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Hepatocyte growth factor enhances proteolysis and invasiveness of human nasopharyngeal cancer cells through activation of PI3K and JNK

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Abstract The hepatocyte growth factor (HGF) receptor, Met, is frequently overexpressed in nasopharyngeal cancer (NPC). Here, we showed for the first time that human NPC cells with high Met expression were more sensitive to the cell motility and invasion effect of HGF. The downregulation of Met by small interfering RNA decreased tumor cell invasion/migration. HGF significantly increased matrix metalloproteinase-9 production. This was inhibited by blocking phosphatidylinositide 3-kinase (PI3K) and c-Jun N-terminal kinase (JNK), but not extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase signaling pathways. We also demonstrated that PI3K induced activation of JNK, with Akt as a potential point of this cross-talk. These results provide new insights into the molecular mechanism responsible for NPC progression and metastasis.
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Keywords: Hepatocyte growth factor; Met oncogene; Nasopharyngeal cancer; Signaling; Invasion; Cell motility

1. Introduction

Nasopharyngeal cancer (NPC) is one of the most common fatal cancers, especially in Southern China and Southeast Asia where the incidence is approximately 30–80/100,000 people per year (30-fold higher than worldwide) [1]. In addition to its rapid growth behavior, NPC has a great tendency to invade adjacent regions and metastasize to regional lymph node and distant organs. As a result, most (60–85%) NPC patients already have advanced disease when diagnosed [1] and this leads to a high rate of treatment failure. Thus there is a need for new therapeutic targets and a better understanding of the mechanisms involved in the spread of NPC.

The expression of hepatocyte growth factor (HGF) receptor, encoded by the Met oncogene, is frequently overexpressed in NPC. Importantly, Met expression is often elevated in NPC metastases than in primary lesions, and higher Met protein expression is associated with shorter patient survival time [2,3]. In addition, HGF is abundantly detected in the interstitial tissues surrounding the tumor [2], which further suggests that NPC could progress or metastasize through activation of the HGF-Met system. However, a role for HGF in NPC cell migration/invasion has not been established and the mechanism by which HGF contributes to NPC invasiveness is also unknown.

In this study, we show for the first time a direct role for HGF in the invasive phenotype and motility of NPC cells. We further demonstrate that the availability of Met receptor modifies NPC cell response to HGF. We have also identified a novel mechanism for HGF-induced invasion and migration, which is mediated by JNK through regulation of matrix metalloproteinase (MMP)-9 in response to PI3K/Akt signaling.

2. Materials and methods

2.1. Cell culture and treatments

The human NPC cell lines, CNE-2, HK-1, and C666-1, were grown in RPMI 1640 supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were incubated in 5% CO_2 at 37 °C. To elucidate the underlying signal transduction mechanism, cells were preincubated for 30 min with or without various inhibitors (50 nM K252a, 10 μM SB-3CT, 25 μM LY294002, 200 nM wortmannin, 20 nM rapamycin, 50 μM PD98059, 10 μM SB203580, or 50 μM SP600125) (Calbiochem) or 10 μg/ml MMP-9 neutralizing antibody (Calbiochem) prior to the addition of human recombinant HGF (R&D Systems). Cells were transfected with dominant negative Akt (Upstate), or Met siRNA (5′-ACUCUAGAUGCUCAGACUU-3′) [4] (Dharmacon) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 24 h, the cells were collected for Western blotting or biological assays.

2.2. Cell invasion and migration assays

Cells (1×10^5) were seeded onto the Matrigel-coated filters (BD Biosciences) and incubated in serum-free medium with 10 ng/ml HGF for 24 h. Non-invading cells on the upper side of the filters were removed with moistened cotton swab. Cells that penetrated the membrane were fixed with ice-cold methanol, stained with 0.5% crystal violet, photographed, and counted under the microscope. To assess cellular migration potential, the protocol described above was used, except that Matrigel was omitted.

2.3. Semi-quantitative RT-PCR

Total RNA was extracted by the Trizol reagent, and RT was performed using the SuperScript II kit (Invitrogen, Carlsbad, CA) following the manufacturer′s instruction. The cDNA was used for subsequent PCR using primers specific for human MMP-2 (5′-GGCCCTGTCA-CTCCTGAGAT-3′, and 5′-GGCATCCAGGTTATGGGGGA-3′), or MMP-9 (5′-CAACATCACCTATTGGATCC-3′ and 5′-CGGGT-GTAGAGTCTCTCGCT-3′), or TIMP-1 (5′-CTGGCTTCTGGCAT CCTGTTG-3′ and 5′-AACTCCTCGCTGCGGTTCTG-3′), or TIMP-2 (5′-GCGGTCAGTGAGAAGGAAGTGG-3′ and 5′-CTTGCACT CGCAGCCCATCTG-3′). β-Actin was an internal standard. The number of amplification cycles during which PCR product formation was limited by template concentration, was determined in pilot experiments.

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2.4. Western blot analysis

Proteins (40 μg) were subjected to SDS–PAGE, and then transferred to nitrocellulose membrane. Immunoblotting was performed with the appropriate primary antibodies, and detected with horseradish peroxidase-conjugated IgG as secondary antibody. Monoclonal antibody to Met (25H2) was from Cell Signaling, monoclonal antibodies to MMP-2 and MMP-9, TIMP-1 and TIMP-2 were from Chemicon. The polyclonal phospho-specific antibodies to $Akt^{(S473)}$, $p70^{S6K(T389)}$, $ERK1/2^{(T202/Y204)}$, p38 $MAPK^{(T180/Y182)}$, $JNK^{(T183/Y185)}$, and polyclonal anti-Akt, anti-ERK1/2, anti-p38 MAPK, and anti-JNK were purchased from Cell Signaling. The c-myc (9E10) antibody was from Sigma. Blots were developed with an enhanced chemiluminescence system (Amersham).

2.5. Gelatin zymography

Aliquots (80 μg) of conditioned media were loaded on SDS–PAGE gels containing 0.1% gelatin under non-reducing conditions. The gels were incubated overnight at 37 °C in the developing buffer containing 50 mM Tris–HCl and 20 mM $CaCl_2$, pH 7.4. They were visualized by staining the gel with 0.5% Coomassie Brilliant Blue R-250 for 6 h and, subsequently, destaining of the gel until bands became clear.

2.6. Statistical analysis

All experiments were repeated at least three times. Data were expressed as means ± S.D. and compared by one-way ANOVA and Tukey's least significant difference post-test (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of Met expression is associated with reduced invasion of NPC cells

CNE-2 used in this study is a representative NPC cell line that is derived from a poorly differentiated EBV-positive NPC which expresses high levels of Met and responds to HGF by phosphorylation of the Met receptor [2,5]. Since Met mediates motile signals, we set out to investigate whether downregulation of Met by siRNA could affect cell invasion and migration in response to HGF. The specificity of Met siRNA to inhibit the Met protein was confirmed by Western blotting (Fig. 1A). HGF significantly stimulated CNE-2 cells to migrate through the Matrigel, suggesting enhanced invasive capacity compared with untreated controls. Importantly, inhibition of Met expression by siRNA completely prevented HGF-induced cell invasion, while nonspecific siRNA had no effect (Fig. 1B). We also assessed in vitro migration in response to Met siRNA using a transwell migration assay. Consistently, cells with silenced Met abolished the HGF-induced cell migration in contrast with cells transfected with nonspecific siRNA (Fig. 1C). Pretreatment of cells with K252a, a chemical inhibitor that suppressed Met kinase activity [6], had a similar inhibitory effect as Met siRNA (Figs. 1B and 1C). In line with this, we also observed pronounced morphological changes as revealed by loss of cell–cell contact and spindle-shaped or fibroblastic cell morphology (scattering), suggesting enhanced spreading and migration capacity of HGF (Fig. S1). There was no significant change in cell proliferation under these conditions by using the MTT assay (Fig. S1). Together, these data indicate that Met overexpression enhances the invasiveness of NPC cells.

3.2. HGF induces MMP-9 expression and enhances its activity

MMP-2 and MMP-9 are well-documented ECM-degrading enzymes and whose activities are associated with NPC tumor invasiveness [7,8]. To investigate whether they play a role in the HGF-stimulated cell invasion, MMP-2 and MMP-9 protein and enzymatic activities were measured by Western blotting and gelatin zymography. As shown, the expression (Fig. 2A) and activity (Fig. 2B) of MMP-2 was not significantly altered by HGF, nor did it affect the expression of TIMP-2 (Fig. 2A). In contrast to MMP-2, both expression and proteolytic activity of MMP-9 was found to be dramatically elevated after HGF treatment (Fig. 2A–C). There was however no change in protein and mRNA levels for TIMP-1 (Fig. 2A and C).

3.3. Inhibition of MMP-9 blocks HGF-enhanced invasion

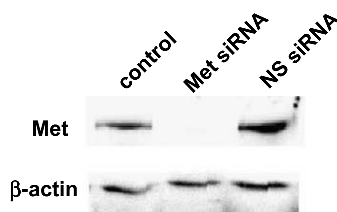
To evaluate the potential contribution of MMP-9 in the HGF-induced invasion of NPC cells, we tested the ability of the potent MMP-9 specific inhibitor (SB-3CT) or MMP-9 neutralizing antibody to inhibit the HGF-mediated effects on the invasiveness of CNE-2. Specific blocking of MMP-9 activity abolished the increase in invaded cells after HGF treatment (Fig. 2D). Similar results were obtained in migration assays (data not shown). MTT assay revealed no significant effect on the growth of treated cells and controls (Fig. S2). These results suggest that MMP-9 plays a critical role in mediating the effects of HGF on the ability of CNE-2 cells to invade the basement membrane matrix.

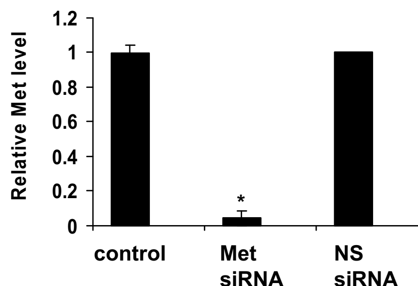
3.4. Activation of the PI3K/Akt and JNK signaling pathways regulates invasion

To investigate the mechanism involved in HGF-induced invasion of CNE-2 cells, we studied the effects of various pharmacological inhibitors on HGF-stimulated invasion. Our data showed that inhibition of PI3K activation by 200 nM wortmannin (data not shown) or 25 μM LY294002 effectively impeded HGF-stimulated invasion (Fig. 3A) and cell scattering (Fig. S3), suggesting that the PI3K pathway is required for NPC cell invasiveness. This result was not due to the inhibition of proliferation (Fig. S3). Akt is the primary signal mediator of PI3K. Activation of PI3K/Akt stimulates multiple signal transduction mechanisms, including $p70^{S6K}$. Although HGF stimulation of NPC induced $p70^{S6K}$ phosphorylation (Fig. 3B), inhibition of the mTOR/$p70^{S6K}$ pathway by 20 nM rapamycin did not affect NPC cellular invasion (Fig. 3A) or scattering (Fig. S3) induced by HGF.

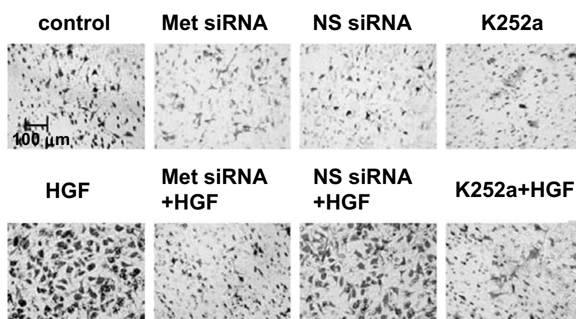
Because studies have shown that the MAPK pathway is critical for the activation of gene expression and cell migration by HGF in some cell types [9,10], we asked if they also play a role in HGF-induced human NPC cell invasion and/or migration. As shown, addition of HGF to the cells significantly increased the phosphorylation of ERK1/2, p38 MAPK, and JNK (Fig. 4A). However, it was only treatment of 50 μM SP600125, a specific JNK inhibitor, significantly reduced the number of invaded (Fig. 4B) and scattered (Fig. S3) cells to near basal levels. SP600125 did not alter cell proliferation (Fig. S3). In contrast, inhibition of HGF-stimulated ERK1/2 and p38 MAPK activation by 50 μM PD98059 or 10 μM SB203580, respectively, had no effect (Fig. 4B and Fig. S3). Similar results were also obtained with a different MEK inhibitor U0126 and with a different p38 MAPK inhibitor SB202190 (data not shown). Interestingly, LY294002 was able to prevent JNK phosphorylation. In contrast, the level of Akt was not affected when the JNK pathway was inhibited by SP600125 (Fig. 4C). These data suggest that PI3K/Akt is an upstream kinase of JNK in NPC.

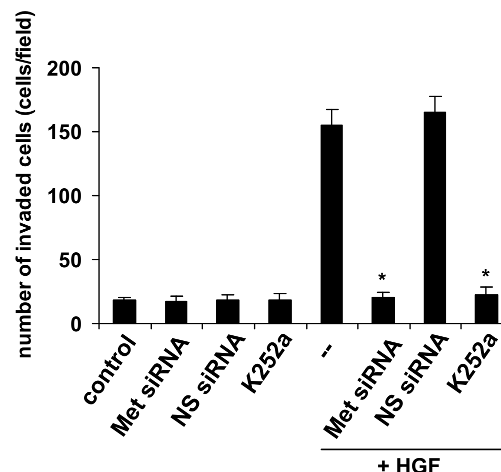
A. Western blot



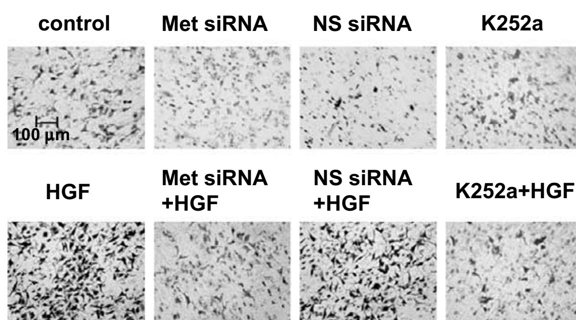


B. Invasion assay





C. Migration assay



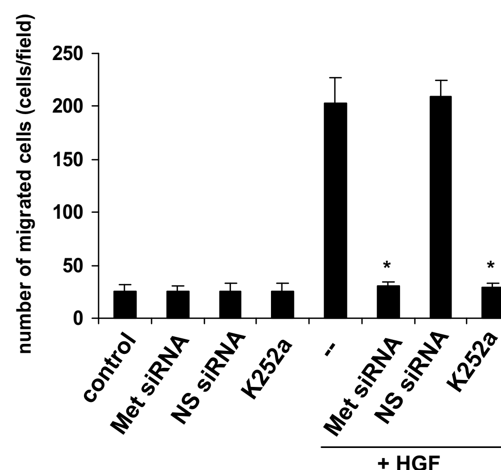
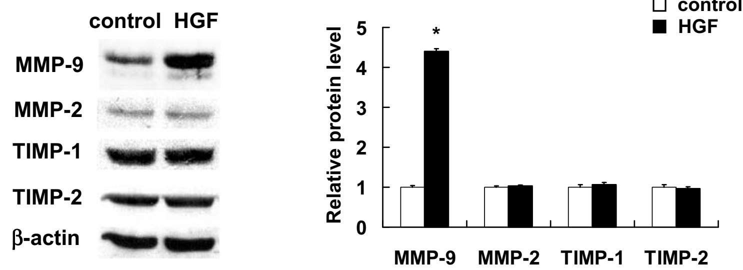
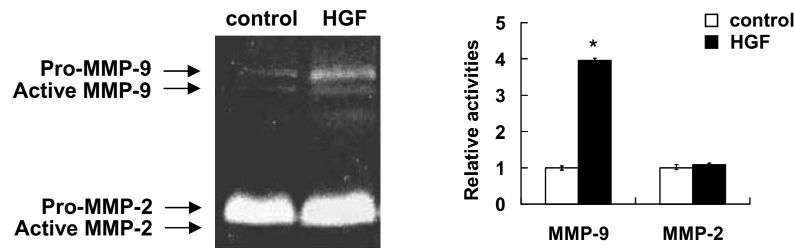
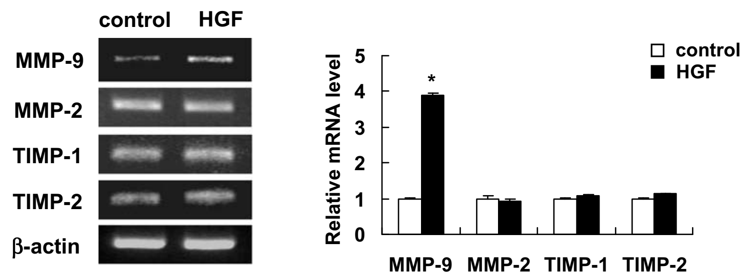


Fig. 1. Met overexpression mediates HGF-induced invasion and migration. (A) CNE-2 cells were transiently transfected with Met siRNA or nonspecific siRNA (NS siRNA) as control. Met expression was analyzed by immunoblotting. (B) Cells were also incubated untreated or treated with HGF, in either the presence or absence of K252a. In parallel experiments, they were subjected to invasion assay through Matrigel-precoated filter and (C) migration assay through uncoated filter. Representative pictures were shown in the left panel. The bar graphs (right panel) represent the mean number of invaded or migrated cells of five fields ± S.D. of triplicate wells from three independent experiments. Bar, 100 µm. $^{*}P < 0.05$ compared with HGF alone.

To confirm JNK is a downstream molecule of Akt that mediates the invasive response, the effects of expressing dominant negative Akt (DN-Akt) on cellular invasion were studied. Specific inactivation of Akt in DN-Akt transfectants was confirmed by Western blotting (Fig. 5A). Expression of DN-Akt prevented the phosphorylation of JNK (Fig. 5A) and decreased HGF-induced MMP-9 production (Fig. 5B) and cell invasion (Fig. 5C), demonstrating that HGF transmits an invasion signal through Akt to JNK. Similar results were also obtained when these experiments were done in HK-1 and C666-1 NPC cell lines, indicating that these findings were not merely due to a cell line-specific phenotype (data not shown).







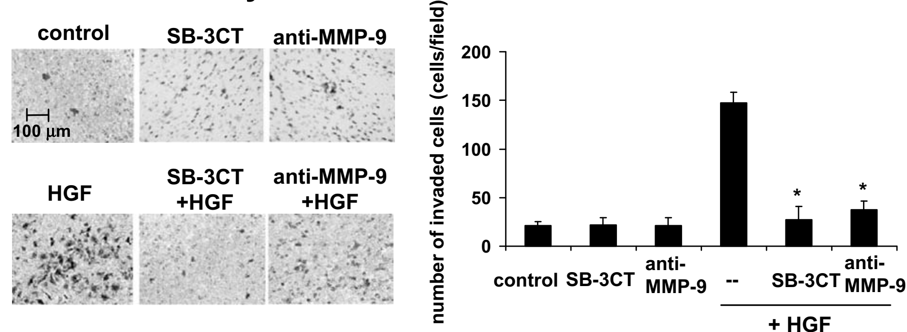
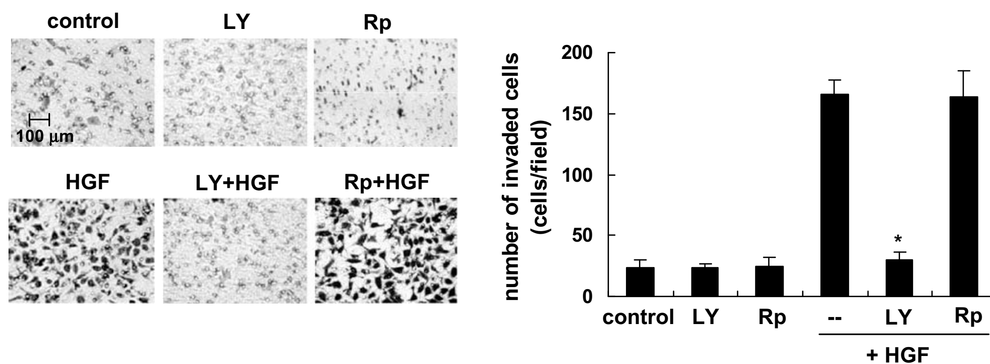


Fig. 2. HGF induces production and synthesis of MMP-9. (A) MMP-2 and MMP-9 proteins were analyzed by Western blot. β-actin was included as loading control. (B) Conditioned medium from HGF-untreated or treated cells were also collected and analyzed for MMP activities by gelatin zymography. (C) Cells were harvested for semi-quantitative RT-PCR with specific primers as described in Materials and methods. The signal intensity was determined by densitometry, and the levels of MMP-9, MMP-2, TIMP-1, and TIMP-2 were normalized against that of β-actin. (D) Cells were left untreated or pretreated with SB-3CT or neutralizing MMP-9 antibody (anti-MMP-9) for 30 min. Cells were then were subjected to invasion assay in the presence or absence of HGF. Representative pictures were shown in the left panel. The bar graph (right panel) represents the mean number of invaded cells of five fields ± S.D. of triplicate wells from three independent experiments. Bar, 100 µm. $^{*}P < 0.05$ compared with HGF alone.

4. Discussion

Distant metastasis of NPC is more frequent than most of other head and neck cancers and it remains the most common reason for treatment failure and poor prognosis of the patients with late-stage NPC [1]. In this study, we explored for the first time a direct role of Met in NPC progression. We found that HGF increased the transformed phenotype of NPC cells by promoting cell migration and invasion. These intriguing findings are relevant to a large number of NPC because the Met receptor for HGF is commonly overexpressed in human NPC and HGF is abundantly detected in the interstitial tissues



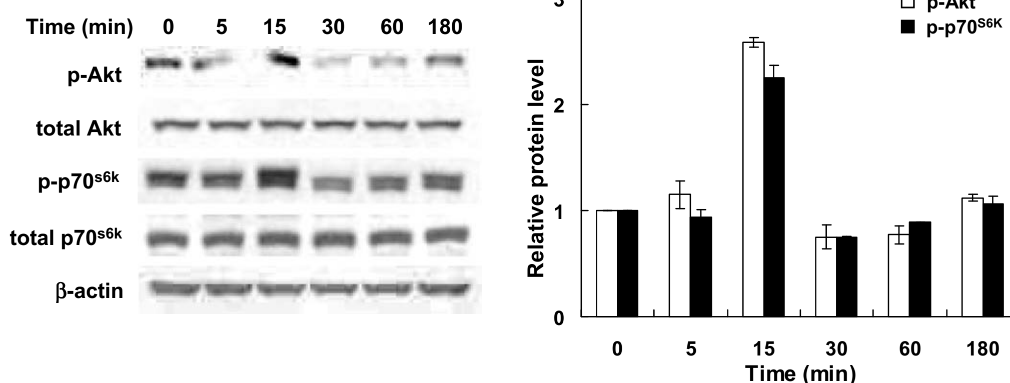
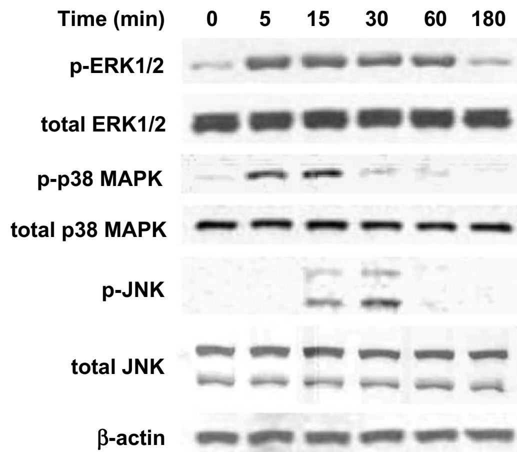


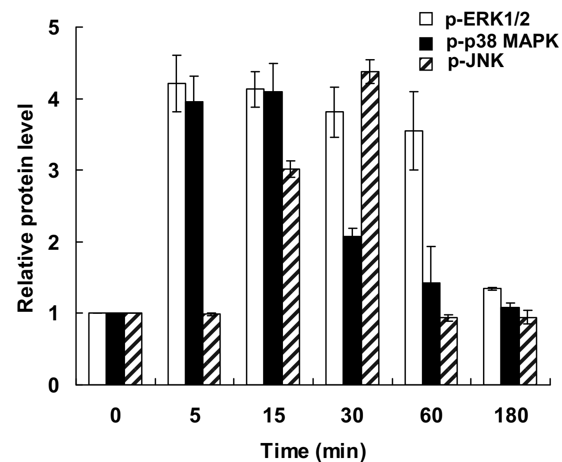
Fig. 3. Inhibition of PI3K inhibits HGF-induced cell invasion. (A) Cells were preincubated with LY294002 (LY) or rapamycin (Rp) for 30 min and then subjected to invasion assay in the presence or absence of HGF. Representative pictures were shown in the left panel. The bar graph (right panel) represents the mean number of invaded cells of five fields ± S.D. of triplicate wells from three independent experiments. Bar, 100 μm. $^{*}P < 0.05$ compared with HGF alone. (B) Cells were exposed to 10 ng/ml HGF for the indicated durations. Activities of Akt and $p70^{S6K}$ were determined by Western blotting. Total non-phosphorylated enzymes and β-actin were used as internal controls. The relative intensity of phosphorylated (p-) forms of Akt and $p70^{S6K}$ was normalized to total values of Akt and $p70^{S6K}$, which were determined by densitometric analysis.

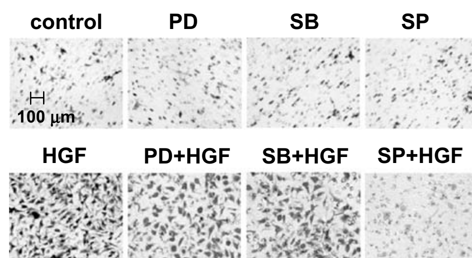
surrounding the tumor [2]. Moreover, we show that the cross-talk between PI3K/Akt and JNK pathways is required for NPC cell invasion, providing an insight into the prospect of developing targeted therapy for NPC.

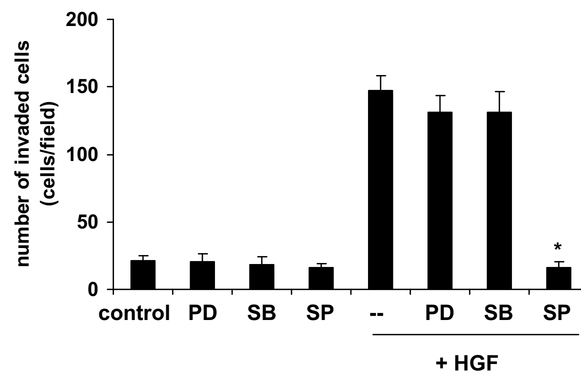
HGF receptor activation stimulates multiple downstream signals, including PI3K/Akt. The most interesting aspect of this work was the finding that while PI3K/Akt and JNK are two parallel pathways in some cell types, we show that they are two related pathways in NPC, since inhibition of PI3K/Akt can alter activation of JNK. The cross-talk between PI3K/Akt and JNK is just emerging. In contrast to most studies [11–13], in which JNK was inhibited when PI3K/Akt was activated, our present studies provide a new mechanism whereby stimulation of PI3K/Akt activated the JNK pathway and this positive regulation is important for the invasive and metastatic growth of NPC. Unlike Akt, involvement of JNK in cell invasiveness or migration has not been reported extensively. JNK-dependent signaling molecules were recently shown to regulate invasive behavior and MMP-9 production of human gliomas [14]. Interestingly, JNK is also shown to be specifically involved in Epstein–Barr viral protein-mediated cellular transformation [15,16]. How JNK controls invasion/cell migration is still not clear. One possibility is through changes in gene expression. JNK has been well established as a key regulator of transcription through AP-1 and SP-1 binding sites [17,18]. The MMP-9 gene shown in this study to be regulated by Met is also, at least in part, regulated transcriptionally through very similar mechanisms [19]. JNK could also regulate cell migration through direct signaling to the cytoskeleton. Earlier studies in *Drosophila* have shown that JNK directly interacts with and phosphorylates the actin-associated Spir 150, a WASP family protein that might connect JNK activity to actin cytoskeleton [20]. A direct link between JNK and actin cytoskeleton has recently been found in mammals, with the observation that JNK phosphorylates paxillin, an actin-associated protein [21]. In addition, it is known that HGF promotes expression of angiogenesis factors interleukin-8 and vascular endothelial growth factor in head and neck cancer cell lines through both MAPK- and PI3K-dependent pathways [22]. Whether blocking the HGF/Met pathway may benefit the tumor treatment in NPC patients via inhibiting angiogenesis in addition to inhibiting tumor cell invasion and migration is worthwhile for future investigation.

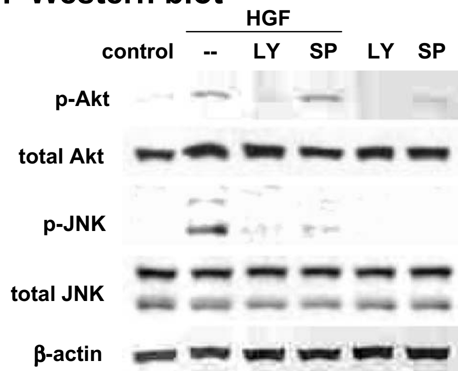
Our data show that HGF plays a role in modulating the expression of MMP-9 in NPC. MMP-9 activity is an important determinant of NPC invasion and metastasis. Upregulation of MMP-9 is found in patients with advanced-stage NPC [8]. Furthermore, it has been reported that several Epstein–Barr viral proteins can activate the MMP-9 [3,23,24]. Besides its role in cell invasion, MMP-9 can promote tumor











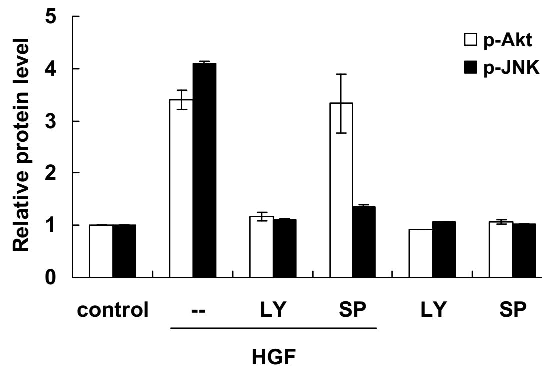


Fig. 4. Inhibition of JNK inhibits HGF-induced cell invasion. (A) Cells were exposed to HGF for the indicated durations. Activities of ERK1/2, p38 MAPK, and JNK were determined by Western blotting using antibodies specific for phosphorylated (p-) forms of ERK1/2, p38 MAPK, and JNK. Total non-phosphorylated enzymes and β-actin were used as internal controls. The signal intensity was determined by densitometry, and the levels of p-ERK1/2, p-p38 MAPK, and p-JNK were normalized against total ERK1/2, JNK, and p38 MAPK. (B) Cells were preincubated with PD98059 (PD), SB203580 (SB), or SP600125 (SP) before they were subjected to invasion assay in the presence or absence of HGF. Representative pictures were shown in the left panel. The bar graph (right panel) represents the mean number of invaded cells of five fields ± S.D. of triplicate wells from three independent experiments. (C) Cells were preincubated with LY294002 (LY) or SP for 30 min and then stimulated with HGF for further 15 min. Whole cell lysates were analyzed by immunoblotting with phosphorylated and total Akt and JNK. Immunoblotting for β-actin was used as loading control. The signal intensity was determined by densitometry, and the levels of p-Akt and p-JNK were normalized against total Akt and JNK. $^{*}P < 0.05$ compared with HGF alone.

migration by influencing cytoskeletal organization through their association with different families of adhesion receptors [25], and angiogenesis via vascular endothelial growth factor production [26]. MMPs expression is often coordinately regulated with production of their endogenous inhibitors, TIMPs. Surprisingly, activation of MMP-9 was not related to a de-

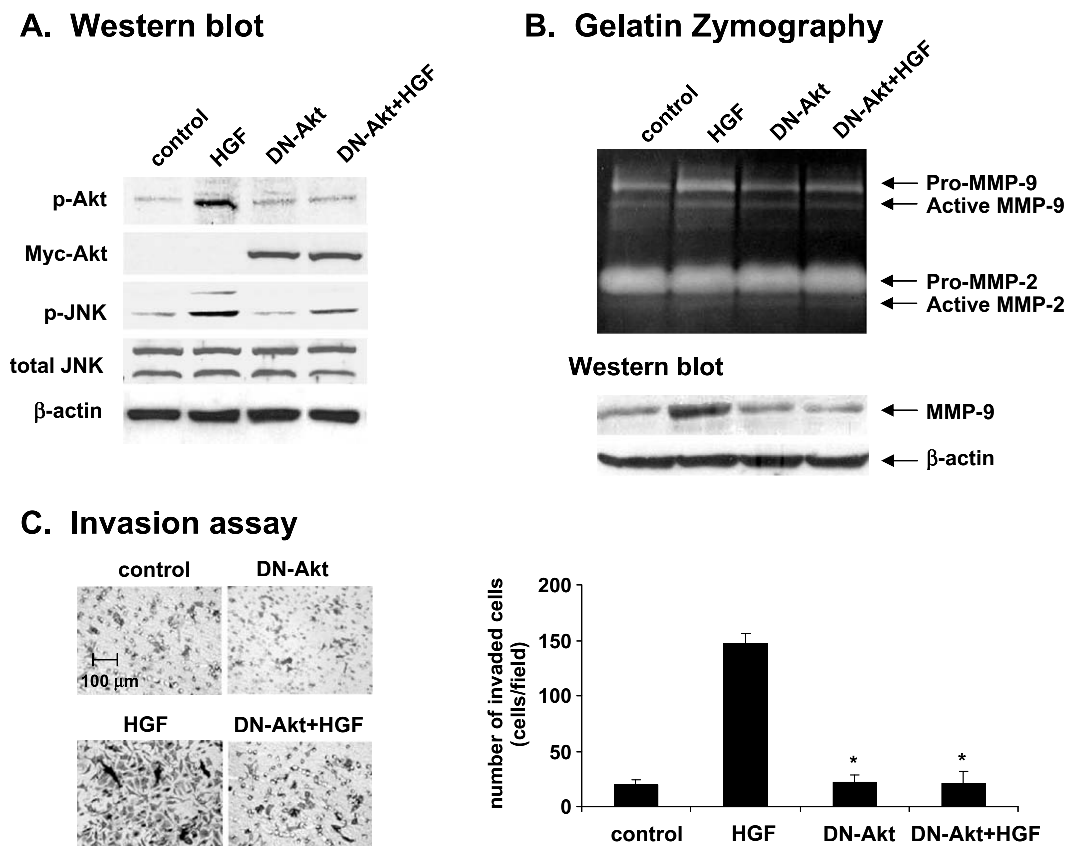


Fig. 5. The effect of HGF on cell invasion requires Akt and JNK. (A) Cells were transiently transfected with myc-tagged dominant negative (DN) mutant of Akt for 24 h, followed by HGF addition for 15 min. Akt and JNK activities were analyzed by immunoblot analysis using phospho-specific (p-) Akt and p-JNK antibodies. Total non-phosphorylated enzymes and β-actin were used as internal controls. (B) The MMP-9 gelatinolytic activities secreted into media were examined by gelatin zymography. MMP-9 protein expression was analyzed by Western blot. (C) Cells were then allowed to invade through the Matrigel-coated filters for 24 h. Representative pictures were shown in the left panel. The bar graph (right panel) represents the mean number of invaded cells of five fields ± SD of triplicate wells from three independent experiments. Bar, 100 μm. $^{*}P < 0.05$ compared with HGF alone.

crease in the specific inhibitor of TIMP-1 in CNE-2 cells. However, as there is an increase in the MMP-9/TIMP-1 ratio, this reflects a net increase in MMP-9 activity, which may also favor ECM degradation.

In summary, our findings emphasize the potential role of overexpression of the Met receptor in promoting cellular migration and matrix-degrading activities in NPC cells. This information provides a mechanistic rationale for the observed Met overexpression in advanced-stage NPC. The cross-talk between PI3K/Akt and JNK represents a new mechanism for enhancing the invasiveness of NPC and may contribute to develop improved and more specific therapeutics for the treatment of NPC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.09.004.

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