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Ligand-independent Activation of MET Through IGF-1/IGF-1R Signaling

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Abstract

The receptor tyrosine kinase, MET, has been implicated in tumorigenesis and metastasis of many solid tumors, by multiple mechanisms, including cross talk with Epidermal Growth Factor Receptor. In this study, we examined the role of Insulin Like Growth Factor Receptor-1 (IGF-1R) signaling in MET activation, focusing on prostate cancer cells. Stimulation of the prostate cancer cell line PC3 with IGF-1 induces a delayed phosphorylation of MET at multiple sites (indicative of full activation), reaching a maximum 18h after IGF-1 addition. MET activation does not require the sole MET ligand Hepatocyte Growth Factor (HGF), but does require transcription to occur. Furthermore, direct injection of IGF-1 is sufficient to induce MET activation *in vivo*, in a PC3 xenograft model. Pharmacologic or genetic inhibition of the tyrosine kinase, Src, abolishes MET phosphorylation, and expression of activated Src is sufficient to induce Met phosphorylation in the absence of IGF-1 stimulation. Activated MET is essential for IGF-1-mediated increased migration of PC3 cells, demonstrating an important biologic effect of IGF-mediated MET activation. Finally, we demonstrate that IGF-1 induced delayed MET activation occurs in multiple cell lines which express both the receptors, suggesting that IGF-1R-mediated MET activation may contribute to tumorigenic properties of multiple cancer types when both growth factor receptors are expressed. The results further suggest that MET may be activated by multiple receptor tyrosine kinase receptors, and dual targeting of these receptors may be important therapeutically.

Keywords

Prostate Cancer; IGF-1R; MET; Src; cross talk

INTRODUCTION

MET is a receptor tyrosine kinase normally activated in development and in tissue regeneration in adults.^{1, 2} However, strong evidence has demonstrated that aberrant expression of c-Met is important in tumorigenesis and metastasis in a number of solid tumors by contributing to increased cellular proliferation, migration, invasion, epithelial to mesenchymal transition (EMT), and cell survival. Several mechanisms have been demonstrated whereby aberrant expression or activation of MET contributes to

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tumorigenesis or tumor progression. Missense mutations in *c-met*, most frequently occurring in papillary renal cell carcinomas,^{3, 4} autocrine or paracrine activation by the sole MET ligand, hepatocyte growth factor (HGF, also known as scatter factor), especially in central nervous system tumors⁴ and overexpression of MET, in several epithelial and mesenchymal cancers including prostate cancer (PCa),⁵ have all been demonstrated to initiate or enhance tumor growth or metastasis. For these reasons, numerous clinical trials in solid tumors are in progress with MET inhibitors or multi-targeted small molecule tyrosine kinase inhibitors that have MET as one of the principal targets.^{6–10}

Canonical activation of MET results from HGF binding, following which MET dimerizes and increases its activity as a kinase, leading to transphosphorylation of two ‘catalytic’ tyrosine residues (Tyr1234 and Tyr 1235) within the kinase activation loop. These phosphorylations trigger a cascade of downstream signaling events responsible for MET function. Additional Met phosphorylations occur (e.g. on Y1349 (carboxyl terminal docking site)), Y1365 (regulating primarily morphogenesis) and Y1003 (regulating primarily MET internalization and MET degradation). These events are critical for full MET function, and have been well described in numerous reviews.^{2, 8, 9, 11}

More recently, numerous studies have demonstrated that MET activation can occur in the absence of ligand binding. Integrin activation, plexins, CD44, some G protein coupled receptors and other receptor tyrosine kinases including EGFR and RET have all been implicated in MET activation without the requirement for HGF binding.^{12–15} In each case, the mechanism of MET activation is poorly understood, but in most, but not all instances, association of these membrane spanning proteins with MET appears to be important for MET activation to occur.

Activation of MET by EGFR has received extensive attention. EGFR and MET are commonly overexpressed in the same tumors^{16, 17} and transactivation of MET by EGFR appears to require EGFR overexpression.¹⁸ In non-small cell lung cancer with EGFR mutations, similar activation of Met occurs. Recently, Dulak et al. demonstrated that EGFR could activate MET in a delayed, transcription-dependent mechanism, requiring transcription of unknown factors but not requiring direct MET/EGFR association.¹⁹ MET amplification occurs in many patients with tumors harboring EGFR mutations after treatment with the EGFR inhibitor, gefitinib, rendering these patients resistant to gefitinib.²⁰ The above studies demonstrate that EGFR/MET cross talk is complex and may occur through multiple mechanisms.

However, whether MET can be activated by signaling through other growth factor receptors aberrantly expressed or activated in tumor cells is unclear, as are potential functional consequences of such activation and possible roles in tumor progression. In this work, we examine the role of IGF-1 in MET activation, focusing first on prostate cancer cells. In prostate cancer, MET expression increases throughout disease progression and metastasis, with higher levels occurring in bone metastases.²¹ MET overexpression correlates with poor prognosis, yet there is no current evidence of autocrine activation of MET in prostate cancer. IGF-1R is also overexpressed and frequently activated in PCa.²² IGF-1R signaling has been linked to PCa risk, and its ligands are overexpressed in PCa bone metastases,²³ promoting aspects of metastatic growth.^{24–28} We therefore hypothesized that some of the pro-tumorigenic/metastatic effects IGF-1R may occur through MET activation. In this work, we demonstrate that IGF-1 leads to a delayed activation of MET in a transcription-dependent mechanism requiring activation of the tyrosine kinase, Src. We further demonstrate that MET is critical for the increased migratory effects associated with activated IGF-1R.

MATERIALS AND METHODS

Cell lines and media

PC3 cells were a gift from Dr. Isaiah J. Fidler's laboratory, DU 145 cells were a gift from Dr. Renata Pasqualini's laboratory and A549 cells were gift from Dr. John Heymach at The University of Texas MD Anderson Cancer Center. HT-29 cells were obtained from American Type Culture Collection (ATCC). PC3 and HT-29 cells were maintained in Dulbecco's modified Eagle medium nutrient mixture F-12 (Hyclone, Logan, UT) with 10% fetal bovine serum (Sigma, St. Louis, MO) and 1% penicillin/streptomycin (Hyclone, Logan, UT). DU 145 cells were cultured in RPMI-1640 media (Sigma) with 10% fetal bovine serum (Sigma), and 1% penicillin/streptomycin (Hyclone). Cells were checked every six months and found to be mycoplasma free. The M.D. Anderson Cancer Center Department of Systems Biology performed fingerprinting analysis to confirm the correct identity of the cell lines. Cell lines were validated by STR DNA fingerprinting using the AmpF STR Identifier kit according to manufacturer's instructions (Applied Biosystems cat 4322288). The STR profiles were compared to known ATCC fingerprints (ATCC.org), and to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (<http://bioinformatics.istge.it/clima/>) (Nucleic Acids Research 37:D925-D932 PMID: PMC2686526). The STR profiles matched known DNA fingerprints.

Reagents and chemicals

IGF-1 (Catalog # 291-G1) and HGF (Catalog # 294-HGN-005) were purchased from R&D Systems (Minneapolis, MN) and dissolved in PBS. Actinomycin D (Catalog # A1410) was purchased from Sigma. Dasatinib (SPRYCEL®) was a gift from Bristol-Myers Squibb (Princeton, NJ).

Immunoblotting

For all *in vitro* studies, cells were serum starved for 24h prior to stimulation with growth factors. For the IGF-1 time-course study, 100 ng/ml of IGF-1 was used in serum free media for different time points. For the IGF-1 dose-dependent study, 0, 10, 25, 50, 100 and 200 ng/ml of IGF-1 were used to stimulate the cells. For the HGF time-course study, 15ng/ml of HGF was used in serum free media. For Dasatinib studies, cells were pretreated with 100 nM Dasatinib before stimulation with 100 ng/ml IGF-1 or 15 ng/ml HGF for 24h and 10min respectively. Protein lysates were prepared using RIPA B lysis buffer (150 mM NaCl; 5 mM ethylenediaminetetraacetic acid [EDTA]; 20 mM sodium phosphate buffer; and 1% Triton X-100 [Sigma]; pH 7.4) along with 1 tablet of protease and phosphatase inhibitor cocktail (Roche, Indianapolis, IN). Immunoblotting was performed as described by Windham et al.²⁹ Briefly, protein lysates were loaded onto an 8% polyacrylamide gel, which was then transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 5% milk in tris-buffered saline (TBS) with Tween-20 (0.1%). Membranes were probed with specific primary antibodies for c-Met (C12; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Met Y1234/35, phospho-Met Y1349 (Cell Signaling, Danvers, MA), Src (EMD Millipore), phospho-Src Y416, Akt, phospho-Akt (S473), MAPK, phospho-MAPK, IGF-1R, phospho-IGF-1R beta Y1135/36 (Cell Signaling), vinculin (Sigma), and horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (Bio-Rad, Hercules, CA).

RNA isolation and quantitative-RT-PCR

RNA was isolated from the cells by using RNeasy mini kit (Catalog # 74104) (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, cells were serum starved 24 hours prior to IGF-1 stimulation and then 100 ng/ml of IGF-1 was added in serum free media for 0, 18 and 24 hours. Total RNA (200 ng) was reverse transcribed by

using a cDNA synthesis kit (Invitrogen, Life Technologies) and Thermoscript RT enzyme (Invitrogen, Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Gene expression was determined by quantitative PCR (qPCR) using KiCq Start SYBR Green kit (Sigma). The primer sequences used for c-Met, HGF and 18S are listed in Supplementary Table 1.

Enzyme-linked Immunosorbent Assay

To examine the endogenous secretion of HGF, PC3 cells were serum starved for 24h and then stimulated with IGF-1 100 ng/ml for 0, 18 and 24h. Cell culture media was harvested and analyzed in triplicate by human HGF Quantikine ELISA kit (Catalog # DHG00, R&D Systems), according to the manufacturer's instructions.

ShRNA and plasmids transfection

Ready-to-transfect short hairpin (sh) RNA–GFP–puromycin constructs against human IGF1-R (#SR302344) and Src (#SR304574) were purchased from OriGene Technologies (Rockville, MD). A universal non-targeting negative control shRNA (#SR30004) was provided by the manufacturer. An Activated Src (Y527F) expression vector was used as described previously.³⁰ Cells were transfected using Fugene 6 reagent (Roche) according to the manufacturer's instructions. Lentiviral shc-*met* constructs were a gift from Dr. Menashe Bar Eli at The University of Texas MD Anderson Cancer Center. Stable clones were selected with puromycin or sorted with GFP. Target knockdown was verified 3–4 weeks after transfection by western blots.

Migration assay

Migration assays were performed using migration (catalog #354578) assay inserts (BD Biosciences, Bedford, MA) as described by Zhang et al.³¹ To perform these experiments, PC3 cells expressing the non-targeting and c-*met* targeting vector were starved for 24h then incubated with IGF-1 100 ng/ml for 24 hours in a culture dish. Cells were trypsinized and for each cell type, a total of 50,000 cells were seeded on top of the inserts in serum-free media. Media containing IGF-1 (100 ng/ml) or HGF (15 ng/ml) was used as chemoattractant and cells were allowed to migrate for 24 hours. The bottom of the insert was then stained with Hema-Stain kit (EMD Millipore). The number of migrated cells in 8 fields (x 3 per condition) for each group was counted under a bright-field microscope. To study the effect of Src inhibition on IGF-1- and HGF- induced migration in PC3 cells, the previous experiment was performed with the addition of dasatinib (100nM) in the media. Each experiment was performed in triplicate.

Statistics

t tests and one-way ANOVA analysis of variance were used for all statistical comparisons, and a *p* value of <0.05 was considered statistically significant. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, CA).

RESULTS

IGF-1 induces a delayed activation of MET in PCa cell lines and xenograft tumors

We focused on Insulin like Growth Factor (IGF) as it is aberrantly expressed in the microenvironment of prostate cancer bone metastases and known to be a poor prognostic marker for PCa survival.³² We first determined whether IGF-1 could activate MET through a direct or indirect mechanism. For our initial analyses, we chose the prostate cancer (PCa) cell line PC3. This cell line was selected because of the high levels of MET and IGF-1R, which are representative of the majority of metastatic PCa tumors.^{21, 33} To determine if

IGF-1 affected MET phosphorylation at the same sites phosphorylated by HGF (which would be indicative of MET activation), we performed a time course in which IGF-1 was added to serum-starved cells and activation of signaling enzymes examined various times thereafter as described in Materials and Methods. As shown in Fig 1A, robust activation of IGF-1R was observed within 10min, with no activation of MET at this time. Activation of Src, Akt, and Erk1/2 were observed with similar kinetics to IGF-1R activation (Fig. 1B). No change in protein expression was observed for any of these signaling enzymes. In contrast to the acute activation of signaling enzymes after IGF-1 addition, MET phosphorylation was observed beginning at 12h, reaching maximal levels at 18h, with sustained phosphorylation for at least 24h after stimulation of PC3 cells with IGF-1 (Fig. 1A). Both Y1234–1235 (tyrosine kinase domain) and Y1349 (multisubstrate docking) sites were phosphorylated (Fig. 1A), suggesting full activation of MET occurs at these later time points. No differences in MET expression were evident by immunoblotting.

To determine if IGF-1 induction of MET phosphorylation was dose-dependent, we stimulated PC3 cells with different doses of IGF-1 ranging from 10–200 ng/ml. Under these conditions, IGF-1R phosphorylation increased at each concentration of IGF-1 (Fig. 1C), indicating activation of IGF-1R was dose-dependent. In contrast, 25ng/ml IGF-1 was sufficient to induce delayed phosphorylation of MET, and no further increases in phosphorylation were observed with higher concentrations of IGF-1. These results suggest a threshold of IGF-1R activation is sufficient to fully induce MET phosphorylation. MET and IGF-1R protein levels remain unchanged under these conditions (Fig. 1C).

To examine the magnitude of MET phosphorylation due to IGF-1 (100ng/ml) relative to HGF (15ng/ml), PC3 cells were stimulated with these growth factors for 10min and 24h and activation of IGF-1R and MET were examined. IGF-1 induced a strong delayed activation of MET at 24h that was only slightly less (~70%) than the rapid (10min) MET phosphorylation by HGF, suggesting that activation of MET by IGF-1 is biologically important (Fig. 1D).

Because previous studies in NSCLC have shown that EGFR/MET cross talk leads to increased c-Met transcription and MET protein expression,¹⁹ we further analyzed MET expression by qRT-PCR. The results demonstrated no significant changes in c-Met transcription between treated and control group (Supplementary Fig. 1) when MET becomes activated, suggesting a potential different mechanism is involved in IGF-1 -induced MET activation as compared to EGF induced activation (in which MET expression was increased).¹⁹ Finally, we examined the effect of IGF-1 stimulation in MET activation in PC3 xenograft tumors. Briefly, 20μl of IGF-1 (100 ng/ml) were injected directly into established PC3 xenografts and tumors were harvested 24h after injection. Analysis showed that treated tumors had significantly higher levels of MET and IGF-1R phosphorylation compared to control tumors, whereas no changes in MET and IGF-1R expression were observed (Supplementary Fig. 2). Thus, IGF-1 induces MET phosphorylation *in vitro* and *in vivo*.

IGF-1 induced delayed MET activation is ligand independent

HGF is the sole ligand of MET. In prostate cancer metastases, HGF is aberrantly secreted in the tumor microenvironment by stromal cells and activates MET through a paracrine loop.³⁴ Although PC3 cells have been reported previously to not express HGF,³⁵ we performed experiments to confirm this finding. To examine if endogenous expression/secretion of HGF could play a role in MET activation, HGF mRNA was examined by qRT-PCR in PC3 cells treated with IGF-1 for 0h, 18h and 24h (time points that correspond to minimal and maximal MET activation by IGF-1). The results show very low levels of HGF mRNA with no statistically significant differences between control (0h, Ct value = 27.07) and treated (18h, Ct value = 26.91 and 24h, Ct value = 26.96) groups (Fig. 2A). To further examine potential

HGF expression, cell culture media from each group was harvested and levels of HGF determined by enzyme-linked immunosorbent assay. Secreted HGF levels were beneath the level of detection in all groups (Fig. 2B), suggesting that PC3 cells do not express HGF, in agreement with studies of Dai et al,³⁵ and thus, autocrine signaling does not play a role in IGF-1R-mediated MET activation. Finally, to ensure the HGF/MET axis was functional, cells were serum-starved and HGF (15 ng/ml) was added and a time course was performed. As expected, robust phosphorylation of MET on Y1234/1235 and Y1349 was observed peaking at 15 min of HGF addition (Fig. 2C), in sharp contrast to the delayed activation of MET observed following IGF-1 stimulation, indicating that MET is completely functional and has not lost the ability to be activated by HGF.

IGF-1-mediated MET phosphorylation requires IGF-1R Activation

To examine whether IGF-1R activation was required for lateral MET phosphorylation, we generated a PC3 cell line in which IGF-1R was stably knocked down by expression of an shIGF-1R construct as described in Materials and Methods. Expression of IGF-1R was reduced >90%. When the shRNA IGF-1R-expressing cell line was stimulated with IGF-1, delayed MET phosphorylation was abolished; demonstrating activation of IGF-1R is required for MET phosphorylation (Fig. 3A).

Src activation is essential for IGF-1-induced delayed MET activation

In the study of Dulak et al.,¹⁹ the tyrosine kinase, Src, was shown to be an essential mediator of lateral activation of MET by EGFR. To examine potential roles of Src family kinases in delayed MET activation by IGF-1R, we first used the multi-targeted SFK inhibitor, dasatinib. We pretreated PC3 cells with 100 nM of dasatinib for 2h before stimulating with IGF-1 and HGF for 24h and 10min respectively. Under these conditions, IGF-1-induced delayed MET activation is abolished, suggesting that activation of a Src family kinase may be required for delayed MET phosphorylation (Fig 3C). In contrast, dasatinib has little effect on HGF-induced direct MET activation (Fig 3C). Decreases in MET activation observed in dasatinib only treated cells (Fig 3C, lane 2) suggest that dasatinib might have a small effect on MET phosphorylation (dasatinib is a multi-kinase inhibitor). To determine if activation of Src (as opposed to other Src family kinases) were essential to delayed MET phosphorylation by IGF-1, we stably expressed an shSrc in PC3 cells. As shown in Fig. 3A, greater than 90% knockdown of Src was observed. As with non-targeting clones, IGF-1 was added and MET phosphorylation was examined 18h later. In PC3 cells expressing a non-targeting shRNA, Met was phosphorylated under these conditions. In contrast, the shSrc cells were unable to induce delayed MET activation, with expression of MET unchanged, suggesting Src is required for IGF-1 induced MET activation (Fig. 3A).

To further determine whether Src activation were required for IGF-1-induced MET phosphorylation, we transfected PC3 ShSrc cells with a plasmid harboring an activated Src mutant (Y527F) and then stimulated them with IGF-1 for 24h. Expression of an activated Src led to MET phosphorylation in the presence or absence of IGF-1 (Fig. 3B), suggesting Src activation alone is necessary and sufficient to trigger the cascade leading to MET activation.

Inhibition of transcription abolishes IGF-1R-mediated MET phosphorylation

Because MET phosphorylation occurs much later than activation of other signaling intermediates (Src, Akt, Erk1/2), we determined if transcription were required for IGF-1R to MET cross talk. For these experiments, PC3 cells were treated with the pan-transcription inhibitor, actinomycin D, alone or in combination with IGF-1 for time points corresponding to the maximal IGF-1-induced MET activation. Lack of synthesis of the precursor of MET (upper band, Fig. 3D) indicates actinomycin D blocked *de novo c-met* mRNA synthesis.

Processed MET (lower band) was still present, as expected. These results suggest no further transcription of MET occurred under these conditions. Successful inhibition of transcription abolished IGF-1-induced MET activation (Fig. 3D), whereas IGF-1-induced IGF-1R activation (which occurs within 10min and does not require transcription) was unaffected. These findings indicate that transcription is essential for IGF-1-induced MET activation. They further demonstrate that while IGF-1 activates downstream signaling enzymes (e.g. Src) very rapidly, activation of Src is insufficient to directly phosphorylate MET, consistent with other observations that MET is not a direct target of Src¹⁹.

MET inhibition abrogates IGF-1-induced migration

Upon activation, MET signaling plays a critical role in prostate cancer cell invasive growth (scattering, migration, invasion and metastasis). We therefore determined if IGF-1-induced MET activation affected cellular migration and invasion. These experiments were performed with a PC3 cell line stably expressing shRNA to *c-met*, constructed as described in Materials and Methods. Expression of the *c-met*-specific shRNA resulted in a 95% reduction of MET protein compared to PC3 cells expressing a non-targeting shRNA (Fig. 4A). A migration assay was then performed with cells expressing MET or in which MET had been knocked down using a modified Boyden Chamber assay as described in Materials and Methods. Cells that had migrated were counted after 24h as described in Materials and Methods. Under these conditions, IGF-1 induced a 2.5 fold increase in migration in cells expressing a non-targeting vector relative to non-stimulated cells ($p < 0.0001$), (Fig 4B), in agreement with data previously published from other groups.³⁶ In contrast, in cells in which MET was decreased by expression of an shRNA, no induction of migration by IGF-1 was observed (Fig. 5B). In cells not stimulated with IGF-1 but in which HGF was used as a chemoattractant, a 3 fold increase in migration was observed ($p < 0.0001$), consistent with the role of activated MET in promoting migration (Fig. 4C). These results suggest that IGF-1-mediated increased migration is mediated by MET activation. Overall, the experiments confirm that delayed phosphorylation of MET leads to a functional MET capable of inducing one of its principal roles, migration. To determine if inhibition of Src affected IGF-1 and HGF-induced migration, migration assays were performed in the presence of dasatinib. Overall, dasatinib reduced migration due to IGF-1 or HGF, demonstrating the importance of SFKs in mediating migration.³⁷ Specifically, in the presence of dasatinib, migration due to IGF-1 was reduced more than 10 fold. In contrast, migration due to HGF was reduced only 3 fold (Fig. 4D), suggesting Src inhibition does not completely inhibit the ability of HGF to induce migration (Fig. 4D).

MET is Activated in Multiple Cell Lines Expressing IGF-1R

We next examined whether IGF-1 induced MET activation occurred in another commonly used metastatic prostate cancer cell line, DU145, which expresses both IGF-1R and MET. As before, analysis of MET expression and phosphorylation was performed after 0h, 18h and 24h of IGF-1 treatment. Kinetics of MET phosphorylation in these cells were similar to that of PC3 (Fig. 5A). Finally, as IGF-1 is abundantly expressed in the serum of patients with other types of cancer, we examined the effect of IGF-1 on HT29 colon cancer cells and A549 lung cancer cells, both of which express IGF-1R and MET to differing levels. Delayed phosphorylation of MET was observed in both of these cell lines (Fig 5B, C), though not to the extent of the prostate cancer cells that express higher basal levels of MET. These results suggest that delayed activation of MET is likely a common cross talk pathway in cells expressing both IGF-1R and MET, and this process may contribute to tumorigenicity in multiple types of cancer.

Integrins do not mediate cross talk between IGF-1R and MET

While several mechanisms (described above) might contribute to non-ligand mediated MET phosphorylation, integrins are an attractive possibility as increased integrin clustering and activation not only leads to MET activation, but also to that of Src and downstream pathways. To examine this possibility, we stably expressed an Sh β 1 integrin in PC3 cells and stimulated them with IGF-1 as described above. MET was phosphorylated in both non-targeting and sh β 1 integrin “knock-down” cells, suggesting that β 1 integrin is not required for delayed MET activation (Supplementary Fig 3).

DISCUSSION

In solid tumors, aberrant expression of multiple protein tyrosine kinase receptors is common. Increasing evidence suggests that “cross talk” among these receptors leads to activation of one receptor through signaling pathways mediated by another, first exemplified by the ability of activated EGFR to lead to MET activation. In this study, we demonstrate for the first time that in tumor cell lines in which both IGF-1R and MET are expressed, IGF-1R activation is sufficient to lead to a delayed phosphorylation of MET in a process that is independent of the MET ligand, HGF. The IGF-1/IGF-1R system was examined in this study because in prostate cancer, IGF-1R is overexpressed³³, correlates with poor prognosis³⁸, and is abundant in prostate cancer bone metastases.³² We demonstrate that IGF-1R activation by its ligand, IGF-1, must occur for MET phosphorylation, but MET phosphorylation requires transcription to occur and is not apparent until 18h after IGF-1 stimulation. Phosphorylation of MET occurs at each tyrosine examined, suggesting that MET becomes fully activated, in accord with our results demonstrating the requirement of MET for IGF-1-mediated migration. As Bauer et al. have shown in pancreatic cancer cells³⁹ and colon cancer cells⁴⁰ that cellular migration due to IGF-1 requires MET activity, we expanded the types of tumor cell lines examined and determined that in HT29 colon cancer cells and A549 lung cancer cells that express both IGF and MET, delayed activation of MET by IGF also occurs (Fig. 5). Thus MET activation may be common in tumor cells expressing IGF-1R and MET where IGF-1 is present in the microenvironment.

Our findings on MET activation are similar in several respects to those of Dulak et al.¹⁹, who studied “lateral” activation of MET by EGFR in non-small cell lung carcinoma cells. In both their studies and ours, the initiating kinase (EGFR in Dulak et al., 2011; IGF-1R in our studies) must be activated for cross talk to occur, and both require Src activation and transcription. However, whereas MET expression is increased in response to EGF, which may be at least a partial mechanism of its activation,¹⁹ we demonstrate that MET levels are unaffected by stimulation of cells with IGF-1.

The requirement of Src activation in activating MET phosphorylation is intriguing, as expression of a constitutively active Src was sufficient to lead to MET phosphorylation in the absence of IGF-1. This result is somewhat surprising, as Src family kinases are both overexpressed and aberrantly active in many solid tumors, including prostate cancer,⁴¹ leading to Phase I and II with Src inhibitors alone⁴² or Phase 1, 2 and 3 clinical trials in prostate cancer with the Src inhibitor, dasatinib, in combination with docetaxel.^{43, 44} Despite the aberrantly high activity of Src in many tumor cells, addition of IGF-1 or HGF further increased Src activity in all cell lines examined, suggesting Src activity may serve as a rheostat for cross talk among some tyrosine kinase receptors. Due to the experimental conditions (transfection of a constitutively active Src) we cannot determine whether or not Src activation was directly responsible for delayed MET activation or not. We speculate that Src needs to mediate a transcriptional event for MET phosphorylation, but this needs to be studied in more detail. Further, expression of activated Src does not lead to as robust MET phosphorylation as addition of IGF-1. These results suggest that expression of activated Src

may be blocking full MET activation, a phenomenon similar to that observed for the ability of Src to activate ERK at low levels, but then to repress growth factor-induced ERK activation.⁴⁵ The results may also explain why inhibition of Src is less effective in inhibiting IGF-1-induced migration than HGF-induced migration.

Several mechanisms for non-ligand mediated MET activation have been described. Dulak et al.¹⁹ speculate that increased integrin production and/or clustering might contribute to EGFR-mediated MET activation, as integrin activation leads to both increased MET and Src family kinase activities. Activation of $\beta 1$ integrins appears very important in metastasis.⁴⁶ In PCa cells, $\beta 1$, and $\beta 3$ integrins are expressed,¹² and we have shown recently that inhibition of $\beta 1$ integrins inhibits prostate cancer metastasis to the bone following intracardiac injection.⁴⁷ However, when we knocked-down $\beta 1$ integrin, IGF-1-induced MET activation still occurred, suggesting either the involvement of $\beta 3$ integrin in Met activation, or that integrin activation is not required in the process of IGF-1-induced MET activation. We are currently investigating other possible mechanisms of MET activation, including up-regulation of plexins, which are overexpressed in prostate cancer and which have also been shown to be able to activate MET in the absence of HGF.^{13, 48} Further work is required to determine the non-liganded “partner” of Met that leads to its phosphorylation and activation.

Cross talk among receptors may be a common occurrence in cancer cells. Numerous studies have demonstrated roles for EGFR in MET biology.^{18, 49–51} For example, HGF has been shown to lead to a transcriptional upregulation of TGF α and EGFR activation in human glioblastoma cells.⁵² In mesothelioma cell lines expressing high levels of EGFR and MET, not only did EGF induce Met phosphorylation, HGF induced EGFR phosphorylation, suggesting bi-directional activation of MET and EGFR may occur, although the mechanisms were not studied.⁵³ How common cross talk occurs among other aberrantly expressed tyrosine kinase receptors remains to be determined, but activation of multiple protein tyrosine kinases has obvious implications for therapeutic strategies using tyrosine kinase inhibitors. Dulak et al.¹⁹ showed that combining EGFR and MET tyrosine kinase inhibitors modestly enhanced tumor inhibition. These results are somewhat surprising if MET were activated only through EGF, as inhibition of EGFR should be able to block MET activation. Rather, the results suggest that the combined effects on increased inhibition may be due to individual effects of the two receptor tyrosine kinases, as well as cross talk between them. In agreement with this latter possibility Wu et al., examining uveal melanoma cell lines, demonstrated that IGF-1R, MET, and EGFR have distinct contributions in cellular migration.⁵¹

Thus, our studies add to an increasing body of work that suggest combining inhibitors of tyrosine kinases might be a good therapeutic strategy when more than one is activated. Activation of MET through multiple ligand- independent mechanisms indicates that MET might be an important downstream node through which other tyrosine kinase receptors mediate their biologic effects. Finally, only a few of the intermediates required for delayed activation of MET are known; thus identification of novel, potentially common intermediates in these processes may lead to identification of novel targets for therapy in which multiple tyrosine kinase receptors are activated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EMT	epithelial to mesenchymal transition
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor 1
IGF-1R	IGF-1 receptor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
PCa	Prostate cancer
CRPC	Castrate resistant prostate cancer

References

1. Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature*. 1995; 376:768–71. [PubMed: 7651534]
2. Trusolino L, Bertotti A, Comoglio PM. MET signalling: principles and functions in development, organ regeneration and cancer. *Nat Rev Mol Cell Biol*. 2010; 11:834–48. [PubMed: 21102609]
3. Schmidt L, Duh FM, Chen F, Kishida T, Glenn G, Choyke P, Scherer SW, Zhuang Z, Lubensky I, Dean M, Allikmets R, Chidambaram A, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nat Genet*. 1997; 16:68–73. [PubMed: 9140397]
4. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol*. 2003; 4:915–25. [PubMed: 14685170]
5. <http://www.vai.org/Met/Index.aspx>.
6. Varkaris A, Corn PG, Gaur S, Dayyani F, Logothetis CJ, Gallick GE. The role of HGF/c-Met signaling in prostate cancer progression and c-Met inhibitors in clinical trials. *Expert opinion on investigational drugs*. 2011; 20:1677–84. [PubMed: 22035268]
7. Liu X, Newton RC, Scherle PA. Development of c-MET pathway inhibitors. *Expert opinion on investigational drugs*. 2011; 20:1225–41. [PubMed: 21740293]
8. Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET incancer: rationale and progress. *Nature reviews Cancer*. 2012; 12:89–103.
9. Eder JP, Vande Woude GF, Boerner SA, LoRusso PM. Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2009; 15:2207–14. [PubMed: 19318488]
10. Yap TA, de Bono JS. Targeting the HGF/c-Met axis: state of play. *Molecular cancer therapeutics*. 2010; 9:1077–9. [PubMed: 20442310]
11. Gao CF, Vande Woude GF. HGF/SF-Met signaling in tumor progression. *Cell research*. 2005; 15:49–51. [PubMed: 15686627]
12. Fornaro M, Manes T, Languino LR. Integrins and prostate cancer metastases. *Cancer Metastasis Rev*. 2001; 20:321–31. [PubMed: 12085969]
13. Giordano S, Corso S, Conrotto P, Artigiani S, Gilestro G, Barberis D, Tamagnone L, Comoglio PM. The semaphorin 4D receptor controls invasive growth by coupling with Met. *Nat Cell Biol*. 2002; 4:720–4. [PubMed: 12198496]
14. Ghatak S, Hascall VC, Markwald RR, Misra S. Stromal hyaluronan interaction with epithelial CD44 variants promotes prostate cancer invasiveness by augmenting expression and function of

- hepatocyte growth factor and androgen receptor. *The Journal of biological chemistry*. 2010; 285:19821–32. [PubMed: 20200161]
15. Lai AZ, Abella JV, Park M. Crosstalk in Met receptor oncogenesis. *Trends Cell Biol*. 2009; 19:542–51. [PubMed: 19758803]
 16. Shattuck DL, Miller JK, Carraway KL 3rd, Sweeney C. Met receptor contributes to trastuzumab resistance of Her2–overexpressing breast cancer cells. *Cancer Res*. 2008; 68:1471–7. [PubMed: 18316611]
 17. Nakajima M, Sawada H, Yamada Y, Watanabe A, Tatsumi M, Yamashita J, Matsuda M, Sakaguchi T, Hirao T, Nakano H. The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. *Cancer*. 1999; 85:1894–902. [PubMed: 10223227]
 18. Jo M, Stolz DB, Esplen JE, Dorko K, Michalopoulos GK, Strom SC. Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. *The Journal of biological chemistry*. 2000; 275:8806–11. [PubMed: 10722725]
 19. Dulak AM, Gubish CT, Stabile LP, Henry C, Siegfried JM. HGF-independent potentiation of EGFR action by c-Met. *Oncogene*. 2011; 30:3625–35. [PubMed: 21423210]
 20. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science*. 2007; 316:1039–43. [PubMed: 17463250]
 21. Knudsen BS, Gmyrek GA, Inra J, Scherr DS, Vaughan ED, Nanus DM, Kattan MW, Gerald WL, Vande Woude GF. High expression of the Met receptor in prostate cancer metastasis to bone. *Urology*. 2002; 60:1113–7. [PubMed: 12475693]
 22. Su AI, Welsh JB, Sapinoso LM, Kern SG, Dimitrov P, Lapp H, Schultz PG, Powell SM, Moskaluk CA, Frierson HF Jr, Hampton GM. Molecular classification of human carcinomas by use of gene expression signatures. *Cancer Res*. 2001; 61:7388–93. [PubMed: 11606367]
 23. Kimura T, Kuwata T, Ashimine S, Yamazaki M, Yamauchi C, Nagai K, Ikehara A, Feng Y, Dimitrov DS, Saito S, Ochiai A. Targeting of bone-derived insulin-like growth factor-II by a human neutralizing antibody suppresses the growth of prostate cancer cells in a human bone environment. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 16:121–9. [PubMed: 20028742]
 24. Papageorgiou E, Pitulis N, Manoussakis M, Lembessis P, Koutsilieris M. Rosiglitazone attenuates insulin-like growth factor 1 receptor survival signaling in PC-3 cells. *Molecular medicine (Cambridge, Mass)*. 2008; 14:403–11.
 25. Ngo TH, Barnard RJ, Leung PS, Cohen P, Aronson WJ. Insulin-like growth factor I (IGF-I) and IGF binding protein-1 modulate prostate cancer cell growth and apoptosis: possible mediators for the effects of diet and exercise on cancer cell survival. *Endocrinology*. 2003; 144:2319–24. [PubMed: 12746292]
 26. Miyake H, Nelson C, Rennie PS, Gleave ME. Overexpression of insulin-like growth factor binding protein-5 helps accelerate progression to androgen-independence in the human prostate LNCaP tumor model through activation of phosphatidylinositol 3'-kinase pathway. *Endocrinology*. 2000; 141:2257–65. [PubMed: 10830316]
 27. Maloney EK, McLaughlin JL, Dagdigian NE, Garrett LM, Connors KM, Zhou XM, Blattler WA, Chittenden T, Singh R. An anti-insulin-like growth factor I receptor antibody that is a potent inhibitor of cancer cell proliferation. *Cancer Res*. 2003; 63:5073–83. [PubMed: 12941837]
 28. Krueckl SL, Sikes RA, Edlund NM, Bell RH, Hurtado-Coll A, Fazli L, Gleave ME, Cox ME. Increased insulin-like growth factor I receptor expression and signaling are components of androgen-independent progression in a lineage-derived prostate cancer progression model. *Cancer Res*. 2004; 64:8620–9. [PubMed: 15574769]
 29. Windham TC, Parikh NU, Siwak DR, Summy JM, McConkey DJ, Kraker AJ, Gallick GE. Src activation regulates anoikis in human colon tumor cell lines. *Oncogene*. 2002; 21:7797–807. [PubMed: 12420216]
 30. Allgayer H, Wang H, Gallick GE, Crabtree A, Mazar A, Jones T, Kraker AJ, Boyd DD. Transcriptional induction of the urokinase receptor gene by a constitutively active Src.

- Requirement of an upstream motif (–152/–135) bound with Sp1. *The Journal of biological chemistry*. 1999; 274:18428–37. [PubMed: 10373450]
31. Zhang J, Park SI, Artime MC, Summy JM, Shah AN, Bomser JA, Dorfleitner A, Flynn DC, Gallick GE. AFAP-110 is overexpressed in prostate cancer and contributes to tumorigenic growth by regulating focal contacts. *The Journal of clinical investigation*. 2007; 117:2962–73. [PubMed: 17885682]
 32. Dayyani F, Parikh NU, Varkaris AS, Song JH, Moorthy S, Chatterji T, Maity SN, Wolfe AR, Carboni JM, Gottardis MM, Logothetis CJ, Gallick GE. Combined Inhibition of IGF-1R/IR and Src Family Kinases Enhances Antitumor Effects in Prostate Cancer by Decreasing Activated Survival Pathways. *PloS one*. 2012; 7:e51189. [PubMed: 23300537]
 33. Hellawell GO, Turner GD, Davies DR, Poulson R, Brewster SF, Macaulay VM. Expression of the type 1 insulin-like growth factor receptor is up-regulated in primary prostate cancer and commonly persists in metastatic disease. *Cancer Res*. 2002; 62:2942–50. [PubMed: 12019176]
 34. Humphrey PA, Zhu X, Zarnegar R, Swanson PE, Ratliff TL, Vollmer RT, Day ML. Hepatocyte growth factor and its receptor (c-MET) in prostatic carcinoma. *The American journal of pathology*. 1995; 147:386–96. [PubMed: 7639332]
 35. Dai Y, Siemann DW. Constitutively active c-Met kinase in PC-3 cells is autocrine-independent and can be blocked by the Met kinase inhibitor BMS-777607. *BMC cancer*. 2012; 12:198. [PubMed: 22639908]
 36. Montagnani Marelli M, Moretti RM, Procacci P, Motta M, Limonta P. Insulin-like growth factor-I promotes migration in human androgen-independent prostate cancer cells via the α 5 β 3 integrin and PI3-K/Akt signaling. *International journal of oncology*. 2006; 28:723–30. [PubMed: 16465378]
 37. Park SI, Zhang J, Phillips KA, Araujo JC, Najjar AM, Volgin AY, Gelovani JG, Kim SJ, Wang Z, Gallick GE. Targeting SRC family kinases inhibits growth and lymph node metastases of prostate cancer in an orthotopic nude mouse model. *Cancer Res*. 2008; 68:3323–33. [PubMed: 18451159]
 38. Chan JM, Stampfer MJ, Ma J, Gann P, Gaziano JM, Pollak M, Giovannucci E. Insulin-like growth factor-I (IGF-I) and IGF binding protein-3 as predictors of advanced-stage prostate cancer. *Journal of the National Cancer Institute*. 2002; 94:1099–106. [PubMed: 12122101]
 39. Bauer TW, Somcio RJ, Fan F, Liu W, Johnson M, Lesslie DP, Evans DB, Gallick GE, Ellis LM. Regulatory role of c-Met in insulin-like growth factor-I receptor-mediated migration and invasion of human pancreatic carcinoma cells. *Molecular cancer therapeutics*. 2006; 5:1676–82. [PubMed: 16891453]
 40. Bauer TW, Fan F, Liu W, Johnson M, Parikh NU, Parry GC, Callahan J, Mazar AP, Gallick GE, Ellis LM. Insulinlike growth factor-I-mediated migration and invasion of human colon carcinoma cells requires activation of c-Met and urokinase plasminogen activator receptor. *Ann Surg*. 2005; 241:748–56. discussion 56–8. [PubMed: 15849510]
 41. Summy JM, Gallick GE. Src family kinases in tumor progression and metastasis. *Cancer Metastasis Rev*. 2003; 22:337–58. [PubMed: 12884910]
 42. Lara PN Jr, Longmate J, Evans CP, Quinn DI, Twardowski P, Chatta G, Posadas E, Stadler W, Gandara DR. A phase II trial of the Src-kinase inhibitor AZD0530 in patients with advanced castration-resistant prostate cancer: a California Cancer Consortium study. *Anti-cancer drugs*. 2009; 20:179–84. [PubMed: 19396016]
 43. Araujo JC, Mathew P, Armstrong AJ, Braud EL, Posadas E, Lonberg M, Gallick GE, Trudel GC, Paliwal P, Agrawal S, Logothetis CJ. Dasatinib combined with docetaxel for castration-resistant prostate cancer: results from a phase 1–2 study. *Cancer*. 2012; 118:63–71. [PubMed: 21976132]
 44. <http://www.clinicaltrials.gov/ct2/show/NCT00744497?term=dasatinib+docetaxel&rank=1>.
 45. Stofega MR, Yu CL, Wu J, Jove R. Activation of extracellular signal-regulated kinase (ERK) by mitogenic stimuli is repressed in v-Src-transformed cells. *Cell growth & differentiation: the molecular biology journal of the American Association for Cancer Research*. 1997; 8:113–9.
 46. Kato H, Liao Z, Mitsios JV, Wang HY, Deryugina EI, Varner JA, Quigley JP, Shattil SJ. The Primacy of β 1 Integrin Activation in the Metastatic Cascade. *PloS one*. 2012; 7:e46576. [PubMed: 23056350]

47. Lee YC, Jin JK, Cheng CJ, Huang CF, Song JH, Huang M, Brown WS, Zhang S, Yu-Lee LY, Yeh ET, McIntyre BW, Logothetis CJ, et al. Targeting Constitutively Activated beta1 Integrin inhibits Prostate Cancer Metastasis. *Mol Cancer Res*. 2013
48. Wong OG, Nitkunan T, Oinuma I, Zhou C, Blanc V, Brown RS, Bott SR, Nariculam J, Box G, Munson P, Constantinou J, Feneley MR, et al. Plexin-B1 mutations in prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:19040–5. [PubMed: 18024597]
49. Bonine-Summers AR, Aakre ME, Brown KA, Arteaga CL, Pietenpol JA, Moses HL, Cheng N. Epidermal growth factor receptor plays a significant role in hepatocyte growth factor mediated biological responses in mammary epithelial cells. *Cancer biology & therapy*. 2007; 6:561–70. [PubMed: 17495520]
50. Bergstrom JD, Westermarck B, Heldin NE. Epidermal growth factor receptor signaling activates met in human anaplastic thyroid carcinoma cells. *Experimental cell research*. 2000; 259:293–9. [PubMed: 10942601]
51. Wu X, Zhou J, Rogers AM, Janne PA, Benedettini E, Loda M, Hodi FS. c-Met, epidermal growth factor receptor, and insulin-like growth factor-1 receptor are important for growth in uveal melanoma and independently contribute to migration and metastatic potential. *Melanoma Res*. 2012; 22:123–32. [PubMed: 22343486]
52. Reznik TE, Sang Y, Ma Y, Abounader R, Rosen EM, Xia S, Laterra J. Transcription-dependent epidermal growth factor receptor activation by hepatocyte growth factor. *Mol Cancer Res*. 2008; 6:139–50. [PubMed: 18234969]
53. Brevet M, Shimizu S, Bott MJ, Shukla N, Zhou Q, Olshen AB, Rusch V, Ladanyi M. Coactivation of receptor tyrosine kinases in malignant mesothelioma as a rationale for combination targeted therapy. *J Thorac Oncol*. 2011; 6:864–74. [PubMed: 21774103]

Figure 1. IGF-1 induces MET activation

A, B. IGF-1 was added (100ng/ml) to PC3 cells and expression and phosphorylation of signaling intermediates examined at indicated times thereafter. C. Indicated concentrations of IGF-1 were added and immunoblotting was performed 24h later. D. IGF-1(100ng/ml) and HGF(15ng/ml) were added to PC3 cells at indicated times and the magnitude of MET activation was examined. For all immunoblots, vinculin was used as a loading control.

Figure 2. Expression of HGF after IGF-1 addition

24h after IGF-1 addition (100ng/ml), HGF expression was examined by: A. qRT-PCR; B. ELISA on HGF was performed as described in Materials and Methods. Data represent Mean and SEM from three independent experiments. C. Exogenous HGF (15ng/ml) was added to PC3 cells and MET phosphorylation was examined at indicated times.

Figure 3. Dependence on IGF-1R, SRC and transcription for MET phosphorylation

A. IGF-1 (100ng/ml) was added to non-targeting cells or cells that stably expressed shIGF-1R or shSrc blocked and MET phosphorylation was examined 24h later. Vinculin was used as a loading control. B. PC3 cells were transfected with a constitutively active SRC (Src Y527F), stimulated with IGF-1 (100ng/ml) phosphorylation of MET (pY1349) was examined 24h later. C. Cells were pre-treated with 100nM of Dasatinib for 2h after which they were stimulated with IGF-1 (100ng/ml) for 24h or HGF (15ng/ml) for 10min. MET phosphorylation was examined. D. Actinomycin D (0.01mg/ml) was added to cells to inhibit transcription. IGF-1 was added (100ng/ml) and MET phosphorylation examined 18h (lanes 3 and 5) and 24h (lanes 4 and 6) later. Vinculin was used as a loading control.

Figure 4. Role of MET in IGF-1-mediated migration of PC3 Cells

A. Stable knockdown of MET by expression of shRNA. **B.** Effect of IGF-1 on migration of Cells. 5×10^4 Cells were plated on top of a Boyden Chamber as described in Materials and Methods. IGF-1 (100ng/ml) was used as a chemoattractant. Migration was compared in *shc-Met* knockdown cells and respective controls. Data represent Mean and SEM from three independent experiments; * $p < 0.0001$ (one-way ANOVA analysis of variance). **C.** Role of MET in migration of PC3 cells. The same experiment was performed as described in B, except that HGF was used as a chemoattractant. **D.** Effect of Dasatinib in IGF-1 and HGF-induced cell migration. Experiments described in B and C were performed in the presence of Dasatinib (100nM). Data represent Mean and SEM from three independent experiments; * $p < 0.0001$ (one-way ANOVA analysis of variance).

Figure 5. Effects of IGF-1 on Met phosphorylation in Different Cell Types

DU145 (prostate Ca) HT29 (colon Ca) and A549 (lung Ca) cell lines, expressing both IGF-1R and MET were stimulated with IGF-1 (100ng/ml) as described in Materials and Methods. Expression and phosphorylation of MET and IGF-1R examined 18 and 24h later. Vinculin was used as a loading control.