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Original Article

MET Suppresses Epithelial VEGFR2 via Intracrine VEGF-induced Endoplasmic Reticulum-associated Degradation

Tom T. Chen [a], Ellen Filvaroff [a,1], Jing Peng [b], Scot Marsters [a], Adrian Jubb [c], Hartmut Koeppen [c], Mark Merchant [b], Avi Ashkenazi [a,*]

[a] Cancer Immunology, Genentech, Inc. 1 DNA Way, South San Francisco, CA 94080, USA
[b] In Vivo Pharmacology, Genentech, Inc. 1 DNA Way, South San Francisco, CA 94080, USA
[c] Research Pathology, Genentech, Inc. 1 DNA Way, South San Francisco, CA 94080, USA

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ABSTRACT

Hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) drive cancer through their respective receptors, MET and VEGF receptor 2 (VEGFR2). VEGFR2 inhibits MET by promoting MET dephosphorylation. However, whether MET conversely regulates VEGFR2 remains unknown. Here we show that MET suppresses VEGFR2 protein by inducing its endoplasmic-reticulum-associated degradation (ERAD), via intracrine VEGF action. HGF–MET signaling in epithelial cancer cells promoted VEGF biosynthesis through PI3-kinase. In turn, VEGF and VEGFR2 associated within the ER, activating inositol-requiring enzyme 1α, and thereby facilitating ERAD-mediated depletion of VEGFR2. MET disruption upregulated VEGFR2, inducing compensatory tumor growth via VEGFR2 and MEK. However, concurrent disruption of MET and either VEGF or MEK circumvented this, enabling more profound tumor inhibition. Our findings uncover unique cross-regulation between MET and VEGFR2—two RTKs that play significant roles in tumor malignancy. Furthermore, these results suggest rational combinatorial strategies for targeting RTK signaling pathways more effectively, which has potentially important implications for cancer therapy.

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1. Introduction

A number of growth factors and cognate receptor-tyrosine-kinases (RTKs) display genetic alterations in cancer and contribute to various aspects of tumor progression (Choura and Rebai, 2011; Takeuchi and Ito, 2011). Hepatocyte growth factor (HGF), also known as scatter factor, signals through the RTK MET, mainly to regulate epithelial-cell functions including motility, invasiveness, survival and proliferation (Gherardi et al., 2012). In contrast, vascular endothelial growth factor (VEGF), particularly VEGF-A, signals through the RTK VEGFR2, primarily to regulate endothelial cell activities that facilitate vasculogenesis, angiogenesis and vascular function (Carmeliet and Jain, 2011; Ellis and Hicklin, 2008; Ferrara et al., 2003). Aberrant MET stimulation in tumor epithelial cells, via activating mutations, gene amplification and/or mRNA and protein overexpression, increases tumor aggressiveness and correlates with poor prognosis (Gherardi et al., 2012; Sadiq and Salgia, 2013). On the other hand, VEGF production by malignant epithelial cells or associated stromal cells enables the formation and maintenance of vascular networks that support tumor growth. Although VEGFR2 is expressed most frequently on tumor endothelial cells (Smith et al., 2010), it can be expressed also by malignant epithelial cells, and promotes their proliferation—for example, in concert with signaling by epidermal growth factor receptor (EGFR) (Goel and Mercurio, 2013; Lichtenberger et al., 2010). Germline variations in the VEGFR2 gene alter expression of VEGFR2 protein in tumor endothelial and epithelial cells, as well as VEGFR2's involvement in tumor vascularization (Glubb et al., 2011).

Beyond individual RTK contributions, evidence suggests that crosstalk between different RTKs augments tumor growth and promotes resistance to conventional or targeted therapies (Chong and Janne, 2013; Engelman et al., 2007; Wilson et al., 2012). MET interacts functionally with several RTKs, including EGFR, ERBB2 and IGF-1R (Bauer et al., 2006; Boon et al., 2002; Engelman et al., 2007; Khoury et al., 2005; Liu et al., 2009; Yamamoto et al., 2006). EGFR stimulation drives MET phosphorylation (Yamamoto et al., 2006), while MET-gene amplification in lung cancer cells harboring resistance-conferring EGFR mutations activates ERBB3–PI3-kinase (PI3K) signaling (Engelman et al., 2007). Combined EGFR and MET inhibition showed enhanced efficacy against human NSCLC tumor xenografts in mice (Turke et al., 2010). In a phase II clinical study, concurrent treatment with the EGFR inhibitor erlotinib and the anti-MET antibody onartuzumab improved survival as compared to erlotinib monotherapy in a subset of NSCLC patients expressing high tumor

* Corresponding author.
E-mail address: aa@gene.com (A. Ashkenazi).
[1] Current address: Celgene, San Francisco, CA, USA.

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levels of MET (Spigel et al., 2013); however, a subsequent phase III trial did not recapitulate this latter finding (ASCO 2014). VEGFR2 and MET also can cross-interact: in glioblastoma multiforme cells, VEGFR2 inhibits MET phosphorylation through enhanced recruitment of protein tyrosine phosphatase 1B, thereby suppressing MET-dependent tumor invasiveness (Lu et al., 2012). It remains unknown, however, whether MET reciprocally regulates VEGFR2—and if so—then how and to what consequence. We demonstrate here that MET suppresses VEGFR2 in epithelial cancer cells expressing both RTKs, through a unique, cell-autonomous mechanism involving intracrine VEGF and endoplasmic reticulum associated degradation or ERAD. MET disruption upregulates VEGFR2, which drives compensatory tumor growth. Importantly, this undesired outcome of MET disruption can be blocked by concurrent inhibition of the MET and VEGFR2 pathways. Our results underscore the potential of combinatorial RTK inhibition to enhance anti-tumor efficacy. More specifically, our data provide translational strategies for increasing the efficacy of therapeutic modalities targeting the HGF–MET and VEGF–VEGFR2 pathways in cancer.

2. Materials and Methods

2.1. Patient Tumor Samples

Formalin-fixed paraffin-embedded tissues from 31 non-small cell lung cancers (9 adenocarcinomas, 3 adenosquamous carcinomas, 13 squamous cell carcinomas and 6 large cell carcinomas) were obtained from multiple sources (Advanced Bioscience Laboratories, Kensington, MD; University of Michigan, Ann Arbor, MI; Cureline, South San Francisco, CA; Cooperative Human Tissue Network, Nashville, TN; ProteoGenex, Culver City, CA; Cytomix (Origene), Rockville, MD; MT Group, Van Nuys, CA, USA). Histological diagnosis was confirmed centrally by a pathologist (H.K.).

2.2. Cell lines and Cell Culture

H441, C829, C32, PC-3, H1838 and H2347 were purchased from ATCC. PSN1, UM-UC-1 and UM-UC-3 cells were purchased from ECACC. RERF_LC, EBC1 and KP4 were purchased from JHSF. The NSCLC cell line LKPH4 was derived from a $KRas^{LSL\text{-}G12D/+}$;$p53^{FL/+}$;Z/EG lung tumor-bearing mouse. H441 cells were cultured in F12/DMEM (50:50) with 10% Fetal Bovine Serum and 2 mM L-glutamine. C829, C32, PC-3, H1838, H2347, PSN1, UM-UCs, RERF_LC, EBC1 and KP4 were cultured in RPMI 1640 with 10% Fetal Bovine Serum and 2 mM L-glutamine. LKPH4 cells were cultured in DMEM high glucose with 10% FBS and 2 mM L-glutamine. Cells grown under hypoxia were incubated for 48 h under an atmosphere of 5% CO_2-balanced N_2 to obtain 1% O_2 at 37 °C. Otherwise grown under normoxia at 37 °C, 5% CO_2.

2.3. Reagents

Recombinant human $VEGF_{165}$ and HGF were generated and purified at Genentech (South San Francisco, CA). Antibodies against human VEGFR2, phospho-VEGFR2 (Tyr1175), MET, phospho-MET (Tyr1234/1235), Akt, phospho-Akt (S473), Rab5/8/9, Cbl, Cbl-b, ubiquitin, Gab1, phospho-Gab1, PLC, phospho-PLC, MEK, phospho-MEK, ERK, phospho-ERK, phospho-S6, EGFR, PERK, CHOP and BIP were from Cell Signaling (Beverly, MA). Anti-ATF6 was from Cosmo Bio Co. (Tokyo, Japan). Antibodies against phosphotyrosine (4G10) and GAPDH were from Millipore (Billerica, MA). Antibodies against actin, tubulin, and VEGFR1 were from Abcam (Cambridge, MA). Mouse anti-VEGF was from Origene (Rockville, MD). Rabbit anti-VEGFR2 (N-terminus) was from Cell Sciences (Canton, MA). FGFR1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against ER marker (P4HB/PDI), Golgi marker (GOLGA2), gp78 (AMFR), and HRD1 (SYVN-1) were purchased from Sigma Aldrich (St. Louis, MO). K48-ubiquitin, XBP-1s, phosphor-IRE1 α antibodies were generated and purified at Genentech. MG132 and Dynasore were from Calbiochem (La Jolla, CA). Small molecule inhibitors (SMI) against MET (GDC0712), PI3K (GDC0941), MEK (cobimetinib), JAK (G00043484), and IRE1α (compound 3 and 4μ8c) were synthesized for Genentech. SU4312 was from Enzo (Farmingdale, NY). Leupeptin and pepstatin A were from Sigma Aldrich. E-64d was from Cayman Chemicals (Ann Arbor, Michigan).

2.4. Mouse Studies

Five million H441.shMet 3.11 cells suspended in HBSS were inoculated subcutaneously in the right flank of CRL nu/nu mice (Charles River Laboratories). When tumors reached an average volume of ~250 mm^3, mice (8 per group) were treated with either 5% sucrose water (provided as drinking water, changed weekly) plus MCT ((0.5% [w/v] methylcellulose, 0.2% [w/v] polysorbate 80 [Tween-80], 0.1 ml, daily, oral gavage), Doxycycline (0.2 mg/ml, dissolved in 5% sucrose water, changed 3×/week), B20-4.1.1 (anti-VEGF antibody, 5 mg/kg, intraperitoneal, 2×/week), or cobimetinib (MEKi, 5 mg/kg, daily orally dosed for the duration of the study), or the combination of Doxycycline plus B20-4.1.1 or Doxycycline plus cobimetinib. Tumor volumes were measured in two dimensions (length and width) using Ultra Cal IV calipers (Model 54 10 111; Fred V. Fowler Company; Newton, MA). The tumor volume was calculated using the following formula: tumor volume (mm^3) = (length × $width^2$) × 0.5. All procedures were approved by and conformed to the guidelines and principles set by the Institutional Animal Care and Use Committee of Genentech and were carried out in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility.

2.5. Generation of Cell Lines Stably Expression Dox-inducible MET shRNA

Two independent MET shRNA were cloned into pHUSH vector as described (Pai et al., 2008). The sequence used in the studies is as follows.

shMET 3	5′-GATCCCC**GAACAGAATCACTGACATA**TTCAAGAGA**TATGTC AGTGATTCTGTTC**TTTTTTGGAAA-3′
shMET 4	5′-GATCCCC**GAAACTGTATGCTGGATGA**TTCAAGAGA**TCATCC AGCATACAGTTTC**TTTTTTGGAAA-3′
shGFP2 EGFP shRNA (sense)	5′-GATCCCCAGATCCGCCACAACATCGATTCAAGAGATCGAT GTTGTGGCGGATCTTGTTTTTTGGAAA-3′

All constructs were confirmed by sequencing. EGFP control shRNA was described previously (Pai et al., 2008). The shRNA containing retrovirus was produced by co-transfecting GP2-293 packaging cells (Clontech Laboratories, Mountain View, CA) with VSV-G (Clontech Laboratories) and pHUSH-MET shRNA constructs. Viral supernatants were harvested 72 h after transfection, and cleared of cell debris by centrifugation for transduction.

H441 cells were maintained in F12/DMEM 50/50 medium containing tetracycline-free FBS (Clontech Laboratories), and transduced with retroviral supernatant in the presence of 4 μg/ml polybrene. 72 h after infection, 2 μg/ml puromycin (Clontech Laboratories) was added to the medium to select stable clones expressing shRNA. Clones were isolated, treated with 0.1 or 1 μg/ml doxycycline (Clontech Laboratories) for 4 days, and inducible knockdown of MET protein was assessed by immunoblot analysis. Cell cycle analyses were performed as described (Pegram et al., 1999).

2.6. Patient-derived Tumor Xenograft Microarray Analysis

Patient-derived lung tumor xenograft samples were analyzed using Affymetrix Human Genome U133 Plus 2.0 arrays. The Bioconductor

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