The Hepatocyte Growth Factor Isoform NK2 Activates Motogenesis and Survival but not Proliferation due to Lack of Akt Activation

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ABSTRACT

Hepatocyte growth factor (HGF) is a pleiotrophic factor involved in cellular proliferation, migration and morphogenesis. HGF is required for normal tissue and organ development during embryogenesis, but in the adult HGF has been demonstrated to drive normal tissue repair and inhibit fibrotic remodeling. HGF has two naturally occurring human isoforms as a result of alternative splicing, NK1 and NK2. While NK1 has been defined as an agonist for HGF receptor, Met, NK2 is defined as a partial Met antagonist. Furthermore, under conditions of fibrotic remodeling, NK2 is still expressed while full length HGF is suppressed. Furthermore, the mechanism by which NK2 partially signals through Met is not completely understood. Here, we investigated the mitogenic, motogenic, and anti-apoptotic activities of NK2 compared with full length HGF in primary human bronchial epithelial cells (BEpC) and bovine pulmonary artery endothelial cells (PAEC). In human BEpC, NK2 partial activated Met, inducing Met phosphorylation at Y1234/1235 in the tyrosine-kinase domain but not at Y1349 site in the multifunctional docking domain. Partial phosphorylation of Met by NK2 resulted in activation of MAPK and STAT3, but not AKT. This correlated with motogenesis and survival in a MAPK-dependent manner, but not cell proliferation. Overexpression of a constitutively active AKT complemented NK2 signaling, allowing NK2 to induce cell proliferation. These data indicate that NK2 and HGF drive motogenic and anti-apoptotic signaling but only HGF drives cell proliferation by activating AKT-pathway signaling. These results have implications for the biological consequences of differential regulation of the two isoforms under pro-fibrotic conditions.

KEY WORDS: pulmonary, tissue repair, HGF isoforms, signal transduction, fibrosis

1. Introduction

Hepatocyte growth factor (HGF) is a mitogenic, motogenic, morphogenic, and anti-apoptotic factor that is primarily synthesized and secreted by cells from mesenchymal origin and acts on cells expressing its receptor, Met [1]. HGF is required for normal organ and tissue development during embryogenesis; in the adult, HGF plays a key role in tissue homeostasis and repair in many organs including lung, heart, kidney, liver, skin, and brain [2-9]. In animal models, administration of HGF protein or the use of HGF gene therapy promotes normal tissue repair in animal models for injury in the lung, liver, kidney, heart, and brain and prevents fibrotic remodeling [10-16].

Full-length HGF is a multidomain protein, synthesized and secreted primarily by cells of mesenchymal origins as a biologically inert 90 kDa precursor and converted extracellularly into the active form through a single cleavage by a tightly regulated serine protease [17, 18]. The active form of HGF is a heterodimer consisting of disulfide-linked α - and β -chains (55-60 and 32-34 kDa, respectively). The α -chain possesses the ability to bind to and activate the receptor and is composed of an amino-terminal hairpin loop (N) domain and four kringle domains (K1-K4). The β -chain has strong homology to the protease domain of serine proteases, although lacking any protease activity, and contains a secondary Met binding site [19, 20].

Upon binding to the receptor, HGF induces activation of Met by auto-phosphorylation of several critical tyrosine residues: a juxtamembrane tyrosine (Y1003) that is responsible for Met endocytic processing and degradation [21, 22]; two tyrosines in the kinase domain (Y1234 and Y1235) that are required for kinase activity of the receptor [23]; and two tyrosines (Y1349 and Y1356) in the multisubstrate docking domain that are responsible for recruitment of multiple adaptor proteins upon phosphorylation [24]. Depending on the proteins recruited to the receptor

in a specific cell type, Met activates a variety of signaling cascades, including Ras/p42/p44 mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, signal transducer and activator of transcription 3 (STAT3), src homology phosphatase 2 (SHP2), phospholipase C-gamma (PLC- γ), src kinase, protein kinase C (PKC) isoforms, and/or small GTP-binding proteins Rap1, Rac, and Rho [25-36]. The identification of individual signaling pathways required for the specific biological activities of HGF is complicated and appears to have substantial overlap [32, 33, 37-40].

There are two other natural isoforms, known as NK1 and NK2, which are truncated peptides resulting from alternative splicing [41]. The NK1 contains the N-terminal hairpin and the first kringle domain; the NK2 variant extends through the second kringle (K2) domain. Compared to HGF, relatively little is known about how the two other isoforms elicit biological effects. NK1 was demonstrated to induce motility, survival, and proliferation, although its potency is lower than that of HGF [42]. In contrast, NK2 has motogenic activity but antagonizes the mitogenic activity of HGF [43, 44], and probably thereby inhibits normal tissue repair *in vivo* and leading to increased fibrosis [45].

We previously demonstrated that the pro-fibrotic factor transforming growth factor-beta1 (TGF- β 1) inhibits the expression of full length HGF, but not that of the NK2 splice variant. The differential regulation occurs through the regulation of miR-199 binding in the 3'UTR of full length HGF, which differs from the 3'UTR of NK2 [46]. Thus, expression of the NK2 isoform predominates under pro-fibrotic conditions, when TGF- β 1 expression is high. The physiological significance of the differential regulation of HGF and the NK2 isoform are not known.

Here, we compared the biological responses of HGF and NK2 in primary lung cell types, to determine whether this preferential expression might alter the biological outcome of NK2

activation of Met. We now demonstrate that although NK2 can prevent apoptosis of endothelial and epithelial cells and promote the migration of these cells, NK2 is not able to stimulate cell proliferation which is essential for normal tissue repair. We further demonstrate that NK2 activates downstream signaling from Met in a manner which fails to activate AKT, a signaling pathway that is pivotal for the proliferative response.

2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS; 100-106) was purchased from Gemini Bio-Products (Woodland, CA). RPMI-1640 medium, fungizone, penicillin/streptomycin, and Dulbecco's PBS were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO), U0126 MEK1/2 inhibitor from Cell Signaling Technology (Danvers, MA), Angiotensin II from Bachem, Inc. (Torrance, CA), and recombinant human HGF from ProSpec-Tany Technogene Ltd. (East Brunswick, NJ). NK2 protein was produced as previously described [47]. The CA-AKT construct was previously described [48].

2.2. Cell Culture

Human BEpC were purchased from Cell Applications, Inc. and cultured in the prepared B/TEpC growth medium (#511-500; Cell Applications, Inc., San Diego, CA). A starving medium was prepared from this medium by mixing it (1:100 v/v) with the basal medium (#510-500; Cell Applications, Inc.). Bovine PAEC were purchased from ATCC (Manassas, VA). Passage 2-8 cells were used for all experiments. PAEC were cultured in RPMI 1640 with 10% FBS (0.1% for low serum media), 1% penicillin/streptomycin and 0.5% fungizone. Cells were

grown in 5% CO₂ at 37°C in a humidified atmosphere cell culture incubator. At least two isolates of each primary cell type were used to confirm the data.

2.3. Cell proliferation assay

Cells were seeded on 30 mm dishes at 50% confluence for BEpC and 30% confluence for PAEC. The following day, medium was replaced with low serum medium. After 24 h, cells were treated with HGF (25 ng/ml) or NK2 (100 ng/ml) for 2-3 days, then trypsinized and counted using an TC20[™] Automated Cell Counter (BioRad Laboratories Inc., Hercules, CA). Control cells were treated with the same amount of HGF dilution buffer (1×PBS containing 0.1% BSA).

2.4. Neutral Comet Assay

The neutral comet assay was used to measure double stranded DNA breaks as an indication of apoptosis. Confluent cells were treated with Ang II (10 µM) for 24 h. Cells were embedded in 1% low-melting point agarose and placed on a comet slide according to the manufacturer's protocol (Trevigen, Gaithersburg, MD). Slides were then placed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% Na-lauryl sarcosinate, 1% Triton X-100, and pH 9.9) for 20 min, washed by immersion in 1×TBE buffer (0.089 M Tris, 0.089 M Boric acid, and 0.002 M EDTA pH 8.0). The nuclei were subsequently subjected to electrophoresis for 10 min at 6 mAmp in a horizontal mini-electrophoresis apparatus (BioRad Laboratories Inc.) with 1×TBE buffer. Then cells were fixed with 75% ethanol for 10 min and air-dried overnight. Cells were stained with 1×Sybr® Green (Molecular Probes, Eugene, OR) and visualized with an Olympus BX61 fluorescence microscope (Olympus, Center Valley, PA) using 10× magnification at 478 nm excitation and 507 nm emission wavelengths. Approximately 100 cells were randomly selected

and microscopically scored according to tail length. Comets were defined as apoptotic cells as described by Krown *et al.*, 1996 [49].

2.5. Cell Migration Assay

Cells were trypsinized, washed, and plated at a density of 3×10^5 cells/well in 12 well/plate Transwell (Corning, Corning, NY) dishes (12- μ m pore size). Cells were allowed to attach for 3 h, then treated in both upper and lower chambers with HGF (25 ng/ml) or NK2 (100 ng/ml) in triplicate wells. After 16 h, cells on the upper side of the membrane was cleaned using a cotton swab and cells that were migrated through the Transwell insert were fixed using 100% methanol for 15 min, stained with crystal violet (Fisher, Fairlawn, NJ), 0.94% in 20% methanol. After staining for 1 h, cells were counted using microscopy.

2.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from cells using an RNeasy Kit (Qiagen, Valencia, CA). Genomic DNA was removed using the RNase-Free DNase Set (Qiagen, Valencia, CA). RNA was quantified spectroscopically (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE) and integrity was assessed by capillary electrophoresis (Experion, Bio-Rad, Hercules, CA). RNA (1.0 μ g) was subjected to reverse transcription with GeneAmp® RNA PCR kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). RT-PCR was performed in triplicates with 1 μ l cDNA using 4 μ M each forward and reverse primer, 200 μ M each dNTP and 1 U *iTaq* DNA polymerase according to the manufacture's protocol (BioRad Laboratories Inc.). The PCR primers for Bcl-x_L mRNA were: 5'-GGTATTGGTGAGTCGGATCG and 5'-GCTGCATTGTTCCCGTAGAG. Reactions were carried out for 25 cycles using the following

conditions: 95°C 1 min; 60°C 45 sec; 72°C 1.5 min. The last cycle extension was for 10 min at 65°C. PCR primers for GAPDH were: 5'- GAAGCTCGTCATCAATGGAAA-3' and 5'- CCACTTGATGTTGGCAGGAT-3'. PCR reactions were analyzed by gel electrophoresis using 1.5% agarose, PCR products were visualized using ethidium bromide (0.1 µg/ml).

2.7. Determination of mRNA Half-life

PAEC were treated with HGF (25 ng/ml) or NK2 (100 ng/ml) for 30 min prior to Ang II (10 µM) exposure. After 8 h, Actinomycin D (5 µg/ml) was added to the medium to block the transcription. Cells were harvested at the indicated times and total RNA was isolated and the level of Bcl-x_L mRNA was determined by semi-quantitative RT-PCR. Determination of internal GAPDH mRNA level was used as control. ImageJ software (<http://www.uhnresearch.ca/facilities/wcif/index.htm>) was used for quantitation.

2.8. RNA-IP

PAEC were treated with NK2 (100 ng/ml) prior to Ang II (10 µM) exposure for 24 h and harvested by centrifugation for 3 minutes at 1000×g. Cells were resuspended in 10 ml of PBS, RNA and protein complexes were cross-linked by addition of formaldehyde (1.0%, v/v) and incubation for 10 minutes at room temperature with gentle mixing. The cross-link was quenched by addition of glycine (0.125 M, pH 7.0) and 5 min incubation at room temperature. Then, cells were washed twice with 10 ml PBS containing protease inhibitor cocktail [Roche Applied Science, San Francisco, CA] and RNase inhibitor [Applied Biosystems], resuspended in 0.2 ml NP-40 buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP40, protease inhibitors, RNase inhibitor) and incubated on ice for 10 min. After 5 min centrifugation at 14,000×g, supernatant was sonicated

three times for 20 s each at output level 6 of a sonic dismembrator, Model 100 (Fisher Scientific). The samples were cleared by centrifugation for 10 min, $14,000\times g$ at 4°C , and diluted 10 fold using IP buffer (16.7 mM Tris, pH 8.1; 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, protease inhibitor, and RNase inhibitor) to a final volume of 1 ml. 10 μl sample was preserved as an input control. Nucleolin antibody (1:1000 dilutions) was added and immune complexes were allowed to form by gentle mixing on a rotating platform at 4°C overnight; then, 50 μl of Sepharose Beads were added and mixed gently for 2 hours followed by centrifugation at $60\times g$ for 2 min at 4°C . The beads were washed for 5 min, each with low salt (20 mM Tris-HCl, pH 8.1; 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), high salt (20 mM Tris-HCl, pH 8.1; 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl), LiCl (10 mM Tris-HCl, pH 8.1; 0.25M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA), and twice with TE buffer. Immune complexes were recovered by adding of 500 μl of elution buffer (1% SDS, 0.1 M NaHCO_3 , RNase inhibitor). NaCl was added to a final concentration of 200 mM then placed at 65°C for 2 h to reverse crosslink. Next, 20 μl of 1 M Tris-Cl pH 6.5, 10 μl of 0.5 M EDTA, and 20 μg of Proteinase K was added to each sample and incubated for 45 min at 42°C . RNA was extracted using phenol:chloroform and glycogen, as a carrier. DNA was digested by DNase I (Qiagen). Bcl-x_L-3'UTR containing the ARE-2 site was amplified by RT-PCR using the primer pairs 5'-ACCTTCCTCAATTGTCGTGG-3' and 5'-GGGGAAAAGGGTCAGAAAC-3'.

2.9. Dual Luciferase Reporter Gene Assay and Transfection

One day before transfection, cells were seeded at 1.4×10^5 cells/well in a 12-well plate. DNA (1 μg /well) was transfected using the FuGENE 6 transfection reagent (Roche Applied Science), according to the manufacturer's instructions, in a serum-free, antibiotic-free medium. Renilla

luciferase control vector (RSV-Luc) was added to the transfection mixture at 1:6 ratio to normalize transfection efficiency. Transfected cells were washed twice with cold PBS, lysed with passive lysis buffer, and assayed for firefly and Renilla luciferase activities using the dual-luciferase assay (Promega, Madison, WI) according to the manufacturer's instructions in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

2.10. Immunoprecipitation

Cells grown to confluence in a 75 cm² flask. Following treatments, cells were washed with 1×PBS and harvested in 1 ml of lysis buffer [20 mM Hepes, pH 7.5; 150 mM NaCl, 1mM DTT, 1% Triton X-100, with a protease and phosphatase inhibitor cocktail (Roche Applied Science)]. Lysates were incubated on ice for 20 min with occasional shaking. Cell debris was removed by centrifugation for 10 min at 14000×g and 4°C. 10 µg anti-Met antibody (#BAF358, R&D Systems, Minneapolis, MN) were added to the cell lysate and incubated for overnight at 4°C on a rotator. Then, 50 µl Dynabeads[®] Protein G (Life Technologies) was added and incubated for 30 min, at 4°C. Beads were separated using DynaMag[™] 2 (Life Technologies). Beads were washed as follows and incubated each time for 20 min at 4°C on a rotator: one time with 1 ml lysis buffer, twice with 1 ml high salt buffer (20 mM Hepes, pH 7.5; 500 mM NaCl, 1% Triton X-100, protease and phosphatase inhibitor cocktail) and twice with 1 ml low salt buffer (10 mM Hepes, pH 7.5; 1% Triton X-100). To elute immunoprecipitated protein, 50 µl SDS sample buffer was added and incubated for 5 min at 95°C.

2.11. Western Blotting

To prepare whole cell lysates, confluent cells grown on 35-mm dishes were washed once with

cold PBS and resuspended in 100 μ l of 1 $\times$ SDS sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% wt/vol SDS; 10% glycerol; 50 mM DTT; 0.01% bromophenol blue). Following cell lysis, DNA was sheared using an ultra sonicator (Heat Systems-Ultrasonics, Plainview, NY) at 50% output control for 15 s at 4°C. Samples were heated for 5 min at 95°C. Cytosolic protein extracts were obtained by incubating the cells in a lysis buffer containing 20 mM Hepes, pH 7.5; 150 mM NaCl, 1mM DTT, 1% Triton X-100, protease and phosphatase inhibitor cocktail (Roche Applied Science) for 10 min on ice. Cell debris was removed by centrifugation for 10 min at 14000 $\times g$ and 4°C. Equal amount of 2 $\times$ SDS sample buffer was added to the sample and heated for 5 min at 95°C. Whole cell lysates or cytosolic protein extracts (10–20 μ g protein/sample) were subjected to SDS polyacrylamide gel electrophoresis and electroblotted onto PVDF membrane (0.2- μ m pore size). Membrane was blocked with 5% blocking reagent (BioRad Laboratories Inc.). Following primary antibodies were used at 1:1000 dilution: anti phospho-Met Y1234/35 (#3126), phospho-Met Y1249 (#3121), phospho-MAPK (#9101), phospho-AKT (#9271), phospho-STAT3 (#9145), AKT (#9272), and STAT3 (#4904) from Cell Signaling Technologies; ERK1 (sc-161), Bcl-xL (sc-634), Met (Sc-161), and β -actin (sc-47778) from Santa Cruz Biotechnology, Inc. Proteins were detected with horse-radish peroxidase-linked secondary antibodies (1:5,000, R&D Systems) and SuperSignal West Pico (or Dura) Chemiluminescent Substrate (Pierce, Rockford, IL). WCIF ImageJ software was used for densitometry analysis.

2.12. Statistical Analysis

Means $\pm$ standard deviations (SD) were calculated, and statistically significant differences between two groups were determined by the Student's *t* test. For three or more groups, statistical analysis was performed using one-way ANOVA, followed by the Bonferroni post-analysis, as

appropriate; $p < 0.05$ was considered statistically significant. For mRNA half-life, linear regression was calculated and confidence intervals determined. Statistical software for all analysis was SigmaStat 3.1 (Point Richmond, CA).

3. Results

3.1. NK2 induces a subset of HGF-responsive autophosphorylation sites in Met

In transformed breast and lung epithelial cells, HGF has shown to stimulate cell motility and growth, while NK2 stimulated only motility but not growth [43]. How do these two isoforms trigger different responses by binding to and activating the same receptor? In order to address this question, we first performed a comparative study of Met autophosphorylation in response to ligand binding. In BEpC, Met activation was induced by HGF (25 ng/ml) or NK2 (100 ng/ml) for 5, 10, 30, and 60 min and immunoprecipitated from cell lysates using a Met-specific antibody followed by immunoblotting using phosphorylation site-specific antibodies. As shown in Fig. 1A, both HGF and NK2 induced phosphorylation of tyrosines Y1234 and/or Y1235 in the tyrosine kinase domain; significant phosphorylation was sustained for at least 30 minutes. The maximal activity of HGF was ~1.5-1.7 times higher than that of NK2. Phosphorylation of Y1349 in the multifunctional docking domain was triggered only by HGF, but not by NK2 (Fig. 1B). HGF and NK2 concentrations used here have been shown to saturate the receptor, therefore above observed difference is not affected by the affinity of the isoforms to bind the receptor [50].

3.2. NK2 activates MAPK, but not PI3K/Akt, signaling pathways

Upon activation, Met triggers various signal transduction pathways depending on the tyrosine

sites phosphorylated [51, 52]. We found that only HGF, and not NK2, was able to phosphorylate Y1349 in the multifunctional docking domain which is known to recruit a number of signaling molecules that mediate different signaling pathways. Therefore, we were interested to know whether this difference between HGF and NK2 was reflected in the downstream signaling. We conducted a time-course study of activation of the key signaling molecules by HGF or NK2 in both cell types, BEpC and PAEC. Signaling molecules were extracted along with other cytosolic proteins and analyzed by Western blot. It revealed that both isoforms activated mitogen-activated protein kinase (MAPK, Fig. 2A and B) within 5 min of induction. Significant activation of MAPK was sustained for at least 60 min in both cell types upon HGF induction. In BEpC with NK2 induction, similar activation was observed (Fig. 2A); however, PAEC was less sensitive to NK2 induction and MAPK activation was detected only in the first two time points (1.2-3.1 fold higher at 5 min and 2-4 fold higher at 10 min, Fig. 2B).

Activation of AKT was induced only by HGF, but not by NK2, in both cell types (Fig. 2C and D). BEpC was more sensitive to HGF than PAEC as noticed by elevated activation of AKT with longer duration (Fig. 2C and D).

We also observed activation of STAT3 in BEpC with either HGF or NK2 induction (Fig. 2E). However, because of a relatively high level of basal activation of STAT3, the activation induced by HGF or NK2 was relatively weak in PAEC (Fig. 2F).

3.3. NK2 promotes motogenesis and survival but not proliferation

The ability of NK2 to only partially activate the Met receptor and a subset of relevant downstream signaling molecules could result in different biological responses from those of HGF. NK2 was demonstrated to antagonize mitogenic activity of HGF in transformed cells [47, 53,

54]. Because of cancer cell lines may have constitutively activated signal transduction pathways that can lead to biological activities [55], it was important to use primary cells to clarify differential cellular activities of NK2 versus HGF. At first, we focused on mitogenic activity. In agreement with our previous findings [43, 56], HGF stimulated proliferation of both BEpC and PAEC, whereas NK2 did not (Fig.3A and B).

We next compared motogenic activities of HGF (25 ng/ml) and NK2 (100 ng/ml). Both isoforms promoted migration of both primary cell types (Fig. 3C and D). In PAEC, the number of migrated cells was significantly increased after NK2-treatment, but the effect of NK2 was far less than that of HGF (Fig. 3C). However, in BEpC, the activity of NK2 to promote cell migration was equivalent to that of HGF (Fig 3D).

Finally, we investigated the anti-apoptotic activity of NK2 versus HGF. We used Angiotensin II (Ang II, 10 μ M), a potent inducer of intrinsic apoptotic pathway in primary lung cells [57]. In our previous work we obtained similar results regarding apoptosis detected with neutral comet assay or DNA laddering assay {Lee, 2010 #3444} {Mungunsukh, 2010 #3472}. We therefore used neutral comet assay and detected 60-80% apoptosis after 24 h of Ang II treatment in both cell types. Pre-treating cells with HGF or NK2 for 30 min prior to Ang II exposure revealed that HGF completely protected BEpC from apoptosis (~100%, Fig. 3E) and prevented PAEC apoptosis by ~75% (Fig. 3F). Interestingly, NK2 also protected both cell types from Ang II-induced apoptosis (~80% in BEpC and ~57% in PAEC; Fig. 3E and F, respectively); to our knowledge this is the first report of the anti-apoptotic activity of NK2.

3.4. Reconstituting AKT activation enables NK2 to stimulate cell proliferation

In agreement with findings obtained using cancer cell lines [43, 53], we demonstrate that only by HGF, but not NK2, stimulate proliferation of primary BEpC and PAEC. We also demonstrate that in these cells, NK2 was not able to activate AKT, one of the key molecules of HGF signaling pathways. Therefore, it was of interest to determine whether overexpression of constitutively active AKT (CA-AKT) would enable NK2 to stimulate cell proliferation. As shown in Fig. 4, only HGF stimulates proliferation of non-transfected cells. However, transfected cells overexpressing CA-AKT show overall increased proliferation and either HGF or NK2-treated cells significantly increased the number of cells, indicating that the failure of NK2 to activate AKT is responsible for its lack of mitogenic activity.

3.5. Differences between NK2 and HGF induced anti-apoptotic signaling

Our experiments (Fig. 3E and F) demonstrated that NK2 activated survival of primary BEpC and PAEC. We sought to determine whether the anti-apoptotic signaling induced by NK2 differed from that of HGF. We previously determined that HGF protects pulmonary cells from Ang II-induced apoptosis by inhibiting the degradation of the mRNA encoding the anti-apoptotic Bcl-x_L protein [40, 58]. Bcl-x_L binds to the pro-apoptotic protein Bax, preventing Bax from oligomerizing and permeabilizing the outer mitochondrial membrane to initiate intrinsic pathway of apoptosis [40]. Ang II was used to induce apoptosis in PAEC and the level of degradation of Bcl-x_L transcript was measured in the presence of actinomycin D to prevent *de novo* transcription [59]. RT-PCR results (Fig. 5A) demonstrate that Ang II induced degradation of Bcl-x_L transcript, detectable within 1 h of actinomycin D exposure. Within 4 h Bcl-x_L transcripts were below detection. However, in cells pre-treated with NK2, Bcl-x_L transcript was significantly protected (Fig 5A).

Our previous work also demonstrated that Ang II induced nuclear translocation of nucleolin leading to its cytosolic deficiency [57]. In the cytosol, nucleolin binds to the 3'UTR of Bcl-x_L mRNA and stabilizes the transcript [57]. Using RNA-IP and RT-PCR, we previously showed that HGF inhibited Ang II-induced nuclear translocation of nucleolin and stabilized Bcl-x_L mRNA [40]. NK2 also prevented Ang II-induced decrease of nucleolin binding to Bcl-x_L mRNA (Fig. 5B). Interestingly, our reporter gene assays indicate that NK2 does not regulate the Bcl-x_L promoter, whereas HGF activated the promoter by more than 2 fold (Fig. 5C).

The protective action of HGF and NK2 on Bcl-x_L transcript was also observed at the protein level. Western blot analysis revealed that both, HGF and NK2 induced significant increase in Bcl-x_L protein concentration in both cell types, although induction by HGF exceeded that of NK2. Remarkably, the induction was completely inhibited by the ERK inhibitor (U0126) indicating that the anti-apoptotic signaling activated by NK2 is ERK/MAPK- pathway mediated (Fig. 6).

4. Discussion

Here we investigated the signal transduction differences between full length human HGF and its naturally occurring human isoform NK2 in primary lung cells. HGF/Met signaling is critical during development and is required for normal tissue repair in adults. Additionally, HGF/Met signaling contributes to oncogenesis in several cancers and promotes tumor growth and cellular invasiveness that is strongly linked to tumor metastasis, and HGF/Met pathway antagonists and partial antagonists are a main focus of cancer therapy [54]. The understanding the molecular mechanisms of signaling by HGF and its isoforms is relevant to the development of an HGF mimetic useful for the promotion of normal tissue repair and the prevention of fibrotic

remodeling as well as for the development of HGF antagonists for anti-tumor therapy. Our study indicates that NK2 induces partial activation of the Met receptor, resulting in partial downstream signaling. The biological outcome of this is the induction of cell survival and motility, but not proliferation by NK2, whereas HGF signaling results in all three biological events.

Full length HGF binding induces multiple tyrosine phosphorylations on Met, including in the kinase domain Y1234/1235 and in the multi-functional docking domain Y1349 sites on Met [23]. In contrast, we now demonstrate that NK2 induces phosphorylation of Y1234/1235, but not of Y1349. Other mutant studies also showed that Met harboring the Y1235D mutation had constitutive kinase activity and increased ligand sensitivity *in vitro* or *in vivo*, but failed to induce cell motility or morphogenesis in response to HGF [60, 61]. Mutational studies also showed that substitution of either Y1235 or Y1234 with phenylalanine severely reduced the *in vitro* kinase activity. Furthermore, the replacement of both Y1234 and Y1235 with phenylalanine resulted in complete loss of the downstream signaling activity by Met [62]. Previous work also demonstrated that Y1349 is required for Met-mediated cell proliferation, and substitution of tyrosine with phenylalanine completely abolished this activity [52], in agreement with our findings with NK2.

Our findings demonstrate that both HGF and NK2 induced ERK/MAPK as well STAT3 activation and promoted cell survival and migration. Previous studies showed that both ERK/MAPK and PI3K/AKT pathways are required for HGF-induced motility, anti-apoptotic activity, and proliferation [25, 39, 43, 56]. However, only HGF induced activation of AKT, which correlated with stimulation of cell growth. This finding provides a potential mechanism for HGF-induced proliferative signaling that is absent in NK2, *i.e.* PI3K/AKT activation. This is consistent with our finding that NK2 does not activate phosphorylation of Y1349, a docking site

for the adaptor protein, Gab-1 [63], which in turn induces activation of PI3K-mediated cell proliferation. In agreement with this, we found that NK2 was able to stimulate proliferation in cells overexpressing constitutively active AKT.

HGF protects a variety of cells from intrinsic apoptosis [40, 64, 65]. Two predominant signaling pathways implicated in survival by HGF are the MAPK/ERK and PI3K/AKT pathways. The MAPK pathway inhibits apoptosis in both transcriptional-dependent and -independent pathways [66]. MAPK regulates the expression of the anti-apoptotic protein Bcl-x_L, the phosphorylation and inactivation of pro-apoptotic Bad, and the activation of anti-apoptotic Bcl-2 through phosphorylation [66-68]. Bcl-x_L binds to the pro-apoptotic proteins Bax and Bak, preventing them from forming pores in the mitochondrial outer membrane to release cytochrome c from the mitochondria and initiate the intrinsic pathway of apoptosis by activating caspase 9 [69]. Our previous studies revealed that HGF-induced pro-survival signaling in primary lung cells was ERK/MAPK-dependent and involved up-regulation of the anti-apoptotic protein Bcl-x_L [40]. Comet assays, that detect apoptosis-related DNA fragmentation, demonstrated that NK2 prevents Ang II-induced apoptosis in both BEpC and PAEC to almost degree as we previously reported for HGF. NK2 prevented degradation of Bcl-x_L mRNA via nucleolin binding, as we reported previously for HGF [40, 57, 70]. We also previously demonstrated that HGF mitigates daunorubicin-induced apoptosis of cultured cardiac muscle cells by transcriptional activation of *Bcl-x_L* in response to activation of the transcription factor GATA-4 in a MAPK-dependent manner [71]. Interestingly, our current data show that in lung cells HGF also induced transcription downstream of the Bcl-x_L promoter, but NK2 was unable to induce activation of this promoter. Because the level of cell survival by NK2 was still similar to that of HGF, this

suggests that stabilization of the Bcl-x_L mRNA by nucleolin mRNA binding is the more critical of the two mechanisms for Bcl-x_L regulation.

In some cell types, the anti-apoptotic mechanism of HGF appears to primarily involve PI3K activation and the induction of the anti-apoptotic proteins of the Bcl-2 protein family [72-74]. AKT promotes phosphorylation and inactivation of pro-apoptotic proteins including Bad, forkhead transcription factors, and the cell cycle inhibitor p27kip [75-77]. In cancer cells, anti-apoptotic signaling induced by HGF can involve PI3K-activation [78, 79], however cancer cells are known to have altered signaling mechanisms [80]. Our current findings with NK2 further suggest that HGF protection against the intrinsic apoptotic pathway does not require AKT activation in the primary lung cells.

5. Conclusions

This study revealed the differences between HGF and its truncated isoform NK2 regarding their signaling mechanism and cellular activity in primary cells, BEpC and PAEC. We previously demonstrated that full length HGF and the NK2 isoform are differentially regulated under pro-fibrotic conditions, resulting in the preferential expression of NK2. Our data now indicate that although NK2 supports cell survival and motility, it fails to promote proliferation, a requirement for normal tissue repair. Thus, overexpression of NK2 and suppression of full length HGF may contribute to repair failure in fibrotic remodeling.

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Figure Legends

Figure 1. NK2 binding induces partial Met phosphorylation. Cells were grown to confluence, treated with HGF (25 ng/ml) and NK2 (100 ng/ml) for the indicated times. In controls, HGF dilution buffer was used. Proteins were extracted using lysis buffer and subjected to immunoprecipitation with an anti-Met antibody and western blotted for phospho-Met (pMet). Membranes were stripped and reblotted for total Met. Band density analysis was performed using Image J software and results were expressed as bar graphs above the western image. Data show mean $\pm$ SEM. * indicates $p < 0.05$ from control, $n = 3$.

Figure 2. NK2 leads to partial signal transduction via Met. Cells were grown to confluence, treated with HGF (25 ng/ml) and NK2 (100 ng/ml) for the indicated times. Controls were incubated for 5 min after addition of HGF dilution buffer. Cytosolic proteins (A-D) or total proteins (E-F) were extracted and separated by 10% SDS-PAGE. Proteins were transferred to a PVDF membrane and western blotted for phospho-ERK/MAPK (A and B), phospho-AKT (C

and D), and phosphor-STAT3 (E and F); membranes were stripped and reblotted for total ERK, AKT and STAT3, respectively. Band density analysis was performed using Image J software and results were expressed as bar graphs above the western image. Data show mean $\pm$ SEM. * indicates $p < 0.05$ from control, $n = 3$. Representative western blots are shown.

Figure 3. NK2 promotes motogenesis and survival, but not proliferation. (A) cells were seeded at 50% confluence for BEpC and 30 % for PAEC. Next day, cells were starved for overnight, then treated with HGF or NK2 for 2-3 days. Cells were counted after trypsinization. (B) 3×10^5 cells were seeded in the upper chamber of a Transwell (12 well plate with 12 μ m pore). After 3 h, cells were treated with HGF or NK2 and allowed to migrate through the pores. After 16 h, cells were removed from the upper side of the membrane; cells migrated to the bottom side of the membrane were fixed, and stained with crystal violet and counted under microscope. (C). Cells were grown to confluence, pretreated with HGF or NK2 for 30 min prior to Ang II (10 μ M) exposure for 24 h. Cells displaying DNA fragmentation was determined by neutral comet assay. Untreated cells were used as controls. In all experiments, HGF and NK2 concentration used 25 ng/ml and 100 ng/ml, respectively. Data show mean $\pm$ SEM. * indicates $p < 0.05$ from control, $n = 3$. Representative western blots are shown.

Figure 4. NK2 stimulated cell proliferation, only in the presence of activated-AKT. PAEC were transfected using a plasmid expressing constitutively active AKT. After 24 h, medium was changed to a starving medium. Next day, cells were treated with HGF (25 ng/ml) and NK2 (100 ng/ml) for the 2 days. Cells were harvested using trypsin and counted. Data show mean $\pm$ SEM. * indicates $p < 0.05$ from control, $n = 3$.

Figure 5. NK2 prevents anti-apoptotic Bcl-x_L from Ang II-induced degradation. (A) PAEC grown to confluence were treated with HGF or NK2 (25 ng/ml) for 30 min prior to Ang II (10 μ M) exposure for 8h. Then, Actinomycin D (5 μ g/ml) was added and RNA was extracted at times indicated. Level of Bcl-x_L mRNA was determined by RT-PCR, normalized to internal GAPDH mRNA level. Relative band density analysis was performed and results were plotted in linear graphs next to the gel image. (B) After Ang II treatment, RNA and protein complexes were cross-linked and immunoprecipitated using an anti-nucleolin antibody. After RNA recovery, 3'UTR of Bcl-x_L mRNA was amplified by RT-PCR, the level was determined using gel electrophoresis and band densitometry. To normalize data, the input RNA was processed likewise. (C) PAEC were transfected with a luciferase reporter construct harboring Bcl-x_L promoter. After 6 h of transfection cells were treated with HGF or NK2 (25 ng/ml) for 16 h. Dual luciferase assay was performed on cell lysates. Data show mean $\pm$ SEM. * indicates $p < 0.05$ from control, $n=3$. Representative gel images are shown.

Figure 6. NK2 increases Bcl-x_L protein level via ERK/MAPK activation. Cells were grown to confluence and exposed to U0126 (10 μ M) for 20 min prior to HGF and NK2 (25 ng/ml) treatment for the indicated times. Cytosolic proteins were extracted and western blotted for Bcl-x_L. Internal β -actin was determined as loading control. Band density analysis was performed and results were expressed as bar graphs above the western image. Data show mean $\pm$ SEM. * indicates $p < 0.05$ from control, $n=3$. Representative western blots are shown.

Graphic abstract:

Normal tissue repair

Fibrotic repair conditions

A

B

A

B

C

D

E

F

Cell Proliferation Assay

Migration Assay

Comet Assay

Cell Proliferation Assay

A RNA half-life

B RNA-IP

C Reporter Gene Assay