylates PIP3 into PIP2 (phosphatidylinositol-4,5-tris-phosphate) and inhibits PI3K/Akt-mediated cellular processes, such as proliferation and migration. PTEN over-expression has been shown to attenuate the migration and invasion of cancer cells [7]. Conversely, PTEN mutation or deficiency results in hyperactivation of PI3K/Akt pathway, thereby triggering the cellular motility or metastasis [8].

Podosome, a spot-like actin-rich structure, participates in extracellular matrix degradation and cell migration [9]. In the physiological settings, podosomes are found in macrophages and immature dendritic cells [10], and can be induced in non-neoplastic fibroblasts cells after stimulation by growth factors such as epithelial growth factor (EGF), vascular endothelial growth factors (VEGF), or transforming growth factor β (TGFβ) [11]. Podosomes

are frequently observed in some highly metastatic cells, such as melanoma and breast cancer cells [12].

Hepatoma-derived growth factor (HDGF), a nuclear targeting mitogen [13], possesses proliferative activity for fibroblasts [14] and hepatocytes [15]. Recent studies indicate that HDGF over-expression is correlated with the onset of various types of tumor cells, such as hepatocellular carcinoma [16], gastric carcinoma [17] and glioblastoma multiforme [18]. Although HDGF has been characterized to be a migratory inducer in smooth muscle cell [19], lung cancer cell [20] and breast cancer cells [21], the molecular signaling underlying HDGF-stimulated cell migration remains unclear.

In the present study, we evaluated the influence of HDGF on cytoskeletal reorganization and podosome rosettes formation in non-transformed NIH/3T3 cells. Subsequently, we elucidated the role of HDGF on PI3K/Akt signaling by genetic manipulation and pharmaceutical approaches.

2. Materials and methods

2.1. Generation of HDGF expression vector, protein and stable HDGF-expressing cell lines

HDGF expression vector and recombinant HDGF protein were generated as previously described [16]. To generate stable HDGF-expressing cells, NIH/3T3 cells were transfected with phCMV3-HDGF using lipofectamine (Invitrogen) and the stable transfectants were selected by medium containing G418 (750 μg/ml; BD Bioscience).

2.2. Cell culture and HDGF treatment

Mouse embryonic fibroblast NIH/3T3 cells were obtained from American Type Cell Collection (Manassas, VA, USA) and maintained in DMEM medium supplemented with 10% calf serum (CS) in humidified atmosphere containing 5% CO_2. To observe the formation of dorsal ruffles and podosome rosettes, NIH/3T3 cells were cultured in DMEM medium containing 0.2% CS for 16 h, and then treated with recombinant HDGF at various doses (0.1–500 ng/ml) for different time intervals.

2.3. Migration assay

The migration assay was analyzed by using a Boyden chamber inserted with polycarbonate membrane (8.0 μm pore size; Neuro Probe, Inc.) [21]. NIH/3T3 cells (3×10^5 cells/ml) were treated with various doses of HDGF in DMEM medium and placed in the upper chamber (50 μl per well). The lower chambers were filled with the DMEM medium containing 10% CS (30 μl per well). After incubation for 6 h, the cells traversed the membrane were fixed, stained and counted in three different fields of magnification under a microscope. To investigate the effect of PI3K inhibition on cell migration, LY294002 (10 μM) was applied in the upper chambers.

2.4. Scratch wound healing assay

HDGF-mediated scratch migration in NIH/3T3 cells was assessed as previously described [22]. To evaluate the effect of LY294002 on HDGF-mediated cell migration, cells were pre-treated with LY294002 (10 μM) for 10 min then cultured in DMEM medium containing 0.2% CS and HDGF (100 ng/ml) for 48 h. The healing of the wound gap was determined at the different time intervals by microscopic analysis.

2.5. Analysis of dorsal ruffles and podosome rossettes

To analyze dorsal ruffles formation, actin staining was performed by using Alexa Fluor 488-phalloidin (Invitrogen) and counted under fluorescent microscope from 200 cells [23]. Briefly, NIH/3T3 cells grown on glass coverslips were supplemented in DMEM containing 0.2% CS for 16 h, then treated with HDGF of indicated doses for additional 6 h. The cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and blocked with PBS containing 2% BSA and 10% CS. The formation of podosome rosettes was determined as previously described [24]. Briefly, the podosome rosettes of NIH/3T3 cells seeded on coverslips coated with collagen I (50 μg/ml; Roche) were analyzed using immunofluorescence staining with anti-cortactin antibody (Santa Cruz Biotechnology) and Alexa Fluor 488-phalloidin by confocal microscope (LSM 510, Zeiss).

2.6. Generation of adenovirus vectors

The E1-, E3-defective, recombinant adenoviruses encoding green fluorescent protein (Ad-GFP) and PTEN (Ad-PTEN) were generated as previously described [25,26]. For adenoviral gene delivery, NIH/3T3 cells were infected at a multiplicity of infection (MOI) of 500 and 2000 for 48 h prior the cells were harvested for subsequent assays.

2.7. Western blot analysis

Western blot analysis was performed as previously described [22]. Blots were probed with the appropriate antibodies and developed using ECL kit (Pierce). Antibody against HDGF was generated as previously described [16]. Antibodies against HA, PI3K p110, PI3K p85, phospho-Akt, and Akt were from Santa Cruz Biotechnology.

2.8. Statistical methods

Data were expressed as mean ± SEM and statistically analyzed using paired Student's t-tests. A P value of less than 0.05 was considered significant.

3. Results

3.1. HDGF stimulates the migration, actin reorganization, podosomes formation in non-transformed NIH/3T3 cells

Although the pro-migratory activities of HDGF have been demonstrated in some tumor cells, it was not clear whether and how HDGF influenced the motility of non-transformed cells. We evaluated the effects of recombinant HDGF on motility in NIH/3T3 cells using Boyden chamber assay. It was found that exogenous HDGF significantly the trans-well migration of NIH/3T3 cells at as low as 10 ng/ml and in a dose-dependent manner (Fig. 1A). In scratch wound assay, HDGF supply (100 ng/ml) significantly enhanced the closure of wound gap at 24 h after protein application (Fig. 1B). Thus, HDGF is a chemotactic factor for NIH/3T3 cells.

We subsequently investigated whether HDGF modulated the actin filament network to promote the migration of NIH/3T3 cells. By staining with Alexa Fluor 488-phalloidin, fluorescent microscopy analysis revealed that exogenous HDGF at 10 ng/ml was sufficient to augment the dorsal ruffles formation. Moreover, such increment was dependent on doses of recombinant HDGF (Fig. 1C; right panel). Because motogenic cytokines stimulates podosomes in NIH/3T3 cells, we investigated whether HDGF
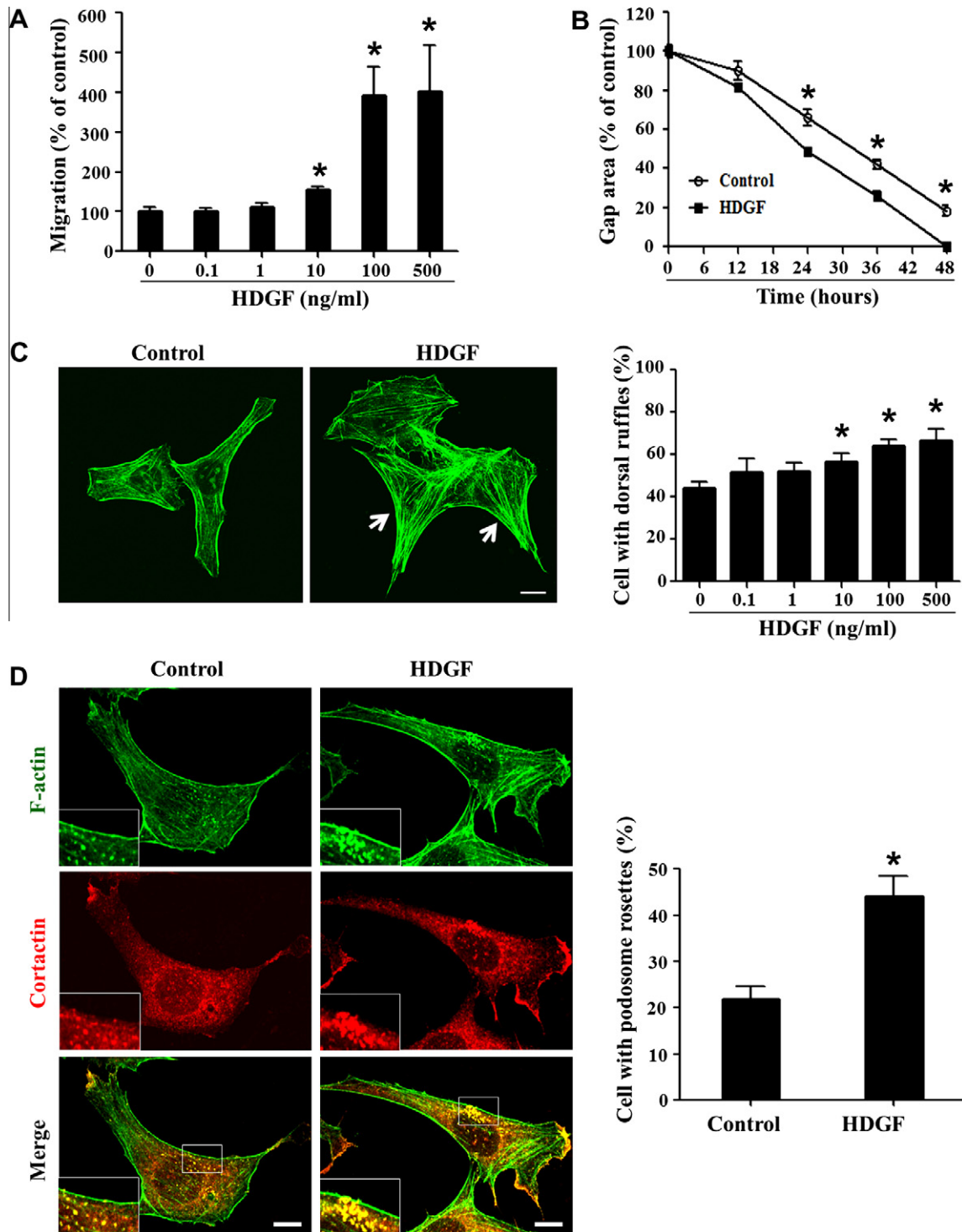
Fig. 1. Effect of HDGF on migration and F-actin cytoskeleton network in NIH/3T3 cells. (A) Dose-dependent effect of HDGF on the trans-well migration of NIH/3T3 cells using Boyden chamber. Data were mean ± SEM of quadruplicates. (B) Effect of HDGF supply (100 ng/ml) on scratch wound healing of NIH/3T3 cells. The percentages of wound healing were determined at the indicated time intervals after injury. (C) Dose-dependent effect of HDGF on dorsal ruffles formation in NIH/3T3 cells. After treatment with HDGF of various doses for 6 h, the dorsal ruffles of NIH3T3 cells were determined by staining with Alexa Fluo 488-phallodin and counted under fluorescent microscope from 200 cells. (left panel) Enhanced F-actin staining and stress fibers-rich structure in HDGF-treated cells (100 ng/ml; indicated by arrows) compared with control. Scale bar: 10 μm. (D) Effect of HDGF (100 ng/ml) on podosome rosettes formation in NIH/3T3 cells. Increased co-localization of F-actin and cortactin was observed in HDGF-treated cells compared with untreated control. Left-corner insets: 2× magnification of the area within the smaller white squares. Scale bar, 10 μm. (Right panel) Statistical analysis. $^*P < 0.05$.

possessed such function to promote the podosome rosettes formation in NIH/3T3 cells. By using Alexa Fluor 488-phalloidin and anti-cortactin double staining, immunofluorescent microscopy studies showed that HDGF supply significantly stimulated the percentages of cells with F-actin- and cortactin-positive podosome rosettes from basal level (21.9 ± 6%) to 44.2 ± 6.2% (Fig. 1D). Together, these results indicate that HDGF not only stimulates the motility, but also modulates the cytoskeletal organization in NIH/3T3 cells.

3.2. HDGF supply induced PI3K up-regulation and Akt activation in NIH/3T3 cells

To elucidate the role of PI3K/Akt pathway in HDGF-mediated cell migration, we examined the influence of HDGF on PI3K complex expression and Akt phosphorylation by immunoblot analysis. It was found that HDGF potently increased the protein levels of PI3K p110 and p85 subunits (Fig. 2A) and enhanced Akt phosphorylation (Fig. 2B) in a dose-dependent manner. Immunoblot analysis was performed to delineate the time required for activation of PI3K/Akt signaling after HDGF treatment. It was shown that HDGF (100 ng/ml) evoked a significantly increased levels of p110 and p85 PI3K subunits (Fig. 2C) and Akt phosphorylation within 1 h (Fig. 2D). Besides, such induction was sustained for at least 24 h. Quantitative RT–PCR analysis revealed that HDGF elicited a significant increment of mRNA levels of PI3K p110 and p85 subunits

within 30 min after application (S1). Thus, HDGF stimulates the PI3K/Akt signaling in NIH/3T3 cells.

3.3. Pharmaceutical inhibition of PI3K/Akt signaling by LY294002 attenuated the HDGF-stimulated migration, dorsal ruffles and podosomes formation

To elucidate whether the activated PI3K/Akt signaling contributed to HDGF-induced cell migration, the motility of HDGF-treated NIH/3T3 cells was evaluated in the absence or presence of PI3K inhibitor, LY294002 using scratch wound assay. It was observed that LY294002 potently abolished the HDGF-stimulated healing of scratch wound in NIH/3T3 cells (Fig. 3A). Besides, we also investigated the influence of LY294002 on HDGF-mediated actin reorganization including dorsal ruffles and podosomes formation. Consistently, LY294002 application significantly perturbed the
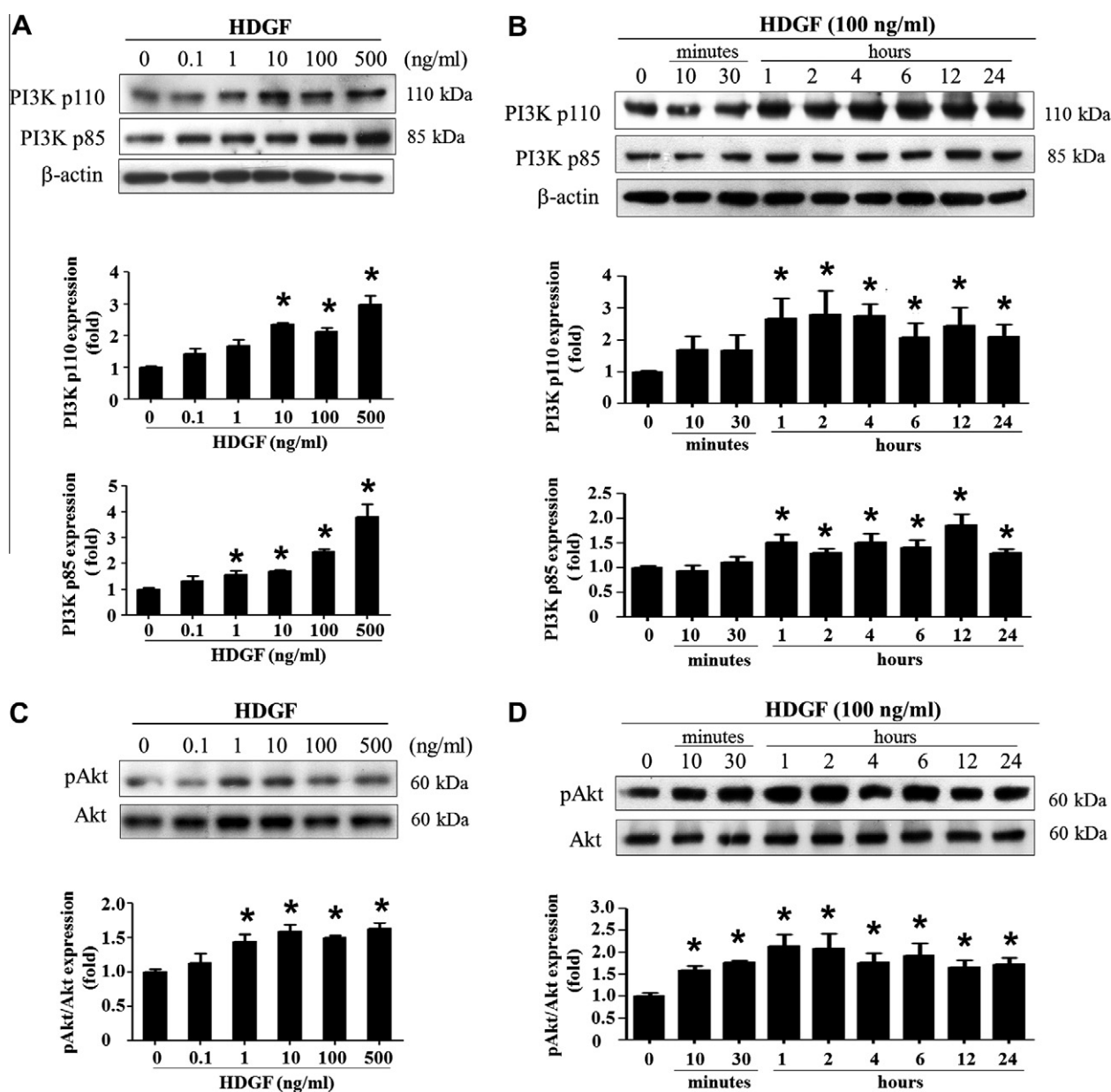

Fig. 2. Dose- and time-dependent effects of HDGF on the PI3K expression and Akt activation by western blot analysis. Determination of the expression levels of PI3K p110 and p85, and Akt phosphorylation. (A, B) Dose effect. Cells were treated with either different concentrations of HDGF for 1 h. (C, D) Time effect. Cells were treated with HDGF (100 ng/ml) for indicated time intervals. *$P < 0.05$.
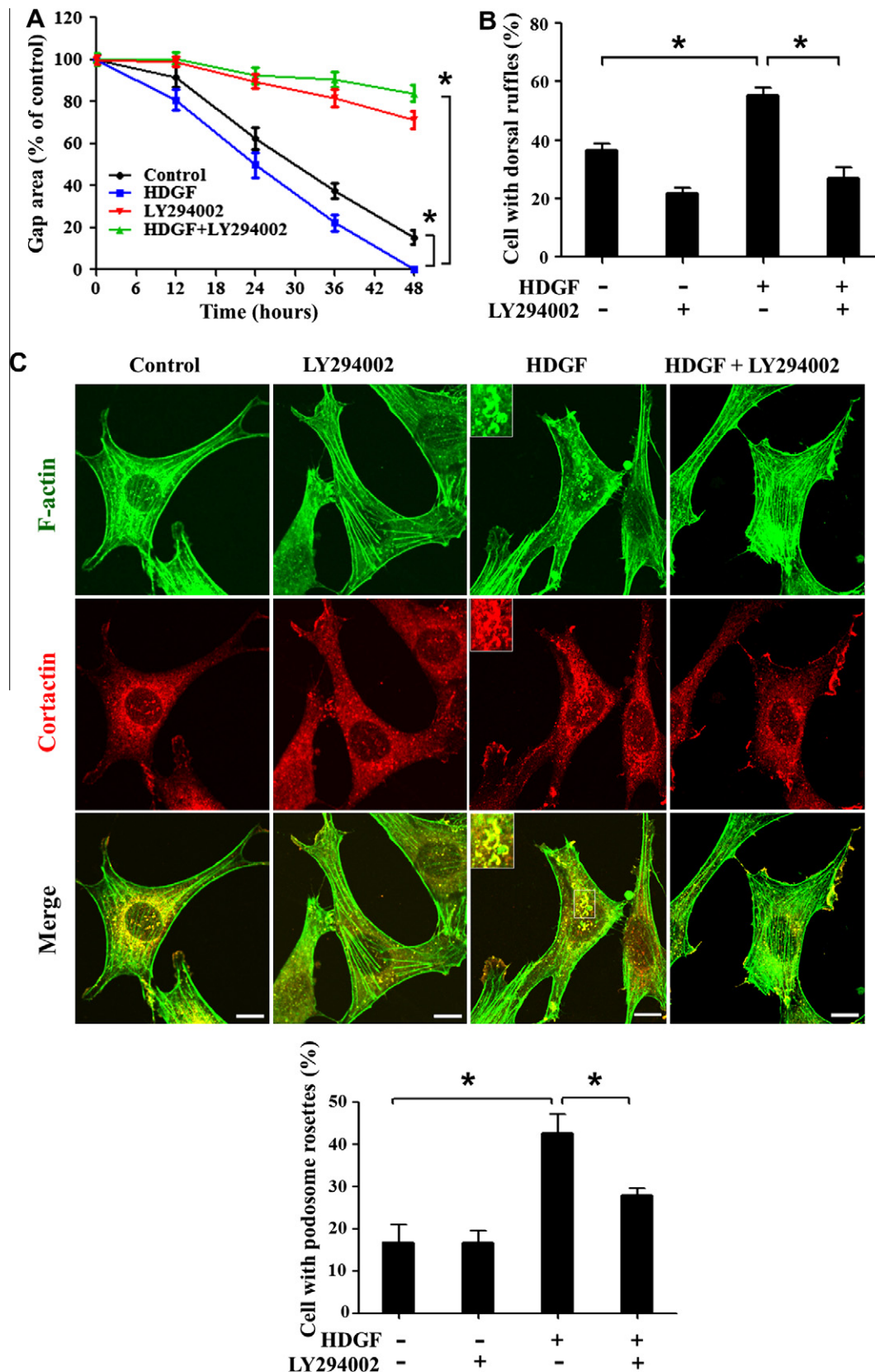
Fig. 3. Effect of LY294002 on HDGF-stimulated cell motility and PI3K/Akt signaling cascade. (A) Effect of LY294002 on HDGF-stimulated wound healing. After induction of scratch injury, cells were treated with HDGF (100 ng/ml) in the absence or presence of LY294002 (10 μM) of indicated time intervals and monitored for healing extent. (B) Effect of LY294002 on HDGF-mediated dorsal ruffles formation. (C) Effect of LY294002 on HDGF-induced podosome formation. The cells with dorsal ruffles and podosome rosette structures were determined by immunofluorescence analysis and quantified from at least 200 cells. $^*P < 0.05$.
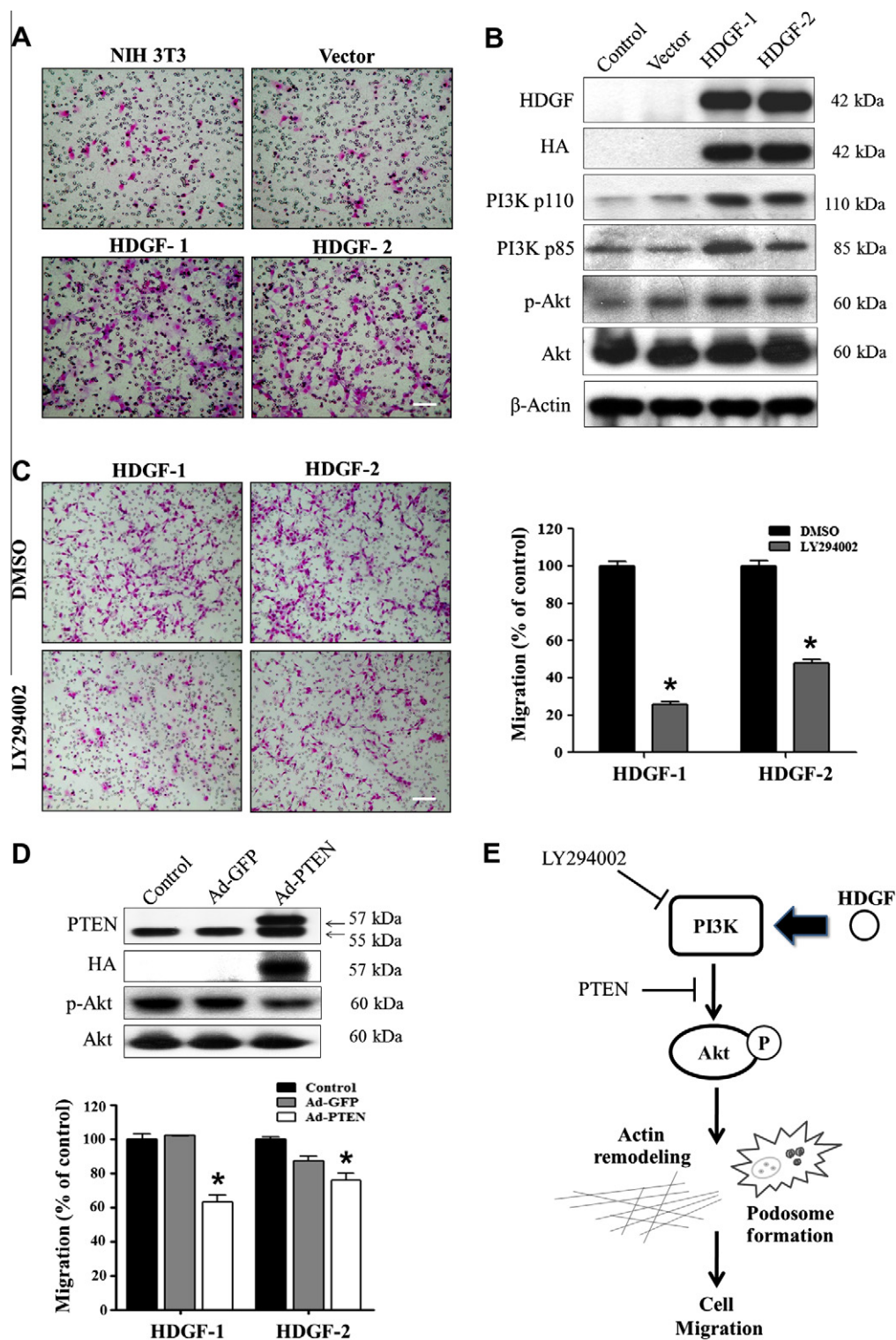
Fig. 4. Effect of LY294002 or PTEN gene delivery on motility and PI3K/Akt signaling in HDGF-overexpressing NIH3T3 transfectants. (A) Enhanced migration in HDGF-overexpressing NIH3T3 transfectants. (B) Increased PI3K expression and elevated Akt activation in HDGF transfectants. The expression levels of the PI3K p110 and p85, and Akt phosphorylation were analyzed by Western blot analysis from triplicate experiments. (C) Effect of LY294002 on motility of HDGF transfectants. (Left panel) Representative migration profiles for LY294002-treated HDGF transfectants. (Right panel) Statistical analysis. (D) Effect of PTEN gene delivery on motility and PI3K/Akt signaling in HDGF transfectants. After infection with adenovirus vectors for 48 h, the PTEN expression and Akt activation were determined by Western blot analysis (upper panel). The size for endogenous and exogenous PTEN was 55 and 57 kDa, respectively. The effect of PTEN gene delivery on migration of HDGF transfectants was determined by trans-well migration assay (lower panel). Data were mean ± SEM of quadruplicates. (E) The scheme for PI3K/Akt signaling in HDGF-induced migration and cytoskeleton remodeling. *$P < 0.05$.

HDGF-induced dorsal ruffles in NIH/3T3 cells (from 55.3 ± 6.3 to 27 ± 9.1%; S2 and Fig. 3B). Furthermore, LY294002 significantly re-versed the HDGF-stimulated podosomes formation (from 42.7 ± 10 to 28 ± 3.8%; Fig. 3C). Together, these results suggest that PI3K/Akt

activation plays a pivotal role in HDGF-induced migration and cytoskeleton re-organization.

3.4. HDGF overexpression in NIH/3T3 cells enhanced the migration and PI3K/Akt activation, which was attenuated by LY294002 treatment or PTEN gene delivery

Because HDGF expression is frequently elevated in many types of cancer, NIH/3T3 transfectants that stably expressed HDGF were generated to study the consequence of HDGF overexpression on cellular motility and PI3K/Akt signaling. By using trans-well assay, it was found that the motility of HDGF-overexpressing transfectants were significantly higher compared with parental and vector control cells by 2- to 4-fold (Fig. 4A). Moreover, the expression of PI3K p110 and p85 subunits and Akt phosphorylation were also significantly elevated in HDGF-transfectants compared with control cells (Fig. 4B). Treatment with LY294002 inhibited the migration of HDGF transfectants by 60–80% inhibition (Fig. 4C). In addition to pharmaceutical blockade, we investigated whether overexpression of PTEN, an endogenous antagonist of PI3K, affected the migration and Akt activation in HDGF transfectants. After adenovirus gene delivery, HDGF transfectants infected with adenoviral encoding PTEN (Ad-PTEN) exhibited significantly reduced migration and Akt phosphorylation (Fig. 4D). Therefore, inhibitors and genetic studies suggest HDGF overexpression augments the motility through activation of PI3K/Akt signaling.

4. Discussion

The present study demonstrates for the first time that HDGF elicits the formation of dorsal ruffles and podosomes in non-transformed NIH/3T3 cells. Besides, exogenous HDGF supply or ectopic HDGF overexpression enhances the cell migration and induces the activation of PI3K/Akt pathway. Conversely, pharmaceutical inhibition of PI3K by LY294002 treatment or gene delivery of PI3K-antagonizing PTEN gene suppressed the HDGF-induced migration and the PI3K/Akt signaling cascade. Together, these results indicate that HDGF promotes cellular migration and cytoskeleton remodeling through PI3K/Akt activation (Fig. 4E).

One novel finding of this study is that HDGF regulates the actin-based filament networks such as dorsal ruffles and podosome rosettes in NIH/3T3 cells. During cell migration, the interaction between actin cytoskeleton and matrix adhesion/degradation have to be tightly regulated and coordinated. By far, growth factors, such as VEGF, EGF and TGFβ, have been shown to induce the podosomes in murine fibroblasts [11]. Thus, our results revealed that HDGF is also a podosome-inducing cytokines. Because the formation of podosome rosettes is correlated with cell invasion, the podosome-stimulating capability of HDGF is consistent with our recent observation that HDGF regulates the metastasis of breast cancer through modulating of epithelial-mesenchymal transition [21].

The mechanism of HDGF-mediated podosome formation remains unclear. In this study, HDGF significantly increased the expression of podosome markers such as cortactin and cytoskeleton-associated kinases PI3K. Cortactin is an actin regulator and co-localized with F-actin during podosome formation [27]. The present study revealed that HDGF supply elicits PI3K up-regulation at mRNA and protein levels and Akt phosphorylation within 30 min after treatment, implicating PI3K activation is one of the early events of HDGF signaling. The activated PI3K signaling may induce the formation of phosphatidylinositols PIP2 andPIP3 [28] and activation of focal adhesion kinase (FAK), which is required for the assembly of podosome formation [29]. Stable expression of c-Src oncogene in NIH/3T3 cells has been reported to induce podosomes formation [30]. In addition, VEGF and TGFβ have been shown to induce podosomes formation via activation of Src and PI3K proteins either in invasive cancer cells [31] or endothelial cells [32,33]. Furthermore, inoculation of NIH/3T3-HDGF transfected cells in the nude mice has been demonstrated that HDGF is able to stimulate VEGF expression and thereby induction of tumorigenesis [14]. Further studies are warranted to delineate the interplay between HDGF, VEGF, Src, and PI3K in podosome formation within the transformed and non-transformed cell models.

In conclusion, the present study demonstrated that HDGF mediates cell migration by activation of the PI3K/Akt signaling cascade, podosome formation, and cytoskeletal reorgnization in non-transformed fibroblasts. Future studies are warranted to elucidate the mechanism underlying HDGF-stimulated podosomes formation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.060.

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