Cytokine 123 (2019) 154762

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Comparative characterization of the HGF/Met and MSP/Ron systems in primary pancreatic adenocarcinoma

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ARTICLE INFO

Keywords:
Pancreatic
Cancer
HGF
MSP
Ron
Met

ABSTRACT

Pancreatic cancer is an aggressive disease with a poor prognosis for which current standard chemotherapeutic treatments offer little survival benefit. Receptor tyrosine kinases (RTK)s have garnered interest as therapeutic targets to augment or replace standard chemotherapeutic treatments because of their ability to promote cell growth, migration, and survival in various cancers. Met and Ron, which are homologous RTKs activated by the ligands hepatocyte growth factor (HGF) and macrophage stimulating protein (MSP), respectively, are over-activated and display synergistic malignant effects in several cancers. Despite the homology between Met and Ron, studies that have directly compared the functional outcomes of these systems in any context are limited. To address this, we sought to determine if the HGF/Met and MSP/Ron systems produce overlapping or divergent contributions towards a malignant phenotype by performing a characterization of MSP and HGF driven signaling, behavioral, and transcriptomic responses in a primary pancreatic adenocarcinoma (PAAD) cell line *in vitro*. The impact of dual Met and Ron expression signatures on the overall survival of PAAD patients was also assessed. We found HGF and MSP both encouraged PAAD cell migration, but only HGF increased proliferation. RNA sequencing revealed that the transcriptomic effects of MSP mimicked a narrow subset of the responses induced by HGF. Analysis of clinical data indicated that the strong prognostic value of Met expression in primary PAAD does not appear to be modulated by Ron expression. The relatively reduced magnitude of MSP-dependent effects on primary PAAD cells are consistent with the limited prognostic value of Ron expression in this cancer when compared to Met. Although HGF and MSP produced a differing breadth of responses *in vitro*, overlapping pro-cancer signaling, behavioral, and transcriptional effects still point to a potential role for the MSP/Ron system in pancreatic cancer.

1. Introduction

Pancreatic cancer is currently the third leading cause of cancer deaths in American men and women with over 40,000 deaths occurring annually in recent years [1]. Because symptoms often do not appear in the early stages of the disease, pancreatic cancer is usually diagnosed after metastasis has occurred when surgical resection is no longer an option [2]. Treatment approaches vary by patient health and disease stage, but often include approaches based on chemotherapeutics such as gemcitabine, nab-paclitaxel, or FOLFIRINOX [2–4]. Unfortunately, pancreatic cancer treatments are rarely curative, and as a result, the 5-year survival rate for patients diagnosed with pancreatic cancer is approximately 8% [1]. Considering the limited effectiveness of the current therapeutic approaches for treating pancreatic cancer, there is a real need to identify and understand alternative therapeutic targets.

Receptor tyrosine kinases (RTKs) are a broad group of transmembrane spanning proteins that are activated via extracellular ligands to engage signaling programs that are often linked to cancer-like behavior

Abbreviations: RTK, receptor tyrosine kinase; HGF, hepatocyte growth factor; MSP, macrophage stimulating protein; MAPK, mitogen-activated protein kinase; Akt, protein kinase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGR1, early growth response protein 1; HEGF, heparin binding EGF like growth factor; HPRT1, hypoxanthine phosphoribosyltransferase 1; FBS, fetal bovine serum; WST-8, (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium); RT-qPCR, Reverse Transcription-quantitative PCR; TBS, tris buffered saline; IEG, immediate early gene; RNA-seq, RNA sequencing; HR, hazard ratio; CI, confidence interval; PAAD, pancreatic adenocarcinoma; TCGA, The Cancer Genome Atlas

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https://doi.org/10.1016/j.cyto.2019.154762
Received 31 March 2019; Received in revised form 13 June 2019; Accepted 14 June 2019
Available online 26 June 2019
1043-4666/ Published by Elsevier Ltd.

such as increased migration, proliferation, and survival [5]. Most RTKs share a similar structural layout that includes an extracellular ligand binding surface, a transmembrane spanning domain, and an intracellular tyrosine kinase domain [5]. In general, the binding of a ligand to the extracellular RTK surface results in the stabilization of receptor oligomers and ultimately the auto-phosphorylation of tyrosines on the intracellular c-terminal domain [5]. These phospho-tyrosines serve as recognition sites for signaling adaptors, particularly those that contain Src-homology-2 binding sites, which serve as nodes for activating many downstream pathways [6]. Although many RTK mediated changes in cellular programs are made via propagation of phosphorylation signal alone, RTKs also influence transcriptomic changes via several transcriptional regulators [7].

Met and Ron are two closely related RTKs that are expressed in pancreatic cancers and have been considered as anti-cancer drug targets [8–10]. Preclinical evidence suggests that Met expression in pancreatic cancer supports tumor growth and metastasis through a mechanism that maintains populations of cancer stem cells [11]. Several studies have demonstrated that inhibiting Ron expression in mouse models of pancreatic cancer inhibits tumor growth, but may trigger Met-dependent escape mechanisms [12,13]. The growth factor ligands hepatocyte growth factor (HGF) and macrophage stimulating protein (MSP) activate Met and Ron, respectively [14]. Met and Ron share approximately 30% sequence identity over the entire receptor surface and approximately 60% sequence identity between the intracellular surfaces [14]. Sequence homology between Met and Ron translates to a high conservation of structural properties, with each receptor consisting of an extracellular alpha chain linked to a transmembrane beta chain via disulfide bonds [15,16]. The ectodomains of both Met and Ron form the ligand binding structures and are comprised of semamorphin, plexin-semamorphin-integrin, and several immunoglobulins-plexins-transcription factor domains [17]. On the intracellular surface of each receptor is a tyrosine kinase domain [17]. Signaling similarities between each system have been highlighted, with the activation of many Met/Ron effectors being dependent on the parallel ability of Met and Ron to activate the Akt and MAPK/Erk signaling arms [8].

The similarities between the HGF/Met and MSP/Ron systems enable several interactions between these systems. Following HGF mediated activation, the Met receptor can directly transphosphorylate the Ron receptor [18]. Similarly, MSP binding to Ron can trigger the transphosphorylation of Met via activated Ron receptors [18]. Because of the interconnection of these two systems, it is not surprising that the elevated expression of both Met and Ron are associated with synergistically coordinating negative patient outlook in several cancer types [19–21]. In pancreatic adenocarcinoma (PAAD), there is evidence that elevated levels of Met expression are associated with poor patient outlook [10]. Although accumulating preclinical evidence suggests a role for the MSP/Ron system in pancreatic cancer, Ron expression alone in primary tumor tissue has not been shown to be a significant marker of patient outlook in PAAD [13,22–25]. Whether a signature of combined high Met and Ron expression can coordinate a synergistic anti-survival effect in pancreatic cancer patients is unknown.

Despite the extensive structural and functional similarities that have been identified between Met and Ron, studies that directly compare the functional properties of the HGF/Met and MSP/Ron systems are sparse. Differential functions of the major signaling adaptor proteins GAB1 and GRB2 have been suggested as a divergent property, although the broad consequence of this difference has not been thoroughly investigated [26]. A second distinguishing property of these two receptors is the high catalytic activity of the kinase domain of Met, which has been measured at five times that of Ron's kinase domain [27].

Because of the need to characterize these new potential therapeutic targets in pancreatic cancer, a comparison of the signaling, behavioral, and transcriptome-wide changes induced in the BxPC-3 human primary PAAD cell line in response to treatment with HGF or MSP was performed. The impact of elevated expression levels of both the Met and Ron receptors in primary tumors of PAAD patients on overall survival times was also determined. It was found that MSP regulated a focused subset of behaviors and genes that were regulated by HGF in PAAD cells. In step with the large effect of HGF relative to MSP *in vitro,* Met expression was significantly associated with reduced PAAD patient survival times independent of concurrent Ron expression. These studies collectively showed that the effects associated with the HGF/Met system in primary PAAD appeared to dwarf those of the MSP/Ron system. However, the observation that both HGF and MSP redundantly drove several cancer-related signaling, behavioral, and transcriptional effects still support a potential role for the MSP/Ron system in pancreatic cancer.

2. Materials and methods

2.1. Cell culture

BxPC-3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in a "complete medium" preparation made from RPMI (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Genesee Scientific, San Diego, CA, USA), 100 U/ml Penicillin-Streptomycin (Life Technologies, Carlsbad, CA, USA), and 5 μg/ml Plasmocin (Invivogen, San Diego, CA, USA). Cells were maintained in a 37 °C 5% CO_2 incubator. "Serum starvation" by incubation of cells in serum free RPMI was used in some experiments to reduce the basal level activation of Met and Ron from serum derived growth factors. Serum free RPMI was prepared similar to complete medium with the exception that FBS was not included.

2.2. Reagents

Human recombinant hepatocyte growth factor (#294-HGF) and Macrophage Stimulating Protein (Cys672Ala mutant, #4306-MS) were obtained from R and D systems (Minneapolis, MN, USA). Phospho-Met (#3077), Met (#3127), phospho-Erk (#4377), phospho-Akt (#4060), glyceraldehyde-3-phosphate dehydrogenase (#5174), and HRP-linked anti-rabbit IgG (#7074) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). A total Ron antibody (#125283) was purchased from abcam (Cambridge, United Kingdom). R-phycoerythrin conjugated Ron and Alexa Fluor® 647 conjugated Met antibodies were purchased from BD Biosciences (San Jose, CA, USA). Crystal violet was obtained from Sigma Aldrich (St. Louis, MO, USA). RNeasy Plus Mini Kit was purchased from Qiagen (Hilden, Germany). Qubit RNA HS Assay Kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). WST-8 reagent (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) was purchased from Dojindo Molecular Technologies (Rockville, MD, USA).

2.3. Survival analysis

Data corresponding to The Cancer Genome Atlas pancreatic adenocarcinoma dataset (Genomic Data Commons) was downloaded from the University of California Santa Cruz Xena browser (https://xenabrowser.net/). mRNA expression data was obtained in HTseq – Counts format and was curated to include only samples corresponding to primary tumor tissue. Raw gene counts were normalized and transformed with DESeq2 using variance stabilizing transformation [28]. Individual samples were coded as "high expressers" for Met or Ron if the expression level for the gene of interest was greater than the median expression level for that gene across all samples. Otherwise samples were designated as "low expressers" for the gene of interest. Clinical data (survival time, time to last follow-up, tumor staging, etc.) corresponding to the samples was downloaded from the Fire Browse interface of the Broad Institute (http://firebrowse.org/). The impact of high levels of Met or Ron expression and select clinical properties on overall

survival was initially examined by applying univariate Cox regression. Clinical factors that were associated with a P-Value < .1 in a univariate analysis were incorporated into a multivariate model for an analysis of the expression signatures of Met and/or Ron on patient survival. Survival analysis was implemented with the R library "survival" [29]. Survival curves were drawn with the R library "survminer" [30].

2.4. Flow cytometry

BxPC-3 cells were cultured in 10% FBS RPMI in a 150 mm dish until 100% confluent. Cells were then rinsed with warm sterile phosphate buffered saline once before dislodging cells from the growing surface with the addition of 0.25% trypsin. Trypsin was then quenched by adding complete medium. Complete medium was removed by centrifugation and cells were resuspended in ice cold flow cytometry (FC) buffer (phosphate buffered saline containing 1% bovine serum albumin). Cells were then counted with a hemocytometer and the concentration of cells was adjusted to 7×10^6 cells/ml with FC buffer. Approximately 7×10^5 cells in a 100 µl volume were transferred to each of four 12 × 75 mm falcon tubes. Cells were kept on ice for the remainder of the experiment. Stain was added to each of the 4 falcon tubes by the following scheme: mock stain (FC buffer), Alexa Fluor® 647 conjugated Met antibody alone (10 µg/ml), R-phycoerythrin conjugated Ron antibody alone (10 µg/ml), and both Alexa Fluor® 647 conjugated Met and R-phycoerythrin conjugated Ron antibodies (each at 10 µg/ml). Cells were stained at 4 °C in the dark for 30 min. Stain solutions were removed from the cells via centrifugation and cells were washed three times with FC buffer before fixing cells in 2% formaldehyde in phosphate buffered saline for 15 min at 4 °C. Flow cytometry was performed on a BD FACSCalibur flow cytometer (BD Biosciences, CA, USA) operated with CellQuest software (BD Biosciences). Cells were first separated from debris by gating according to forward scatter and side scatter properties. The expression of Met and/or Ron on BxPC-3 cells was then evaluated by comparing fluorescent signals from Alexa Fluor® 647 and R-phycoerythrin. Approximately 15,000 events were obtained per stain condition and data was analyzed on the FCS Express software platform (De Novo Software, CA, USA).

2.5. RNA sequencing

BxPC-3 cells were cultured in 12 well plates until 90% confluent in 10% FBS RPMI before replacing complete medium with serum free RPMI for an overnight serum starvation period. The following morning, treatments of HGF (80 ng/ml), MSP (100 ng/ml), or phosphate buffered saline (N = 3/group) were added to the culture medium before returning the cells to the incubator. At 1 and 4 h timepoints, plates representing each treatment condition were removed from the incubator and total cell RNA was isolated with an RNeasy Plus Mini Kit according to the manufacturers' instructions. Quantification of RNA was performed with a Qubit RNA HS Assay Kit before analyzing RNA integrity with a Fragment Analyzer (Advanced Analytical Technologies Inc, Ankeny, IA, USA). RNA samples with RQN values greater than 8 were submitted to the University of Oregon Genomics & Cell Characterization Core Facility (https://gc3f.uoregon.edu/illumina-sequencing) for library preparation with a KAPA mRNA HyperPrep Kit (Roche, Basel, Switzerland) and sequencing on an Illumina HiSeq 4000 (San Diego, California, USA) in a 100 bp paired end run with a target depth of 40 million reads per sample. Raw sequence data was assessed for quality using FastQC (https://www.bioinformatics.babraham.ac.uk/). Reads were aligned to the human genome (Ensembl release 94) with STAR [31]. Gene count matrices were generated from mapping reads with the featureCounts command of the Subread package [32]. The R library "EdgeR" was used to perform differential gene/pathway expression analysis using a quasi-likelihood pipeline [33,34]. The R library "ggplot2" was used for data visualization [35].

2.6. Reverse transcription-quantitative PCR (RT-qPCR)

cDNA was synthesized from 1 µg of RNA using a High-Capacity-cDNA Reverse Transcription Kit purchased from Thermo Fisher Scientific (Waltham, MA, USA) according to the manufacturers' instructions. The reverse transcription reaction was performed on a Bio-Rad iCycler (Berkley, CA, USA) programmed to run at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. Quantitative PCR was then performed using a DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Fisher Scientific) according the manufacturers' instructions. Samples were prepared by combining 0.1 µl of cDNA with 2x SYBR green dye. Forward and reverse primers (Invitrogen, Carlsbad, CA, USA) were added at a final concentration of 0.5 µM. The PCR was performed by programming an Applied Biosystems 7500 Fast Real-Time PCR machine (Thermo Fisher Scientific) to run one initial cycle at 95 °C for 7 min and 40 annealing/extension cycles at 95 °C for 15 s and 60 °C for 1 min. Expression levels of target transcripts (EGR1 and HBEGF) were determined with the Δ Δ CT method using HPRT1 as an endogenous control. Preliminary RT-qPCR experiments were carried out to validate that all primer pairs had efficiency rates between 90 and 110%. Primer specificity was assessed with qPCR endpoint melt curves. PCR products were run on a 2% agarose gel to further ensure primer specificity. The following primer sets were used for RT-qPCR: (HPRT1) Forward 5′-CAGCCCTGGCGTCGTGATTA-3′/Reverse: 5′- TGATGGCCTCCCATCT CCTT-3′, (HBEGF) Forward 5′- ATCGTGGGGCTTCTCATGT-3′/Reverse 5′- CCAGCCGATTCCTTGAGCA-3′, (EGR1) Forward 5′-ACCTGACCGCA GAGTCTTTTCC-3′/Reverse 5′-AGGCCACTGACCAAGCTGAA-3′.

2.7. Immunoblotting

BxPC-3 cells were cultured in 6 well plates until 90% confluent before removing 10% FBS RPMI and replacing with serum free RPMI for a 24-hour period. The following day, wells were spiked with treatments of phosphate buffered saline control or a dose range of HGF or MSP (N = 3/group) before returning cells to the incubator for a 30 min treatment period. After treatment, plates were removed from the incubator, placed on ice, and protein was harvested with radio-immunoprecipitation assay buffer supplemented with MS-SAFE protease/phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Protein samples were diluted to equivalent protein concentrations in Laemmli sample buffer (Bio-Rad laboratories, Berkley, CA, USA) and boiled for 10 min at 100 °C. Equal volumes of these samples were loaded alongside a pre-stained protein ladder (Thermo Fisher Scientific, Waltham, MA, USA) on a 4–12% pre-cast polyacrylamide gel (Bio-Rad), and resolved via electrophoresis in TMOPS buffer (Bio-Rad) at 200 V for 1 h. Resolved proteins were transferred from the gel to a nitrocellulose membrane in Towbin transfer buffer at 15 V overnight at 4 °C. The following morning, membranes were incubated in tris buffered saline (TBS) blocking solution containing 5% bovine serum albumin and 0.1% TWEEN® 20 at 25 °C for 1 h with rocking. A solution of primary antibody diluted to the manufactures' recommendation in blocking buffer was prepared. The membranes were incubated in a primary antibody solution overnight with rocking at 4 °C. The following morning, the primary antibody was removed with TBS washes before incubating the membrane for 1 h at 25 °C with a horseradish peroxidase conjugated secondary antibody diluted in blocking buffer at 1:3000. After washing the membrane with TBS to remove the secondary antibody solution, SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was added to the blot to develop a light signal for imaging on a chemiDoc (Bio-Rad). The location of signal from protein targets was compared to the pre-stained protein ladder to validate target size. All membrane image analysis was performed with ImageJ (National Institutes of Health, Bethesda, MD, USA) by quantifying target signal with densitometry and normalizing to GAPDH as a loading control.

2.8. Proliferation assay

6000 BxPC-3 cells suspended in 10% FBS RPMI were seeded in each well of a 48 well plate and allowed to incubate overnight to facilitate attachment to the growing surface. The following day, 10% FBS RPMI medium was removed from the cells and replaced with serum free RPMI supplemented with phosphate buffered saline vehicle control or a dose range of HGF or MSP (N = 4/group). The plates were returned to the incubator for a 48-hour treatment period. To assess apparent proliferation, WST-8 reagent was then added to each well and the plates were returned to the incubator for 2.5 h for the development of signal produced by the reduction of WST-8 by dehydrogenase activity of viable and recently viable cells. At study endpoint, plate absorbance was read at 450 nm on a Synergy H1 Hybrid Reader (Biotek, Winooski, VT, USA).

2.9. Transwell® migration

20,000 BxPC-3 cells suspended in 0.1% FBS RPMI were seeded in the upper chambers of Transwell® inserts resting in a 24 well plate (Costar, Washington D.C., USA). Treatments of phosphate buffered saline control or a dose range of HGF or MSP were prepared in 500 µl of serum free medium and added to the corresponding lower chambers of inserts in triplicate before returning the plates to the incubator for a 24-hour treatment period. After the treatment period, plates were removed from the incubator and placed on ice. Inserts were removed from the plate using a forceps and medium was carefully removed from the upper chamber via aspiration with a micropipette. The upper chamber of the insert was then gently swabbed with a phosphate buffered saline soaked cotton applicator to remove non-migrating cells. To fix cells that had migrated across the Transwell® membrane, inserts were submerged in chilled methanol for 15 min before allowing fixed inserts to air dry for 1 h at 25 °C. Cells were then stained by submerging inserts in 0.5% crystal violet for 30 min, after which stained inserts were rinsed thoroughly with distilled water and allowed to air dry overnight. Stained inserts were imaged at 100x magnification with a Nikon Eclipse Ti-E microscope (Minato, Tokyo, Japan). The % stained area on each membrane was determined by quantifying pixels that corresponded to a violet stain color with ImageJ.

2.10. Statistical methods

Prism version 6.01 (GraphPad, San Diego, CA, USA) was used for graphing and statistical analysis of signaling and behavioral studies. One-way Analysis of Variance was used to identify overall significance between groups followed by a Dunnett's test for multiple comparisons to identify significance of pairwise comparisons.

3. Results

3.1. BxPC-3 cells uniformly co-express the Met and Ron receptors

The expression of Met and Ron are detectable in many pancreatic cancer cell lines and pancreatic cancer tumor samples and their expression has been individually associated with aggressive phenotypes in multiple cancers [9,10,13,36,37]. The co-expression of Met and Ron is suspected to result in the amplification of malignant phenotypes resulting from Met/Ron cross-talk and coordinated signaling between these receptors [8]. Although the human primary PAAD-derived BxPC-3 cell line has been described as expressing both the Met and Ron receptors, to our knowledge, the co-expression of these receptors at the single-cell level has not been evaluated [13]. To address this, we first evaluated Met and Ron expression at the cell population level in BxPC-3 cells (Fig. 1). Immunoblotting experiments on BxPC-3 cell lysates indicated that our culture of cells express the Met and Ron receptors at the population level. These expression patterns were further dissected on the single-cell level by analyzing a confluent culture of BxPC-3 cells with flow cytometry. Results from this experiment revealed that nearly all examined cells co-express the Met and Ron receptors. Little to no BxPC-3 cell sub-populations expressed either the Met or Ron receptor alone or were devoid of Met and Ron expression.

3.2. HGF and MSP supported cancer-related signaling in BxPC-3 cells

HGF and MSP bind to the receptors Met and Ron, respectively, resulting in receptor autophosphorylation and the recruitment of adapter proteins to receptor phospho-sites, which function to translate receptor level activation to the activation of downstream effectors like Akt and Erk [8]. To determine if HGF and MSP could produce signaling responses classically associated with Met and Ron activation, the ability of both these growth factors to induce the phosphorylation of select protein targets in the BxPC-3 cell line was tested. BxPC-3 cells were treated with vehicle control or a dose range of HGF or MSP *in vitro* for 30 min and the phosphorylation of Akt and Erk, shared downstream targets of Met and Ron, was measured. Ron phosphorylation in response to MSP treatment was not measured due to difficulties validating the specificity of commercially available phospho-Ron antibodies. A range of 10–80 ng/ml HGF was utilized for signaling and all other dose-response type experiments in this study. A slightly higher range of MSP concentrations ranging from 12.5 to 200 ng/ml were used throughout this study. We predicted that a higher concentration of MSP, relative to HGF, may be necessary to produce observable effects in our assays based on the knowledge that the concentrations of MSP in human plasma are generally much higher than that of HGF [38].

HGF produced significant, dose-dependent increases in the activation of Met at concentrations between 10 and 80 ng/ml (Fig. 2). 10 ng/ml HGF produced significant phosphorylation of Erk over a vehicle treated control, with further increases in HGF concentration between 20 and 80 ng/ml providing only modest further increases in the phosphorylation of this target. Peak Erk phosphorylation in response to HGF at 80 ng/ml was five-fold that of basal levels. Since Erk is a recognized effector of Ron, it served as a surrogate readout of Ron activation in this study. Similar to HGF, MSP produced phosphorylation of Erk, most noticeably between 25 and 100 ng/ml, where phosphorylation peaked at two-fold that of levels observed in the vehicle treated control. These results indicate that HGF and MSP can both activate Erk in these cells, with HGF producing a quantitatively larger response compared to MSP at the concentrations tested.

3.3. HGF but not MSP drives BxPC-3 cell proliferation

Upregulation of Erk signaling can result in the activation of classic cell cycle regulating transcription factors such as Myc, which are often associated with aberrant cell cycle regulation in cancer cells [39]. Since both Met and Ron activation were observed to increase Erk phosphorylation in BxPC-3 cells, we wondered if this Erk activation would translate to a pro-proliferative effect in these cells. To answer this question, BxPC-3 cells were treated with vehicle control or a dose range of HGF or MSP *in vitro* and proliferative responses were measured with a WST-8 based cell viability assay after a 48-hour treatment time.

Our results indicated that HGF produced a significant dose-dependent increase in BxPC-3 cell proliferation at concentrations between 10 and 40 ng/ml with 40 ng/ml yielding an approximate doubling in apparent viable cell number over vehicle treated cells alone (Fig. 3). HGF's pro-proliferative effect appeared to plateau at 40 ng/ml. MSP concentrations between 25 and 200 ng/ml, however, did not provide any significant pro-proliferative effect under the conditions tested. MSP concentrations between 25 and 100 ng/ml resulted in a slight, non-significant reduction in apparent viable cell number. This outcome suggests that despite the shared ability of HGF and MSP to phosphorylate the pro-proliferative factor Erk, HGF and MSP have divergent effects on cell proliferation when applied to BxPC-3 cells under

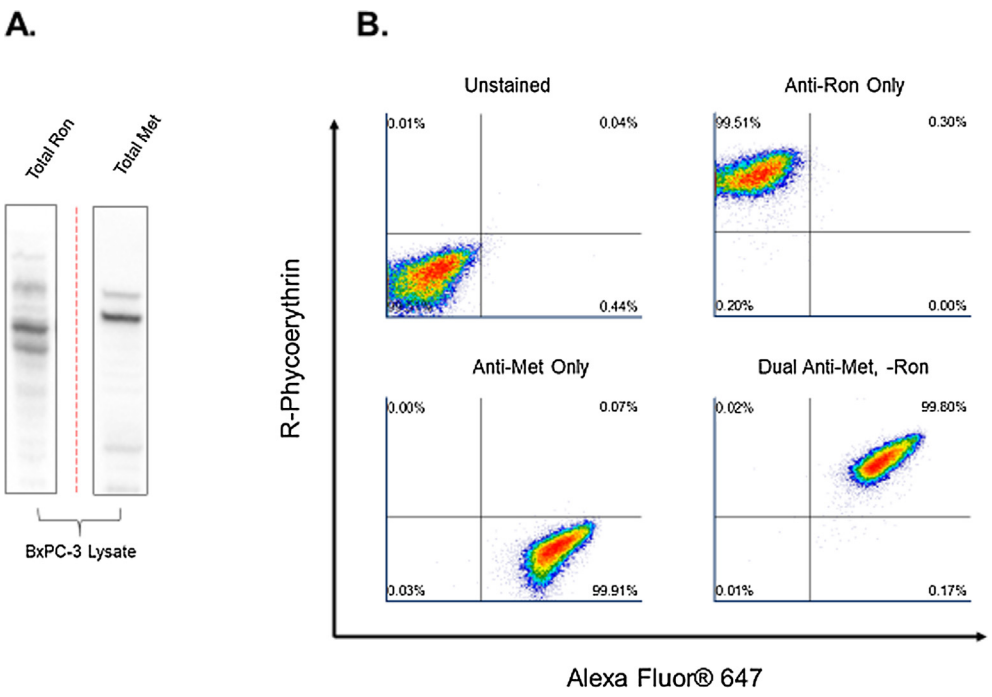

Fig. 1. Met and Ron protein expression profile in BxPC-3 cells. Total protein isolated from untreated BxPC-3 cell lysates was subjected to immunoblotting. **(A)** Images depict blot lanes probed with a total Ron antibody (left) and total Met antibody (right). Lanes were imaged from two separate blots which are delimited by a red dashed line. Untreated BxPC-3 cells were stained with fluorophore conjugated Met and/or Ron antibodies and subjected to flow cytometry analysis to determine the level of Met and Ron co-expression within single cells of the population. **(B)** Density plots depict the percentage of total BxPC-3 cells that express/co-express Met and/or Ron: unstained cells (upper-left), Ron expression (upper-right), Met expression (lower-left), dual Met/Ron expression (lower-right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

comparable conditions.

3.4. HGF and MSP induce BxPC-3 cell migration

Metastasis is a hallmark of late-stage pancreatic cancer and most patients present with metastatic disease upon diagnosis [2]. Although metastasis is coordinated through a cascade of events, tumor cell migration is requisite for the metastatic process [40]. Both MSP and HGF were originally discovered as pro-migratory factors [41,42]. MSP was found responsible for the chemotaxis of mouse peritoneal macrophages and HGF was discovered for its role in enhancing motility in renal epithelial cells [41,42]. Given the importance of metastasis in pancreatic cancer, the role of migration in metastasis, and the association of HGF and MSP with inducing migratory cell behaviors, we sought to characterize the effects of MSP or HGF treatment on the migration of BxPC-3 cells *in vitro* using a Transwell® migration assay platform.

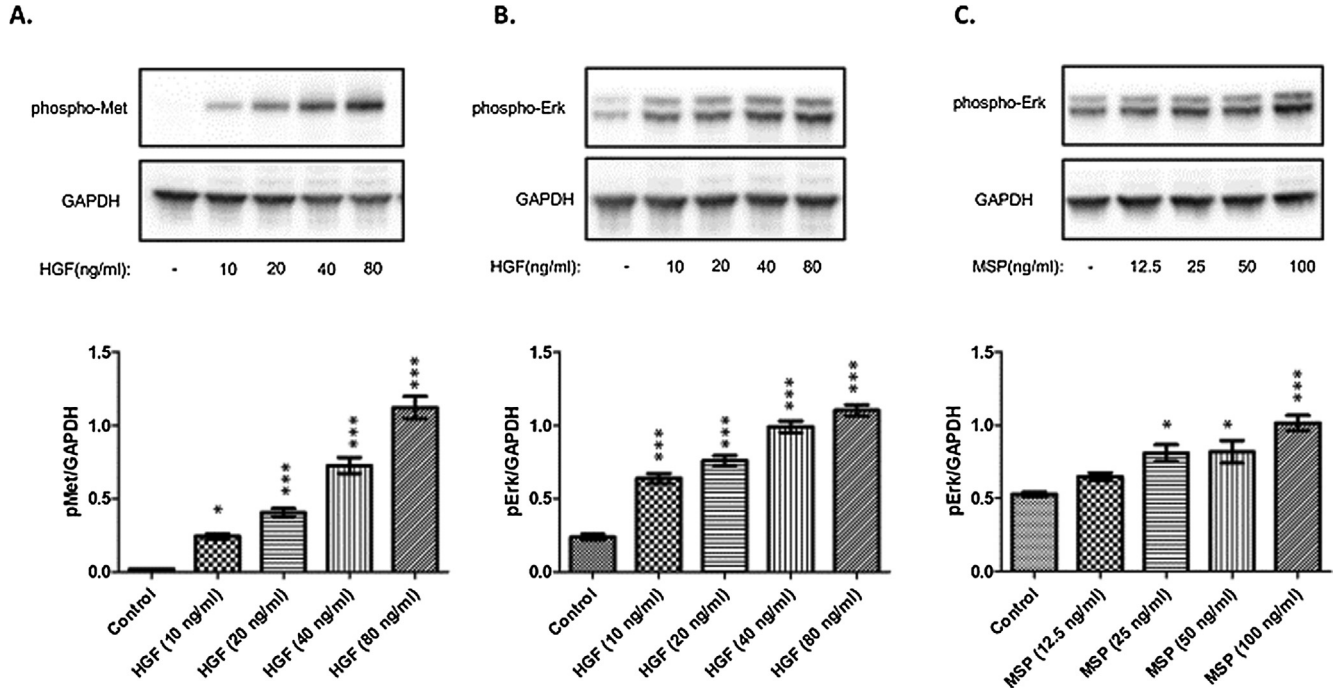

Fig. 2. Changes in cell signaling induced by HGF or MSP *in vitro.* BxPC-3 cells were treated with vehicle or a dose-range of HGF or MSP for 30 min before determining the phosphorylation of Met or Erk, relative to GAPDH via immunoblotting. Blots shown are representative of 3 independent experiments. **(A)** All tested concentrations of HGF produced a dose-dependent increase in Met phosphorylation relative to GAPDH, with each concentration showing a significant response over control. **(B)** HGF triggered significant phosphorylation of Erk when normalized to GAPDH and compared to control at each concentration tested. **(C)** MSP produced significant phosphorylation of Erk relative to GAPDH at concentrations between 25 and 100 ng/ml when compared against control. All plots show means ± S.E.M from 3 independent experiments; *P < .05, **P < .01, ***P < .001 compared against control.

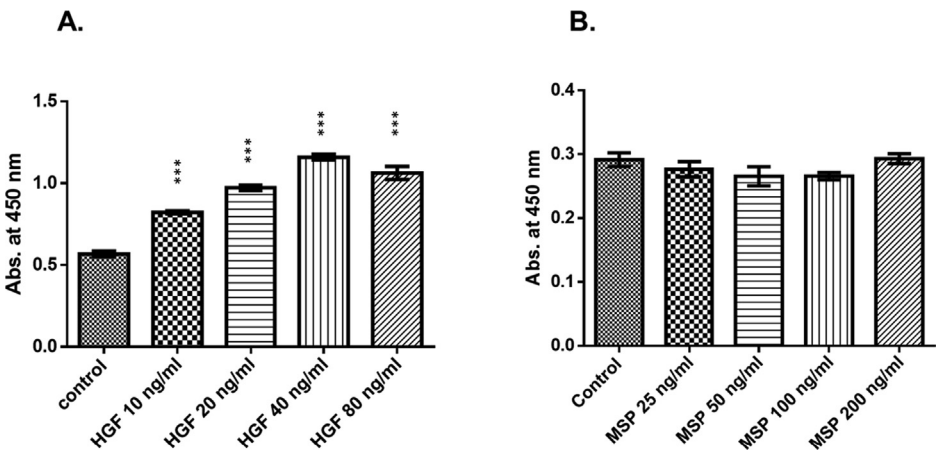

Fig. 3. HGF but not MSP produced a proliferative effect on BxPC-3 cells *in vitro*. Cells were treated with vehicle or a dose range of HGF or MSP for a 48-hour period before measuring proliferation with WST-8. **(A)** Concentrations of HGF between 10 and 80 ng/ml produced a significant increase in proliferation relative to control. **(B)** Cells treated with a range of MSP concentrations did not proliferate at a rate that was significantly different from control. All plots show means ± S.E.M from 4 independent experiments; ***P < .001 when compared against control.

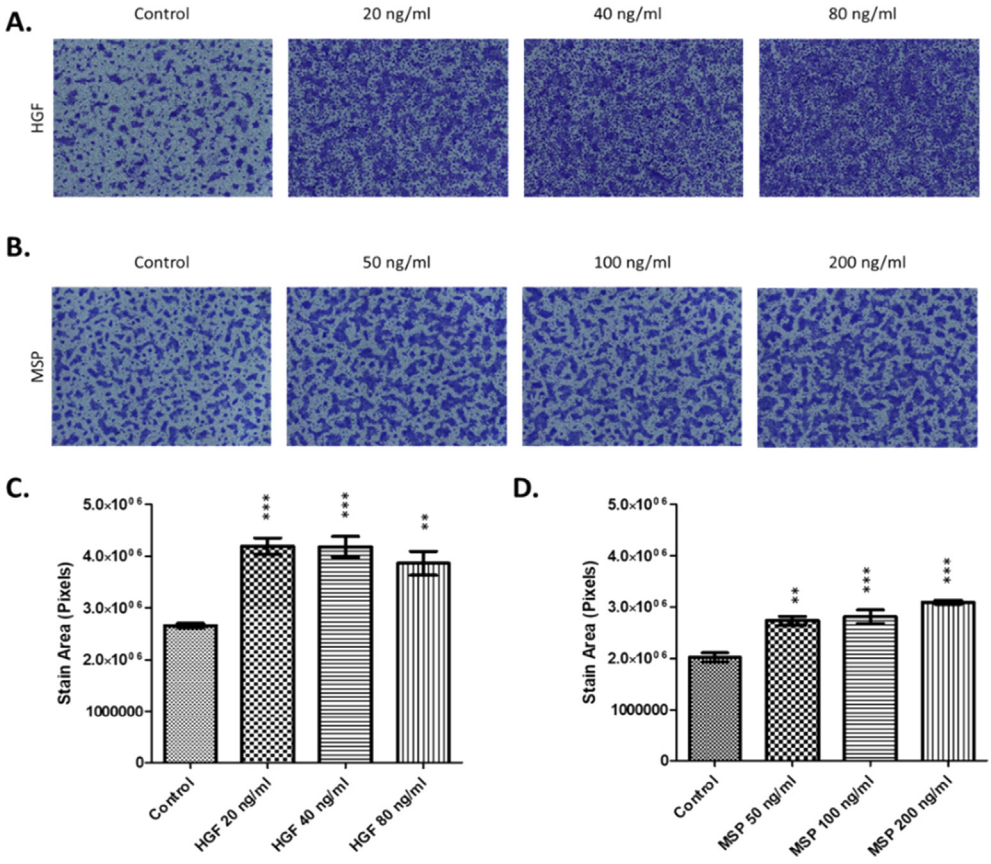

Fig. 4. Both HGF and MSP induced migration in BxPC-3 cells *in vitro*. Cells were seeded in Transwell® inserts and treated with vehicle control, or a dose range of HGF or MSP. After 24 h, migration was determined by staining cells that transversed the insert membrane. Levels of migration were quantified by automated counting of stained cells. **(A)** Stained membranes from cells treated with HGF or vehicle control. **(B)** Membranes of stained cells treated with vehicle control or MSP. **(C)** Quantification of stained membranes shows a significant migratory response to HGF treatment at each concentration. **(D)** Quantification of stained cells indicate that MSP produced a significant, slightly dose-dependent increase in migration at all tested concentrations. All plots show means ± S.E.M from 3 independent experiments; **P < .01, ***P < .001 compared against control. Images show a 3x3 stitch at 100x magnification.

Cells were treated with vehicle control or a dose range of HGF or MSP *in vitro* for a 24-hour period before quantifying the number of migrated cells (Fig. 4). HGF produced a significant, peak migratory response that was maintained between 20 and 80 ng/ml. MSP also produced a significant response in cell migration, which was a dose-dependent increase between 50 and 200 ng/ml. Although the magnitude of HGF and MSP's pro-migratory effects were similar, with each growth factor producing an approximate maximum 1.5-fold increase in migration over control, the morphology of the colonies was decidedly different. BxPC-3 cells treated with MSP appeared to retain a colony forming phenotype while cells that migrated in response to HGF treatment exhibited a loss of cell:cell contact and displayed a more "scattered" phenotype. Cell scattering is a well-known behavioral response to HGF treatment [43]. These results indicate that BxPC-3 cells have a quantitatively similar but qualitatively different phenotypic response to treatment with HGF or MSP in a Transwell® migration assay.

3.5. HGF produces a broad transcriptomic response profile in BxPC-3 cells when compared to MSP

Initial cell responses to RTK activation often involve modifications and translocations at the protein level [5]. These cellular changes can ultimately modify transcription factors, such as ELK1, which regulate the expression of so-called immediate early genes (IEG)s, a collection of genes historically associated with growth factor stimulation [44]. IEGs are often transcriptional regulators, like FOS and EGR1, with short lived transcripts that typically peak within 1 h of RTK activation [44]. In the hours following peak IEG expression, a more stable population of transcripts appear that are often more associated with committing to phenotypic/functional changes such as migration [44].

To compare changes induced in the transcriptional state of pancreatic cancer cells over time by HGF or MSP treatment, BxPC-3 cells were treated with vehicle, HGF (80 ng/ml), or MSP (100 ng/ml) for 1 or

Table 1
Gene regulation by condition.*

Condition	Sig. DE	Downregulated	Upregulated	Not Sig. DE
HGF 1 h	3367	1319	2048	9286
MSP 1 h	127	8	119	12,526
HGF 4 h	9932	5155	4777	2721
MSP 4 h	187	44	143	12,466

* vs time matched control.

4 h before stopping the reaction and isolating RNA for mRNA sequencing and differential gene expression analysis. Using this approach, we isolated and detected transcripts corresponding to 12,653 different genes. When compared to a time matched control, HGF significantly affected the expression of 3367 genes at 1 h (Table 1). The number of genes significantly affected by HGF approximately tripled to 9932 genes at 4 h. In comparison, MSP treatment significantly affected 127 genes and 187 genes at 1 and 4 h, respectively. Select significantly differentially expressed genes were chosen to validate the RNA sequencing data via RT-qPCR (Supplementary Table S1). To provide an overview of differences between groups and to gage the reproducibility of the transcriptomic responses, normalized gene counts were analyzed with principal coordinate analysis and hierarchical clustering. These preliminary analyses indicated that samples generally aggregated by treatment group in an unsupervised manner, suggesting the existence of reproducible, significant differences between treatment groups (Supplementary Fig. S1). Taken together, these results suggest that HGF affects a broader set of genes at each time point when compared to MSP and that the number of genes that respond to each growth factor increased over the course of the experiment. Finally, compared to HGF, MSP showed a bias towards gene upregulation at both timepoints, an imbalance that was not observed with HGF treatment.

The level of redundancy between gene sets regulated by HGF or MSP was determined by separately comparing genes that were up- or down-regulated in response to either treatment (Fig. 5). Nearly all genes that were regulated by MSP at a given timepoint were also regulated by HGF in the same regulatory direction at either 1 h, 4 h, or both 1 and 4 h. MSP upregulated two genes at 4 h (MANEAL and RAB7B) that were not detected in any gene set regulated by HGF. These data indicate that a clear majority of MSP regulated genes were a subset of genes that were also affected by HGF treatment. Moreover, the observation that both growth factors often regulated shared gene sets in the same "direction" further highlights the parallel properties of the transcriptomic response to HGF and MSP treatment.

To connect genes that significantly responded to treatment with either growth factor to broader biological roles, gene sets were analyzed for the representation of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways [45]. The top 5 most significantly represented pathways in the upregulated and downregulated gene sets for each growth factor treatment are shown in Fig. 6. In this subset, HGF and MSP shared regulation of the MAPK/Erk and ribosome biogenesis pathways. HGF also regulated the NF-κB and Hippo transcriptional regulation pathways at 1 h and affected several pathways associated with RNA processing and protein production at 4 h. At both timepoints MSP affected pathways that are categorized as immune/infectious disease related. The outcome that both HGF and MSP support significant engagement of the MAPK/Erk pathway at the transcript level in combination with the redundant capacity of HGF and MSP to activate this pathway at the phospho-protein level (Fig. 2), further reinforces the involvement of HGF and MSP in regulating this cancer-related pathway.

3.6. Elevated Met expression is a strong and independent marker of poor patient survival compared to Ron

High levels of Met expression are associated with poor outlook in PAAD as defined by both disease-free and overall survival times [10]. Although preclinical evidence suggests that the MSP/Ron system may be a contributing factor in driving pancreatic cancer, outlook in PAAD does not appear to be highly dependent on the expression level of Ron in primary tumors [13,22–25]. A signature of both high Met and high Ron expression levels is associated with synergistically imparting a negative impact on patient outlook in bladder, colorectal, and breast cancers [19–21]. However, to our knowledge, it is unknown whether a similar pattern of dual Met and Ron expression is associated with interdependently coordinating an anti-survival effect in PAAD. To directly compare the impact of Met and Ron expression patterns on pancreatic cancer patient prognosis, we performed survival analysis using mRNA expression data from clinical samples of PAAD. Survival analysis was performed using publicly available data from The Cancer Genome Atlas (TCGA) project [46]. We first assessed the impact of high Met expression, high Ron expression, and several other clinical factors on overall survival times of PAAD patients using a univariate Cox regression (Supplementary Table S2, Fig. S2). Consistent with previously published data, high levels of Met expression were strongly associated with reduced patient survival time (HR 3.935, 95% CI (2.138–7.242), $P < .0001$) [10]. High levels of Ron expression trended toward imparting an anti-survival effect, but the effect was modest compared to that of Met and did not reach statistical significance (HR 1.566, 95% CI (0.9244–2.652), $P = .0954$). In this preliminary analysis, we found the most significant clinical predictors of overall survival to be N stage (lymph node spread) and histological grade [47,48]. Clinical factors with a P-Value of ≤ 0.1 were incorporated into a multivariate model to assess if dual Met and Ron expression synergistically coordinates an anti-survival effect in PAAD (Table 2, Fig. 7). Results indicated that high levels of Met expression were associated with a comparable level of reduced patient survival with or without the concomitant expression of Ron (HR 3.1473, 95% CI (1.4118–7.016), $P = 0.00506$) and (HR 3.2350, 95% CI (1.3415–7.801), $P = 0.00894$), respectively. Overall,

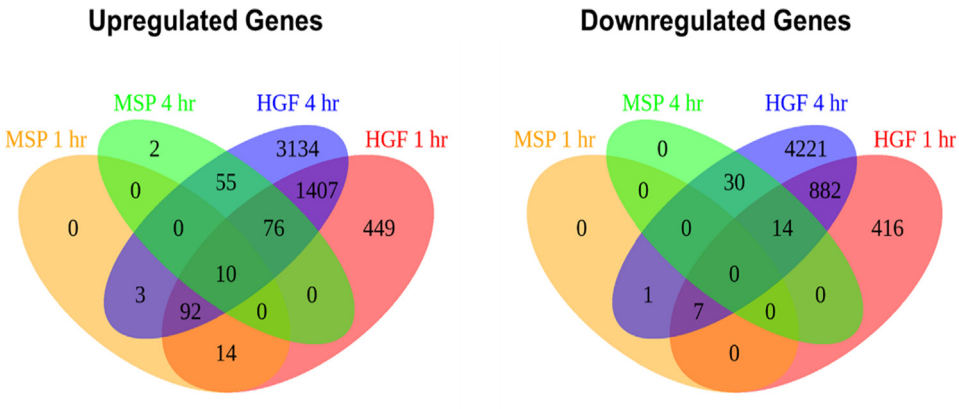

Fig. 5. MSP or HGF treatment altered gene expression in BxPC-3 cells *in vitro*. Cells were treated with vehicle, MSP (100 ng/ml), or HGF (80 ng/ml) for 1 and 4 h treatment periods before isolating RNA for mRNA sequencing and subsequent differential gene expression analysis. Significantly differentially expressed genes in each group were determined by comparing each growth factor treatment against a time matched control. Genes with a Benjamini-Hochberg adjusted p value of < 0.05 were considered significant. Significantly differentially expressed gene sets were subdivided into up- and down-regulated sets for comparison with a Venn diagram.

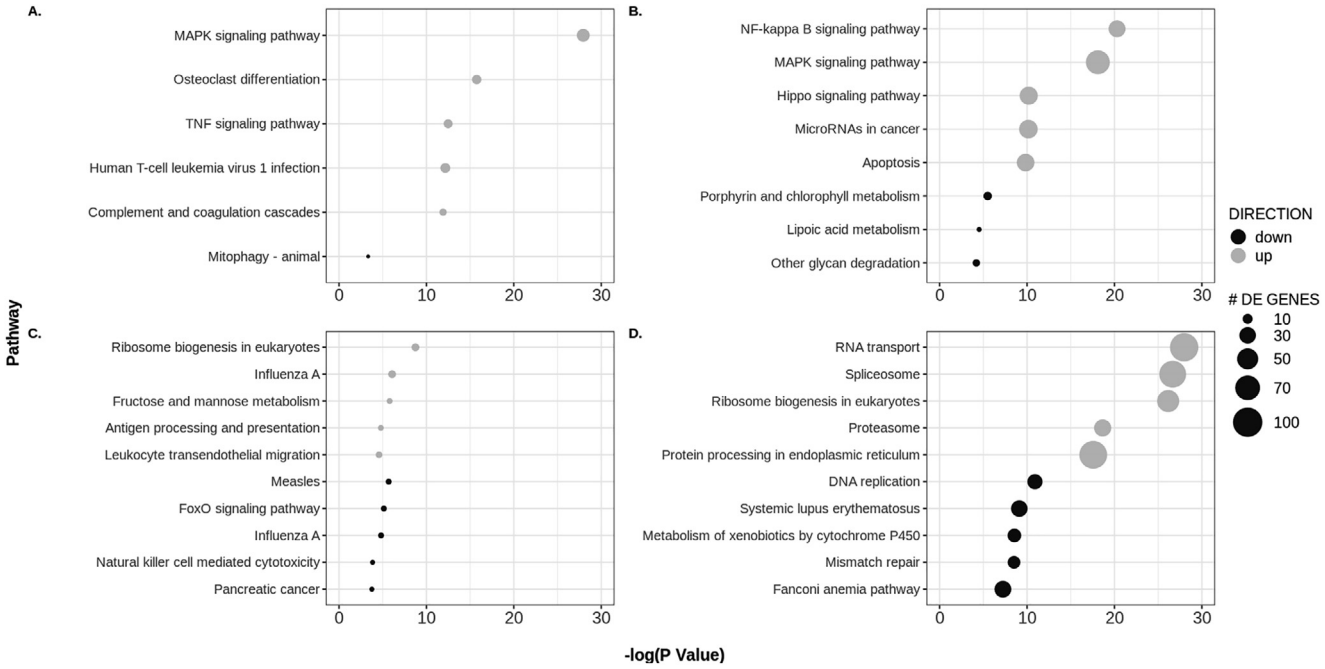

Fig. 6. KEGG pathway analysis of HGF and MSP mediated transcriptional responses in BxPC-3 cells *in vitro*. Genes that were significantly regulated in response to HGF or MSP treatment at either 1 or 4 h were further divided into up- and down-regulated sets for KEGG pathway analysis. Pathways with P-Values < 0.05 were considered significant. The top 5 most significant KEGG pathways in each gene set were plotted against the negative log transform of their P-Value. Bubble areas are set relative to the number of genes in each pathway that were significantly regulated by each treatment. Figure subpanels make the following comparisons against a time matched control: **(A)** MSP at 1 h. **(B)** HGF at 1 h. **(C)** MSP at 4 h. **(D)** HGF at 4 h.

Table 2
Overall survival of pancreatic cancer patients.

Property	*Samples*	*HR*	*95% CI*	*P-Value*
N Stage				
N0	48	1	–	–
N1	116	2.1056	(1.0892–4.071)	0.02683
N1B	4	5.7177	(1.0808–30.248)	0.04023
Grade				
G1	28	1	–	–
G2	92	2.4881	(0.8704–7.112)	0.08894
G3	48	3.3535	(1.1393–9.871)	0.02804
Gene Signature				
Met(−) Ron(−)	52	1	–	–
Met(+) Ron(−)	30	3.2350	(1.3415–7.801)	0.00894
Met(−) Ron(+)	30	0.7766	(0.2490–2.422)	0.66309
Met(+) Ron(+)	58	3.1473	(1.4118–7.016)	0.00506

HR, Hazard ratio; CI, Confidence interval.

these results reinforce that Met is a strong predictive marker of poor patient outlook in PAAD compared to Ron and suggest that the prognostic value of Met may be independent of Ron expression levels. Moreover, unlike the trends observed in bladder, colorectal, and breast cancers, the co-expression of Met and Ron in this dataset does not appear to reflect a synergistic mechanism of reducing PAAD patient survival times [19–21].

4. Discussion

Pancreatic cancer is an aggressive disease with poor patient survival outcomes for which surgical tumor resection remains the only potentially curative treatment option [2]. Unfortunately, the majority of pancreatic cancer patients do not qualify for a surgical approach and ultimately succumb to their disease [2]. To extend patient survival times beyond those acquired by traditional non-targeted therapeutic approaches, several RTKs have received attention as therapeutic targets

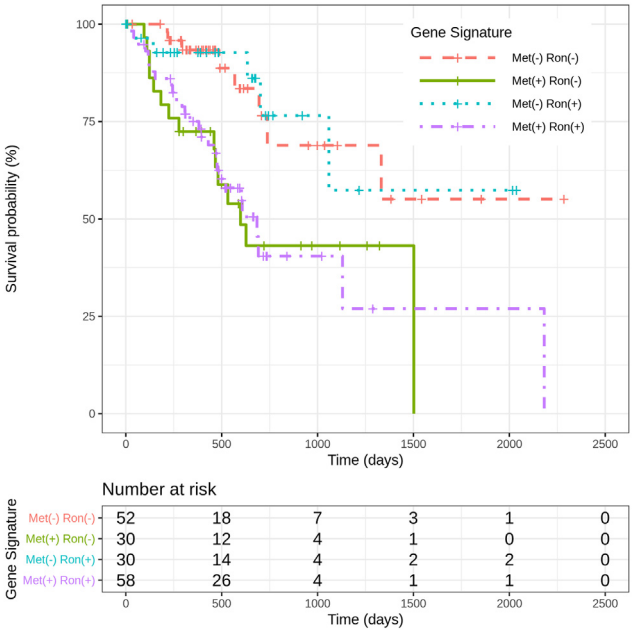

Fig. 7. Kaplan-Meier survival curves associated with Met and Ron expression in the primary tumors of PAAD patients. Survival curves were produced by analyzing publicly available TCGA data. A population of patients was subdivided into groups that express varying levels of Met and/or Ron mRNA. Survival analysis was performed using a multivariate Cox regression to include relevant clinical factors. Survival curve steps represent a death event. Hash marks correspond to censor events. "Number at risk" table indicates the sample sizes at each respective time in the above plot.

[49]. This approach stems from evidence that RTK systems are often dysregulated and/or highly expressed in cancers and are associated with encouraging cell survival, proliferation, and migration [49,50]. Among these potential targets is the HGF/Met RTK system, which has

recently been linked to poor PAAD patient prognosis through elevated Met expression levels [10]. The lesser studied MSP/Ron system, which is a close relative of the HGF/Met system based on sequence and structure, is also expressed in pancreatic cancer [9]. Despite preclinical evidence suggesting a role for the MSP/Ron system in pancreatic cancer, unlike Met, the prognostic value of elevated Ron expression alone in primary PAAD has not been demonstrated [13,22–25]. The current study focused on conducting an exploratory characterization of signaling, behavioral, and transcriptional responses of a human PAAD cell line to stimulation with HGF or MSP. Additionally, we evaluated dual Met and Ron expression profiles in the primary tumors of PAAD patients to determine if these related systems synergistically catalyze a reduction in overall survival times.

Flow cytometry-based analysis of Met and Ron expression in BxPC-3 cells revealed that nearly all (approximately 99.8%) of these cells simultaneously express the Met and Ron receptors. Although not directly addressed in this study, under these conditions it is possible that Met and Ron cross-talk is occurring in these cells. Direct reciprocal cross-talk between Met and Ron has been described in a scenario where cells co-express these receptors [18]. The functional relevance of this is demonstrated by the observation that at least part of the tumor promoting properties of Met can be mitigated by disrupting the potential interaction between Met and Ron [51]. Additionally, the indication that nearly all cells in our culture system co-express the Met and Ron receptors suggests that receptor availability was not a limiting factor in the responses produced by HGF or MSP treatment in the experiments described in this work.

We found that both HGF and MSP treatment had overlapping effects on the MAPK/Erk signaling pathway while diverging in behavioral outcomes. Both MSP and HGF produced redundant activation of the pro-proliferative MAPK/Erk signaling pathway via the phosphorylation of Erk. The phosphorylation of Erk in response to RTK stimulation can be linked via signal propagation through the Ras-Raf-MEK-Erk cascade [52,53]. In this cascade, Erk represents a branching point, as Erk is capable of acting on many substrates [54]. This variety of substrates allows cells to produce varied responses through Erk activation which are partially influenced by input from other pathways [39]. Consistent with this is the evidence that while Erk phosphorylation appears to be necessary for cells to undergo proliferation, Erk phosphorylation alone is not sufficient to trigger cell division [39]. Our cell behavior studies support this, as regardless of the shared ability of MSP and HGF to phosphorylate Erk, only HGF was able to increase BxPC-3 cell proliferation. In contrast to their pro-proliferative differences, both HGF and MSP drove cell migration at a comparable level. This response may be dependent on the shared ability of each growth factor to increase Erk phosphorylation, which has been associated with pro-migratory behavior [55].

Transcriptomic data revealed that MSP impacted a subset of the genes that were regulated by HGF. The large majority of genes that were regulated by both MSP and HGF were regulated in the same direction, indicating a redundant rather than antagonistic transcriptomic effect between the growth factors. Within the narrow set of genes that MSP regulated, the MAPK/Erk pathway was the most significantly upregulated at 1 h post-treatment. Interestingly, MSP produced significant engagement of many immune/infectious disease related pathways. Although a pancreatic cancer cell line was studied here, many of MSP's effects were initially characterized on immune cells [42]. The treatment of pancreatic cancer cells with MSP may drive the activation of pathways that are also represented in immune cells, although the potential relevance of this in pancreatic cancer has not been studied. Similar to MSP, the MAPK/Erk pathway was one of the most significantly upregulated pathways in response to HGF treatment at 1 h. These results, in combination with the observation that both MSP and HGF treatment resulted in Erk phosphorylation point to a strong, redundant role of each of these growth factors in supporting the activation of this pathway. Additionally, HGF showed greater significant upregulation of the ribosome biogenesis pathway at 4 h compared to MSP. Ribosome biogenesis is a rate limiting factor for the division of eukaryotic cells, which must generate large amounts of protein in advance of cell division to ensure the correct size of daughter cells [56]. Upregulation of the proteasome pathway, which was observed solely in response to HGF at 4 h, is similarly associated with cell division through many mechanisms, including the increased degradation of cyclin dependent kinase inhibitors [57,58]. Although not addressed in this study, the more significant upregulation of these cell division related pathways in response to HGF treatment may account for the observation that HGF, but not MSP, was able to drive BxPC-3 cell proliferation in our assays.

Although we observed many similarities in the responses of BxPC-3 cells to treatment with HGF or MSP, we also noted a strong difference in the "scope" of effects that each growth factor produced. HGF acted as a pleiotropic growth factor capable of producing a broad transcriptomic impact, which translated to the engagement of several cancer-like behaviors. By comparison, MSP's response encompassed a focused gene set that drove some, but not all the behavioral effects produced by HGF treatment. Although the HGF/Met and MSP/Ron systems exhibit a high level of sequence and structural homology, the differences in response to HGF or MSP treatment observed here were not entirely surprising as it has been recognized that the activation of apparently similar RTK systems are capable of producing diverse functional outcomes [59]. Although the basis of this is not well understood, one hypothesis is that these divergences may rely on specific combinations of pathway activation, the duration and magnitude of effector activation, or a combination thereof [7,60]. The previously identified weak kinase activity of Ron compared to Met would also be consistent with this hypothesis and is offered as an additional possible explanation for the differing breadth of HGF and MSP responses observed in this study [27].

Analysis of the relative impact of high Met or Ron expression in the primary tumors of PAAD patients bolstered the current hypothesis that high Met expression is a strong prognosticator of overall survival compared to Ron [10,22,23]. Although the co-expression of Met and Ron is associated with synergistically reducing patient survival in several other cancers, we did not find a synergistic anti-survival effect when both these receptors are highly expressed in primary PAAD [19–21]. The strong anti-survival effect associated with Met expression alone was not substantially affected by the concurrent expression of Ron, which led us to conclude that Met is a strong marker of negative outlook in PAAD independent of Ron. The strong relative association of Met expression with reduced survival in PAAD is consistent with the relatively small impact MSP treatment exerted on BxPC-3 cells compared to HGF. The observation that MSP mimicked only a small subset of HGF dependent effects on these cells may be mechanistically related to the divergence of the prognostic value of Met and Ron expression in primary PAAD.

Although this study provides a preliminary comparative assessment of the impact produced by the HGF/Met and MSP/Ron systems in PAAD, several limitations allow room for future studies. Although the BxPC-3 cell line was chosen for these experiments because it is a widely published upon cell model of primary PAAD, several other cell models of pancreatic cancer exist [61]. A more comprehensive picture of the impact produced by HGF and MSP in pancreatic cancer can be gotten by extending these studies to include other available pancreatic cancer cell lines. Cell lines which represent different mutations commonly observed in pancreatic cancer, such as KRAS, TP53, and SMAD4, in addition to cells which were established from metastatic sites rather than the primary tumor may respond to these growth factors differently [62]. Moreover, we recognize that our survival analysis was limited to samples derived from the primary tumor of PAAD patients and that Ron expression may develop to be a more significant prognostic marker in a different context. Particularly because the MSP/Ron system drove pro-cancer effects that overlapped those driven by the HGF/Met system, Ron expression may be a more significant marker of survival following

the treatment of patients with a Met inhibitor. Additionally, because of the strong pro-migratory effect MSP induced in BxPC-3 cells, the expression of Ron in samples derived from metastases, rather than the primary tumor, may reveal a closer relationship between Ron expression and patient prognosis in pancreatic cancer.

In conclusion, HGF produces a broader scope of cell behavior and transcriptional responses in a PAAD cell line when compared to MSP. The strong response of these cells to HGF treatment is consistent with the status of Met as a strong predictor of patient outlook in PAAD, a property of Met that appears to be unaffected by concomitant Ron expression. Although the elevated expression of Ron in primary PAAD tumors is not associated with poor prognosis, the ability of MSP to drive cancer-related effects like those triggered by HGF still suggests the potential for the MSP/Ron system to support a pro-cancer phenotype in pancreatic cancer.

Data statement

Raw reads from RNA sequencing experiments are hosted at the Gene Expression Omnibus under accession # GSE129075.

Funding

This work was supported by Athira Pharma, Seattle, WA.

CRediT authorship contribution statement

Brett R. Vanderwerff: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Kevin J. Church:** Conceptualization, Supervision, Funding acquisition. **Leen H. Kawas:** Conceptualization, Supervision, Funding acquisition. **Joseph W. Harding:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing, Funding acquisition.

Acknowledgments

We thank Dr. William C. Davis for his guidance and involvement in conducting flow cytometry experiments.

Declaration of Competing Interest

Joseph Harding is a founder and director of Athira Pharma. Leen Kawas is the chief executive officer of Athira Pharma. Kevin Church is an employee of Athira Pharma. Athira Pharma provided funds for the experiments described in this work.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154762.

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