CD44 regulates Hepatocyte Growth Factor-mediated vascular integrity: Role of c-Met, Tiam1/Rac1, dynamin 2 and cortactin


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Running Title: HGF/c-Met/CD44 regulation of vascular integrity

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The preservation of vascular endothelial cell (EC) barrier integrity is critical to normal vessel homeostasis with barrier dysfunction a feature of inflammation, tumor angiogenesis, atherosclerosis and acute lung injury. Therefore, agents that preserve or restore vascular integrity have important therapeutic implications. This study explored the regulation of hepatocyte growth factor (HGF)-mediated enhancement of EC barrier function via CD44 isoforms. We observed that HGF promotes c-Met association with CD44v10 and recruitment of c-Met into caveolin-enriched microdomains (CEM) containing CD44s (standard form). Treatment of EC with CD44v10 blocking antibodies inhibited HGF-mediated c-Met phosphorylation and c-Met recruitment to CEM. Silencing CD44 expression (siRNA) attenuated HGF-induced recruitment of c-Met, Tiam1 (a Rac1 exchange factor), cortactin (an actin cytoskeletal regulator) and dynamin 2 (a vesicular regulator) to CEMs as well as HGF-induced trans-EC electrical resistance (TER). In addition, silencing of either Tiam1 or dynamin 2 reduced HGF-induced Rac1 activation, cortactin recruitment to CEM and EC barrier regulation. To validate these in vitro findings in an in vivo murine model of inflammatory lung injury, we observed that both HGF and the CD44 ligand, high molecular weight hyaluronan (HMW-HA)-mediated protection from lipopolysaccharide (LPS)-induced pulmonary vascular hyper-permeability were significantly reduced in CD44 knockout mice. Taken together, these results suggest that CD44 is an important regulator of HGF/c-Met-mediated in vitro and in vivo barrier enhancement, a process with essential involvement of Tiam1, Rac1, dynamin 2, and cortactin.


Introduction

Endothelial cells (EC) constitute the inner lining of all blood vessels and regulate the interface between the circulating blood and the
vessel wall including vascular barrier regulation, passive diffusion and active transport of blood-borne substances, regulation of vascular smooth muscle tone and blood coagulation properties (1,2). Disruption of this semi-selective cellular barrier is a critical feature of inflammation as well as an important contributing factor to atherosclerosis and tumor angiogenesis (3,4). Our prior studies indicated that hepatocyte growth factor (HGF) binding to its receptor tyrosine kinase, c-Met, promotes EC barrier function (5), however the exact mechanism by this occurs is incompletely defined.

The HGF receptor tyrosine kinase, c-Met, is a disulfide linked α–β heterodimeric that has previously been identified as a proto-oncogene (6,7). The 170 kDa c-Met precursor is glycosylated and then cleaved into a 50 kDa extracellular α chain and a 140 kDa membrane-spanning β chain. The c-Met protein serves as the high-affinity receptor for its natural ligand, hepatocyte growth factor (HGF, also called scatter factor), a paracrine factor produced by stromal and mesenchymal cells, acting on c-Met-expressing cells including EC (7,8). Activation of the HGF/c-Met signaling pathway, which requires phosphorylation of various specific tyrosine residues on c-Met itself, leads to cellular responses including increased proliferation, scattering (cell-cell repulsion), increased motility, invasion, and branching morphogenesis (7,8).

Interestingly, the cell surface receptor for hyaluronan, CD44, which we have also shown to serve as a barrier regulatory receptor (9), has also been implicated in the regulation of c-Met signaling (10-13), although the precise mechanism of this interaction is unknown. Further, the role of CD44 in HGF/c-Met-mediated EC barrier enhancement is completely unexplored.

There are multiple CD44 isoforms which result from extensive, alternative exon splicing events (14,15) with the alternative splicing often occurring between exons 5 and 15 leading to a tandem insertion of one or more variant exons (v1-v10) within the membrane proximal region of the extracellular domain (16,17). The variable primary amino acid sequence of different CD44 isoforms is further modified by extensive N- and O-glycosylations and glycosaminoglycan (GAG) additions (17,18).

In endothelial cells, as in many other cell types, there exist specialized plasma membrane microdomains containing a specific scaffolding protein called caveolin-1. We and others have previously shown that hyaluronan recruits CD44 into these caveolin-enriched microdomains (CEMs) (9,19-21) and in this locale, CD44 interacts with the underlying actin cytoskeleton, most likely through cytoskeletal binding proteins critical for CEM association and function (9,21,22). We have previously shown that high molecular weight hyaluronan (HMW-HA) promotes EC barrier enhancement via CD44-mediated PI3 kinase/Rac1 signaling in CEM (9).

Rac1-mediated cytoskeletal reorganization is involved in a number of models of EC barrier enhancement (3,4,9,22,23) with Rac1 activation the consequence of specific Rho family guanine nucleotide exchange factors (GEFs) which catalyze the exchange of Rac1-GDP (inactive) for Rac1-GTP (active) including Tiam1 (T-lymphoma invasion and metastasis gene 1) (24,25). Tiam1 is recruited to CEMs and is essentially involved in hyaluronan-induced Rac1 activation and EC barrier function (22).

Cortactin is a ~80-85 kDa actin-binding protein we have shown to be critically involved in EC cortical cytoskeletal rearrangements (26-28) and agonist-mediated EC barrier enhancement (29,30). Further, cortactin is an important downstream effector of CD44 signaling (31,32) with hyaluronan inducing cortactin translocation to the plasma membrane, a process that requires Rac1 activation (31).

Dynamin 2 is a ~96 kDa GTPase implicated in lipid raft internalization, modulation of cell shape, regulation of podosomal adhesion (33). Dynamin 2 regulates cortical actin dynamics through its interaction with cortactin (33) and has been implicated in Rac1 cellular localization and activation (34). Recently, it has been shown that dynamin 2 can directly bind to caveolin-1 (35).
In this study, we extend our earlier work to explore CD44 as an important regulator of HGF/c-Met-mediated EC barrier enhancement both in vitro and in vivo. Our novel findings indicate that HGF/c-Met-mediated, CD44-regulated, CEM signaling promotes Tiam1/dynamin-2-dependent Rac1 activation and peripheral recruitment of cortactin, processes essential for EC barrier integrity. Understanding the mechanism(s) by which HGF promotes increased EC barrier function may lead to novel treatments for diseases involving vascular barrier disruption including inflammation, tumor angiogenesis, atherosclerosis and acute lung injury.

Experimental Procedures

Cell Culture and Reagents – Human pulmonary microvascular EC (HPMVEC) were obtained from Cambrex (Walkersville, MD) and cultured as previously described (4) in EBM-2 complete medium (Cambrex) at 37°C in a humidified atmosphere of 5% CO₂, 95% air, with passages 6-10 used for experimentation. Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO). Reagents for SDS-PAGE electrophoresis were purchased from Bio-Rad (Richmond, CA), Immobilon-P transfer membrane from Millipore (Millipore Corp., Bedford, MA), and gold microelectrodes from Applied Biophysics (Troy, NY). Recombinant human hepatocyte growth factor (HGF), rabbit anti-vWF (Factor VIII) antibody, goat anti-CD44var (v3-v10) antibody and mouse anti-KDR (VEGF receptor 2) antibody were purchased from Chemicon International (Temecula, CA). Rat anti-CD44 (IM-7, common domain) antibody was purchased from BD Biosciences (San Diego, CA). Rabbit anti-phospho-c-Met (Tyr1234/1235), rabbit anti-phospho-c-Met (Tyr1349) and mouse anti-c-Met antibodies were purchased from Cell Signaling Technology (Boston, MA). Rabbit anti-CD44v3, anti-CD44v6 and anti-CD44v10 antibody were purchased from Calbiochem (San Diego, CA). FITC-conjugated anti-CD44 (HCAM) antibody was purchased from Abcam (Cambridge, MA). Rat anti-phospho-threonine antibody was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Rabbit antidyamin 2, rabbit anti-Tiam1 and rabbit anti-caveolin-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-Rac1 and mouse anti-cortactin antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Mouse anti-β-actin antibody, lipopolysaccharide (LPS) and Optiprep™ were purchased from Sigma (St. Louis, MO). Secondary horseradish peroxidase (HRP)-labeled antibodies were purchased from Amersham Biosciences (Piscataway, NJ).

Caveolin-enriched microdomain (CEM) isolation – Caveolin-enriched microdomain known as detergent-resistant membranes (DRM) or lipid rafts were isolated from HPMVEC as we previously described (9,22). Triton X-100-insoluble materials were mixed with 0.6 ml of cold 60% Optiprep™ and overlaid with 0.6 ml of 40%-20% Optiprep™ and the gradients centrifuged (35,000 rpm) in SW60 rotor for 12 h at 4°C and different fractions were collected and analyzed.

Immunoprecipitation and Immunoblotting – Cellular materials from treated or untreated HPMVEC were incubated with IP buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 1% Nonidet P-40 (NP-40), 0.4 mM Na₃VO₄, 40 mM NaF, 50 μM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture 3). The samples were then immunoprecipitated with either anti-CD44 or anti-dynamin 2 IgG followed by SDS-PAGE in 4-15% polyacrylamide gels, transfer onto Immobilon™ membranes, and developed with specific primary and secondary antibodies. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences). In some cases, standardized average grey values (S.A.G.V., processed from ImageQuant™ software (Amersham Biosciences) were obtained for immunoreactive bands for quantification.

Construction and Transfection of siRNA against c-Met, CD44, Tiam1, Cortactin,
Dynamin 2, Rac1 and the S1P1 Receptor - The siRNA sequence(s) targeting human c-Met, CD44, Tiam1, cortactin, dynamin 2, Rac1 and the S1P1 receptor were generated using mRNA sequences from Gen-Bank™ (gi:42741654, gi:30353932, gi:897556, gi:20357555, gi:32451864, gi:29792301, gi:13027635 respectively). For each mRNA (or scramble), two targets were identified. Specifically, c-Met target sequence 1 (5'-AAAGATAAAACCTCTCAATAG-3'), c-Met target sequence 2 (5'-AACCTCTCATATAAGGCCG-3'), CD44 target sequence 1 (5'-AAATACAACCGCCGTTTGA-3'), CD44 target sequence 2 (5'-AAAATGTCGCTACAGC-3'), Tiam1 target sequence 1 (5'-AAACAGCTTCAGCCTGAC-3'), Tiam1 target sequence 2 (5'-AATGCTCTGAAATCCTAGTC-3'), cortactin target sequence 1 (5'-AAATGCCGAGTTTTTGCACT-3'), cortactin target sequence 2 (5'-AAACAGAACATGCCGAGTTTT-3'), dynamin 2 target sequence 1 (5'-AACATGCCGAGTTTTTGCACT-3'), dynamin 2 target sequence 2 (5'-AACACGAACATGCCGAGTTTT-3'), Rac1 target sequence 1 (5'-AAAACTTGCCTACTGATCAGT-3'), Rac1 target sequence 2 (5'-AACTTGCCTACTGATCAGTTA-3'), S1P1 target sequence 1 (5'-AAGCTACACAAAAAGCCTGGA-3'), S1P1 target sequence 2 (5'-AAAAAGCCTGGATCACTCATC-3'), scrambled sequence 1 (5'-AAGAGAAATCGAAACCGAAAA-3') and scramble sequence 2 (5'-AAGAACCCAATTAAGCGCAAG-3') were utilized. Sense and antisense oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). For construction of the siRNA, a transcription-based kit from Ambion was used (Silencer™ siRNA construction kit). Human lung EC were then transfected with siRNA using siPORTamine™ as the transfection reagent (Ambion, TX) according to the protocol provided by Ambion. Cells (~ 40% confluent) were serum-starved for 1 hour followed by incubated with 3 μM (1.5 μM of each siRNA) of target siRNA (or scramble siRNA or no siRNA) for 6 hours in serum-free media. The serum-containing media was then added (10% serum final concentration) for 42 h before biochemical experiments and/or functional assays were conducted.

Preparation and Quantitation of High MW Hyaluronan (HA) – The method of preparation is similar to that described previously (9,36). Briefly, 500 mg of rooster comb HA (~1 million Da polymers (37) was dissolved in distilled water and centrifuged in an Ultrafree-MC™ Millipore 100,000 Da MW cutoff filter (Bedford, MA) and the flow through (less than 100,000 Da) was discarded. High MW HA was quantitated using an ELISA-like competitive binding assay with a known amount of fixed HA and biotintylated HA binding peptide (HABP) as the indicator (38). HMW-HA with DNA standards were run on 4-20% SDS-PAGE gels and stained with combined Alcian blue and silver staining to further determine HA purity and size (39).

Determination of threonine phosphorylation of CD44 – Solubilized CEM proteins in IP buffer (see above) were immunoprecipitated with rat anti-CD44 antibody followed by SDS-PAGE in 4-15% polyacrylamide gels and transfer onto Immobilon™ membranes (Millipore Corp., Bedford, MA). After blocking nonspecific sites with 5% bovine serum albumin, the blots were incubated with either rat anti-CD44 antibody or rabbit anti-phosphothreonine antibody followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit or goat anti-rat IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

Determination of Complex Formation between CD44 and c-Met - Solubilized CEM proteins in IP buffer (see above) were immunoprecipitated with rat anti-CD44 antibody or anti-c-Met antibody followed by SDS-PAGE in 4-15% polyacrylamide gels and transfer onto Immobilon™ membranes.
After blocking nonspecific sites with 5% bovine serum albumin, the blots were incubated with either rat anti-CD44 antibody or mouse anti-c-Met antibody followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-mouse or goat anti-rat IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

**Measurement of EC Electrical Resistance** – EC were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes, and TER measurements performed using an electrical cell-substrate impedance sensing system obtained from Applied Biophysics (Troy, NY) as previously described in detail (4). TER values from each microelectrode were pooled at discrete time points and plotted versus time as the mean ± S.E.

**Rac1 Activation Assay** – Rac1 activity assays in human lung EC were performed as described previously (40).

**Determination of Complex Formation between Dynamin 2 and Cortactin/Caveolin-1** – Solubilized CEM proteins in IP buffer (see above) were immunoprecipitated with rabbit anti-dynamin 2 antibody followed by SDS-PAGE in 4-15% polyacrylamide gels and transfer onto Immobilon™ membranes (Millipore Corp., Bedford, MA). After blocking nonspecific sites with 5% bovine serum albumin, the blots were incubated with either rabbit anti-dynamin 2 antibody, mouse anti-cortactin antibody, rabbit anti-caveolin-1 antibody or rabbit anti-Tiam1 antibody followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit or goat anti-mouse IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

**Animal Preparation and Treatment** – Male C57BL/6J and CD44 knockout mice (8-10 weeks, Jackson Laboratories, Bar Harbor, ME) were anesthetized with intraperitoneal ketamine (150 mg/kg) and acetylpromazine (15 mg/kg) according to approved protocols. LPS (2.5 mg/kg) or saline (control) were instilled intratracheally and four hours later, HGF (50 μg/kg) or saline control delivered intravenously through the internal jugular vein. The animals were allowed to recover for 24 hours followed by bronchoalveolar lavage protein analysis and/or lung immunohistochemistry.

**Murine Lung Immunohistochemistry** – To characterize protein expression in mouse lung vascular endothelial cells (EC), lungs from C57BL/6J control (untreated) mice were formalin fixed, 5 micron paraffin sections were obtained, hydrated and epitope retrieval performed (DakoCytomation Target Retrieval Solution, pH=6.0, DakoCytomation, Carpinteria, CA). The sections were then histologically evaluated by either FITC-conjugated anti-CD44 antibody or anti-c-Met or anti-Factor VIII (vWF) antibody and secondary secondary fluorescent antibody (Alexa Fluor™ 610 (for vWF) and 350 (for c-Met), Molecular Probes (Invitrogen, Carlsbad, CA)). Negative controls for immunohistochemical analysis were performed by the same method as above but without primary antibody. Immunofluorescent stained sections were photographed (100x) using a Leica Axioscope (Bannockburn, IL).

**Determination of Bronchoalveolar Lavage Protein Concentration** – Bronchoalveolar lavage (BAL) was performed by an intratracheal injection of 1 cc of Hank’s balanced salt solution followed by gentle aspiration. The recovered fluid was processed for protein concentration (BCA Protein Assay Kit; Pierce Chemical Co., Rockford, IL) as previously described (41).

**Statistical Analysis** - Student's t test was used to compare the means of data from two or more different experimental groups. Results are expressed as means ± S.E.
Results

Role of CD44 in HGF/c-Met-mediated human EC barrier enhancement.

Hepatocyte growth factor (HGF) binding to its plasma membrane receptor tyrosine kinase, c-Met, induces a variety of cellular functions (7,8,42) including endothelial cell (EC) barrier enhancement (5). However, the mechanism by which this occurs remains poorly defined. CD44, a major hyaluronan (HA) receptor localized in caveolin-enriched microdomains (CEM), has been implicated in regulating HGF/c-Met signaling (10-13). We, therefore, examined the role of CD44 in HGF-induced EC barrier regulation.

Our data indicate that there are two main CD44 isoforms expressed in human pulmonary EC, CD44v10 (~120 kDa) and CD44s (standard form, ~85 kDa)(Figure 1-A). In the absence of HGF (control), CD44s, but not CD44v10 or c-Met, is localized to caveolin-enriched microdomains (CEM, also termed detergent-resistant membranes or lipid rafts). HGF (25 ng/ml) treatment of human EC induces recruitment of ~70% of total CD44v10 and ~55% of total c-Met into CD44s-containing CEM (Figure 1-B,C).

Prior studies of CD44 involvement in regulating HGF-induced c-Met signaling (10-13) demonstrated that CD44 variant isoforms can bind HGF (10) and regulate c-Met autophosphorylation (Tyr1234/1235) suggesting CD44 can act as a co-receptor for c-Met (12,13). Using CD44v10 blocking antibody treatment of human EC indicates that CD44v10 regulates HGF-mediated c-Met tyrosine phosphorylation (Tyr1234/1235) by ~50% (Figure 2-A,B) and recruitment of c-Met into CEM (Figure 2-C). As shown in Figure 3-A, the c-Met recruited to CEM is active (tyrosine phosphorylated). Figures 3-B,C indicate that HGF induces a time-dependent association of c-Met with CD44v10 followed by CD44s as well as CD44 activation (defined by CD44 serine phosphorylation) in CEM (20,43-45). Abolishing the potential for CEM formation with methyl-β-cyclodextrin (M CD), a plasma membrane cholesterol-depletion agent, or reducing the expression of c-Met or CD44 (via siRNA) attenuates HGF-induced increases in human EC barrier function (Figure 4). These results appear to be specific for HGF as silencing CD44 expression did not alter the barrier enhancing effects of another CEM-regulated agonist, sphingosine 1-phosphate (S1P) (Figure 4-E) (32). Further, silencing CD44 expression blocked c-Met autophosphorylation (Figure 5-A). These results demonstrate an essential role for CD44 and CEM in HGF-induced c-Met activation and EC barrier regulation.

Role of Tiam1, cortactin and dynamin 2 in HGF/c-Met-mediated human EC barrier enhancement.

Considering our results indicating that CD44 regulates HGF-induced EC barrier enhancement (Figure 4), we examined whether Tiam1, cortactin and/or dynamin 2 are involved in HGF-induced increases in EC barrier integrity. Figure 5-B indicates that Tiam1, cortactin and dynamin 2 are present in modest amounts within CEM in control EC with increased recruitment to these caveolin-enriched plasma membrane microdomain structures following HGF (25 ng/ml). Silencing CD44 (siRNA) expression attenuates the HGF-induced recruitment of these molecules to CEM (Figure 5-B) with silencing either Tiam1 or dynamin 2 expression abolishing cortactin localization to CEM (Figure 6-A, B). Immunoprecipitation of dynamin 2 from CEM indicates that cortactin and caveolin-1, but not Tiam1, are complexed with dynamin 2; and HGF-treatment of human EC enhances this association (Figure 6-B). Finally, silencing Tiam1, cortactin or dynamin 2 expression attenuates the EC barrier-enhancing effects of HGF (Figure 6-E) indicating the critical involvement of these molecules in this response.

Role of Rac1 in HGF/c-Met-mediated human EC barrier enhancement.

Rac1 activation is required for the barrier-enhancing properties of sphingosine 1-phosphate, (30), simvastatin (46), hyaluronan (9), ATP (47) and HGF (5). However, the mechanism of HGF-induced Rac1 activation in human EC remain poorly defined. Figure 7 indicates that HGF (25 ng/ml) induces Rac1 activation which is required for HGF-induced human EC
barrier enhancement. The inhibition of CEM formation with methyl-β-cyclodextrin (MβCD), a plasma membrane cholesterol-depletion agent, or silencing (siRNA) c-Met, CD44, Tiam1 or dynamin 2 expression inhibits HGF-induced Rac1 activation. In contrast, silencing cortactin expression does not affect HGF-mediated Rac1 activation.

**Role of CD44 in HGF-mediated regulation of lung vascular integrity in vivo.** Consistent with our in vitro results in human pulmonary microvascular EC, immunohistochemical studies reveal that C57BL/6J wild type murine lung endothelium has colocalized expression of CD44 and c-Met (Figure 8-A). We next examined whether HGF was an effective barrier protective agent in an in vivo model of lipopolysaccharide (LPS)-induced murine lung vascular permeability. LPS administered via an intratracheal route induces murine inflammation and increased vascular leakiness as measured by the protein concentration in bronchoalveolar lavage (BAL) fluid (Figure 8-B,C) (48). Intravenous injection of either the CD44 ligand, high molecular weight hyaluronan (HMW-HA, 1.5 mg/kg)(Figure 8-B) or HGF (50 μg/kg)(Figure 8-C) four hours after LPS delivery attenuated C57BL/6J wildtype mouse pulmonary hyper-permeability. In contrast, this potent protective effect of both HMW-HA and HGF on LPS-induced inflammatory lung injury was markedly attenuated in the CD44 knockout mouse indicating the protective effect of HGF in LPS-induced pulmonary hyper-permeability is dependent upon regulation by CD44.

**Discussion**

This study presents several novel observations including the finding that CD44 isoforms temporally regulate HGF/c-Met mediated EC barrier integrity in vitro. Further, CD44 regulates HGF-mediated EC barrier integrity in vivo. HGF binding to c-Met induces CD44v10-dependent c-Met translocation to caveolin-enriched microdomains (CEM) and temporal association of activated (tyrosine phosphorylated) c-Met with CD44s (serine phosphorylated). CD44 activation was required for HGF-induced recruitment of Rac1 GEF, Tiam1, and the vesicular regulator, dynamin 2, to caveolin-enriched microdomains. Both Tiam1 and dynamin 2 contribute to HGF-induced Rac1 activation and recruitment of the cortical actin cytoskeletal regulatory protein, cortactin, to CEM. Further, dynamin 2 forms a complex with caveolin-1 and cortactin within these CEM structures, events which appear to contribute to HGF/c-Met-mediated CD44 regulation of EC barrier function (Figure 9).

Prior studies of CD44 involvement in regulating HGF-induced c-Met signaling (10-13) demonstrated that the CD44v3 isoform can bind HGF (10) suggesting CD44 could act as a co-receptor for c-Met. CD44v10, like CD44v3, contains glycosaminoglycan (GAG) attachment sites which are involved in recognition of ligands other than hyaluronan (HA) (49) indicating that CD44v10 could directly or indirectly bind to HGF. Further, the extracellular domain of the CD44 isoform, CD44v6, regulates c-Met autophosphorylation (12,13) while the cytoplasmic domain of CD44v6 regulates HGF-induced Sos, Ras, MEK and Erk MAP kinase activation through involvement of ezrin, radixin and moesin (ERM) proteins (12,13). We have extended these observations to show that CD44v10 regulates c-Met CEM localization and CEM localized CD44 regulates Tiam1/dynamin 2-mediated cortactin recruitment and Rac1 activation. Interestingly, we also observed an HGF-induced time-dependent association of c-Met with CD44s (standard form) within CEM. Whether CD44v10 and/or CD44s can also regulate HGF-induced Ras pathway signaling in human EC is currently being investigated in our laboratory.

We observed evidence for a complex formed between activated (tyrosine phosphorylated) c-Met and activated (threonine phosphorylated) CD44v10 and CD44s in CEM fractions following HGF treatment. It remains unknown whether these interactions are direct or mediated through an adapt protein. Further, the mechanism of differential association of c-Met with CD44
isoforms is currently being investigated in our laboratory. Using site-specific phospho-
antibodies, we observed HGF-induced c-Met phosphorylation on Tyr residues 1234/1235 and 1349. Phosphorylation of Tyr1234/1235 within the catalytic domain of c-Met is critical for c-Met kinase activity while phosphorylation of Tyr1349 induces direct binding of Grb-2-associated binder-1 (Gab1) (6-8,42). CD44 induces serine/threonine phosphorylation and plasma membrane recruitment of Gab1 (50). Whether Gab1 is involved in the c-Met/CD44 complex formation is currently being investigated in our laboratory. The CD44 cytoplasmic domain can be phosphorylated at Ser291, Ser316, Ser323 and Ser325 by various serine/threonine kinases including ROCK and PKC which regulates CD44 association with the cytoskeletal protein, ankyrin, and ezrin, radixin and moesin (ERM) proteins (18,20,43-45). We are also investigating these proteins as potential linkers between CD44 isoforms and c-Met.

Our results demonstrated that HGF promotes CD44-dependent Tiam1/Rac1 activation in CEM fractions. Previous reports that Tiam1 is involved in cell-cell adhesion are consistent with our findings that Tiam1 expression is required for HGF/c-Met-induced Rac1 activation and EC barrier enhancement (51,52). HGF induced significant recruitment of Tiam1 into CEM fractions and increased Tiam1-dependent total cellular Rac1 activation, indicating either post-translational modifications of Tiam1 or that localization within CEMs enhances Tiam1 activity. Tiam1 is phosphorylated by serine/threonine kinases including Ca2+/calmodulin-dependent protein kinase II and protein kinase C-dependent mechanisms (53,54). Studies examining HGF-induced Tiam1 post-translational modification are currently in progress.

We have demonstrated that dynamin 2 is complexed with caveolin-1 and cortactin which is augmented in HGF-challenged EC. Further, dynamin 2 is required for HGF-induced, c-Met- and CD44-dependent Rac1 activation. These results are in agreement with previous reports indicating dynamin 2 regulates cortical actin dynamics through its interaction with cortactin (33), regulates Rac1 cellular localization and activation (34) and binds to caveolin-1 (35). In this report, we provide the novel observation that the dynamin 2/caveolin-1/cortactin complex can regulate HGF-induced EC barrier enhancement.

Our recent data indicates that high MW hyaluronan promotes cortical actin rearrangement and increased barrier function in EC through a process dependent on Rac1 activation (9). Actin cytoskeletal rearrangement is required for EC barrier function since actin depolymerizing and microfilament disrupting agents (i.e. cytochalasin B and latrunculin A) abolish this effect (4,30). Overexpression of a dominant active form of Rac1 in EC promotes cortical actin "ring" formation associated with barrier enhancement while silencing (siRNA) Rac1 expression inhibits EC barrier enhancement (4,30). Our results indicate that CD44 is required for HGF-induced Rac1 activation thus implicating CD44 as a crucial mediator between c-Met signaling and cytoskeletal reorganization.

In our studies, we use intratracheal administration of lipopolysaccharide (LPS) to induce vascular hyper-permeability. LPS induces a delayed EC barrier disruptive response by activating a receptor complex of TLR4, CD14 and MD2 with consequent Rho-dependent NF-κB activation, cytokine production and generation of low MW HA degradation fragments (55-61). In particular, RhoA is important in LPS-mediated regulation of IL-8 production (62). Further, LPS induces RhoA/Rho kinase (ROCK)-mediated myosin light chain phosphorylation (63,64) with inhibition of ROCK attenuating LPS-induced acute lung injury (65).

The CEM protein, caveolin-1, inhibits the pro-inflammatory effects of LPS (66). Two downstream targets of ROCK in CEM that can potentially regulate LPS-induced EC barrier disruption include the actin- and phospholipid-binding protein, myristoylated alanine-rich C-kinase substrate (MARCKS) (67-69) and the sodium-hydrogen exchanger 1 (NHE1) (37). ROCK can also act cooperatively with PKCs.
to induce MARCKS phosphorylation (67). LPS induces MARCKS phosphorylation (70) which inhibits its association with the plasma membrane and promotes cytosolic localization (71-73). LPS can also regulate NHE1 (74), a protein implicated in cell stress responses through regulation of intracellular pH and actin cytoskeletal dynamics (75,76).

In vivo, CD44 is expressed in a variety of cells including endothelial, epithelial and immune cells (18,20,77-79). CD44 knockout mice develop lung fibrosis, inflammatory cell recruitment and accumulation of hyaluronan fragments at sites of lung injury (78). In addition to being an important regulator of immune cell function, our previous published data indicate that CD44 is involved in regulating epithelial barrier function (79). Therefore, the reduction in HGF protection from LPS-induced injury in the CD44 knockout mouse can potentially involve immune and epithelial cell involvement in addition to endothelial regulation.

In summary, utilizing both in vitro and in vivo models of pulmonary vascular permeability, we have demonstrated that CD44 regulates HGF-induced vascular integrity via a mechanism we speculate to involve scaffolding of key CEM components (Tiam1, cortactin, dynamin 2 and Rac1) by CD44 isoforms but essential to the HGF response. These results further indicate that HGF may serve as a potentially useful therapeutic treatment for diseases characterized by high permeability states.

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References


Figure Legends

Figure 1 – Analysis of HGF-induced c-Met recruitment to human EC caveolin-enriched microdomains (CEM). Panel A: EC were grown to confluency, lysates obtained and run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-CD44 (IM-7, common domain), anti-CD44 variant (v3-v10), anti-CD44v3, anti-CD44v6 or anti-CD44v10 antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown). Panel B: EC were grown to confluency, serum starved for one hour and either left untreated (control) or treated with 25 ng/ml HGF (5 minutes) or treated with the lipid raft abolishing, cholesterol depletion agent, methyl-β-cyclodextrin (MβCD, 5 mM) for one hour prior to 25 ng/ml HGF treatment (5 minutes). Cellular material was solubilized in 4°C 1% Triton X-100 and soluble and insoluble fractions were obtained. The Triton X-100 insoluble fraction was overlaid with 60%, 40%, 30% and 20% Optiprep™ and centrifuged at 35,000 rpm in SW60 rotor for 12 h at 4°C. The Triton X-100 soluble material and Optiprep™ fractions were run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-caveolin-1 (B-a), anti-c-Met (B-b), anti-CD44 (IM-7, common domain)(B-c), anti-CD44v10 (B-d) or anti-VEGF receptor 2 (B-e) antibody. The 20% Optiprep™ (*) fraction is the caveolin-enriched microdomain (CEM) fraction. Experiments were performed in triplicate with highly reproducible findings (representative data shown). Panel C: Graphical quantitation of immunoreactive bands from experiments depicted in Panel B which were analyzed using ImageQuant™ software (see Materials and Methods). % of Total Protein in CEM on the y-axis refers to (S.A.G.V. 20% Optiprep™ immunoreactive band divided by (S.A.G.V. 20% + 30% + 40% + 60% Optiprep™ immunoreactive band of interest + S.A.G.V. Triton X-100 insoluble material immunoreactive band band of interest)) multiplied by 100.

Figure 2 – The effect of CD44v10 on HGF-induced c-Met activation and recruitment to CEM. EC were grown to confluency, serum starved for one hour, and either left untreated (control) or treated with normal rabbit IgG (pre-immune, 10 μg/ml) or anti-CD44v10 antibody (10 μg/ml) followed by no treatment or treatment with 25 ng/ml HGF (5 minutes) and EC lysates or CEM (lipid raft) fractions (20% Optiprep™ layer) prepared as described in the Materials and Methods. Panel A: EC lysates were run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phospho-tyrosine1234/1235-c-Met (A-a), anti-c-Met (A-b) or anti-actin (A-c) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown). Panel B: Graphical quantitation of immunoreactive bands from experiments depicted in Panel A which were analyzed using ImageQuant™ software (see Materials and Methods). % c-Met Phosphorylation on the y-axis refers to (S.A.G.V. phospho-tyrosine1234/1235 c-Met immunoreactive band divided by S.A.G.V. c-Met immunoreactive band) multiplied by 100. Panel C: CEM (lipid raft) fractions (20% Optiprep™ layer) were run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-c-Met (C-a), anti- CD44 (IM-7, common domain)(C-b), anti-CD44v10 (C-c), anti-VEGF receptor 2 (C-d) or anti-caveolin-1 (C-e) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown).

Figure 3 - Analysis of HGF-induced c-Met/CD44 interactions. EC were grown to confluency, serum starved for one hour and either left untreated (control) or treated with 25 ng/ml HGF (5, 15 or 30 minutes) and CEM (lipid raft) fractions (20% Optiprep™ layer) prepared as described in the Materials and Methods. Panel A: The CEM fractions were run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phospho-tyrosine1234/1235-c-Met (A-a), anti-phospho-tyrosine1349-c-Met (A-b), anti-c-Met (A-c), anti-CD44 (IM-7, common domain) (A-d), anti-
CD44v10 (A-e), anti-VEGF receptor 2 (A-f) or anti-caveolin-1 (A-g) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown). Panel B: EC lysates were solubilized in IP buffer A (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 1% Nonidet P-40 (NP-40), 0.4 mM Na₃VO₄, 40 mM NaF, 50 μM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture 3) and immunoprecipitated with anti-c-Met antibody. The resulting immunobeads were run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-CD44 (IM-7, common domain)(B-a), anti-phospho-serine (B-b) or anti-c-Met (B-c) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown). Panel C: The CEM fractions were solubilized in IP buffer A (see above) and immunoprecipitated with anti-CD44 (IM-7, common domain) antibody. The resulting immunobeads were run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-c-Met (C-a), anti-phospho-serine (C-b) or anti-CD44 (IM-7, common domain) (C-c) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown).

**Figure 4 - The effect of CEM, c-Met and CD44 on HGF-induced human EC barrier enhancement.** Panel A: Immunoblot analysis of siRNA-treated or untreated human EC. Cellular lysates from untransfected (control, no siRNA), scramble siRNA (siRNA that does not target any known human mRNA), c-Met siRNA or CD44 siRNA-transfection were analyzed using immunoblotting with anti-c-Met (A-a), anti-CD44 (IM-7) antibody (A-b) or anti-actin antibody (A-c) as described in Materials and Methods. Experiments were performed in triplicate, each with similar results and representative data is shown. Panel B: EC were plated on gold microelectrodes, serum-starved for one hour and treated with either PBS, pH=7.4 (control) or 5 mM methyl-β-cyclodextrin (MβCD, a cholesterol depletion agent that abolishes CEM formation) 30 minutes prior to PBS, pH=7.4 or 25 ng/ml HGF addition. The arrows indicate the times of MβCD and HGF addition. The TER tracing represents pooled data ± S.E. from three independent experiments as described in Materials and Methods. Panel C: EC were plated on gold microelectrodes and treated with scramble siRNA (control) or c-Met siRNA for 48 hours. EC were then serum starved for one hour followed by addition of 25 ng/ml HGF. The arrow indicates the time of HGF addition. The TER tracing represents pooled data ± S.E. from three independent experiments as described in Materials and Methods. Panel D: EC were plated on gold microelectrodes and treated with scramble siRNA (control) or CD44 siRNA for 48 hours. EC were then serum starved for one hour followed by addition of 25 ng/ml HGF. The arrow indicates the time of HGF addition. The TER tracing represents pooled data ± S.E. from three independent experiments as described in Materials and Methods. Panel E: Graphical representation of percent maximal sphingosine 1-phosphate (S1P)-induced change in EC permeability. EC were plated on gold microelectrodes and treated with no siRNA, scramble siRNA, CD44 siRNA or S1P₁ receptor siRNA for 48 hours. EC were then serum starved for one hour followed by addition of 1 μM S1P. The bar graphs represent pooled TER data ± S.E. at 30 minutes after addition of agonist from three independent experiments as described in Materials and Methods.

**Figure 5 - The role of CD44 in HGF-induced recruitment of c-Met, Tiam1, cortactin and dynamin 2 to human EC CEM.** EC were treated with scramble siRNA (control) or CD44 siRNA for 48 hours. EC were then grown to confluency, serum starved for one hour and either untreated (control) or treated with 25 ng/ml HGF for 5, 15 or 30 minutes. Panel A: EC lysates were run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine(C₁₂₀₁/₁₂₀₃)-c-Met (a,c), anti-c-Met (b,d) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown). Panel B: CEM (lipid raft) fractions (20% Optiprep™ layer) prepared as described in the Materials and Methods were run on SDS-
PAGE, transferred to nitrocellulose and immunoblotted with anti-c-Met (a,f), anti-anti-Tiam1 (b,g), anti-cortactin (c,h), anti-dynamin 2 (d,i) or anti-caveolin-1 (e,j) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown).

**Figure 6 - The effect of Tiam1, cortactin and dynamin 2 on HGF-induced human EC barrier enhancement.** Panel A: Immunoblot analysis of siRNA-treated or untreated human EC. Cellular lysates from untransfected (control, no siRNA), scramble siRNA (siRNA that does not target any known human mRNA), Tiam1 siRNA, dynamin 2 siRNA or cortactin siRNA-transfection were analyzed using immunoblotting with anti-Tiam1 (A-a), Anti-dynamin 2 (A-b), anti-cortactin (A-c) or anti-actin (A-d) antibody. Experiments were performed in triplicate each with similar results. Representative data is shown. For Panels B and C, EC were then grown to confluency, serum starved for one hour and either untreated (control) or treated with 25 ng/ml HGF for 5, 15 or 30 minutes and CEM (lipid raft) fractions (20% Optiprep™ layer) were then prepared as described in the Materials and Methods. Panel B: EC were treated with scramble siRNA (control) dynamin 2 siRNA or Tiam1 siRNA for 48 hours. The CEM fractions were run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-cortactin (B-a,c,e) or anti-caveolin-1 (B-b,d,f) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown). Panel C: CEM fractions were solublized in IP buffer A (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 1% Nonidet P-40 (NP-40), 0.4 mM Na₃VO₄, 40 mM NaF, 50 μM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture 3) and immunoprecipitated with anti-dynamin 2 antibody. The resulting immunobeads were run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Tiam1 (C-a), anti-cortactin (C-b), anti-caveolin-1 (C-c) or anti-dynamin 2 (C-d) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown). Panel D: Graphical quantitation of immunoreactive bands from experiments depicted in Panel C which were analyzed using ImageQuant™ software (see Materials and Methods). % Protein Association with Dynamin 2 on the y-axis refers to (S.A.G.V. immunoreactive band of interest divided by S.A.G.V. dynamin 2 immunoreactive band) multiplied by 100. Panel E: Graphical representation of percent maximal HGF-induced change in EC permeability. EC were plated on gold microelectrodes and treated with scramble siRNA (control), Tiam1 siRNA, dynamin 2 siRNA or cortactin siRNA for 48 hours. EC were then serum starved for one hour followed by addition of 25 ng/ml HGF. The bar graphs represent pooled TER data ± S.E. at 30 minutes after addition of agonist from three independent experiments as described in Materials and Methods.

**Figure 7 - The effect of Tiam1, cortactin and dynamin 2 on HGF-induced Rac1 activation.** Panel A: EC were either untreated, treated with scramble siRNA, c-Met siRNA, CD44 siRNA, dynamin 2 siRNA, Tiam1 siRNA or cortactin siRNA for 48 hours. EC were grown to confluency, serum starved for one hour and either untreated (control) or treated with 5 mM methyl-β-cyclodextrin (MβCD, a cholesterol depletion agent that abolishes CEM formation) 30 minutes prior to PBS, pH=7.4 or 25 ng/ml HGF addition. EC were then solublized in IP buffer and incubated with p21-binding domain (PBD)-conjugated beads to bind activated (GTP-bound form) Rac1. The PBD bead-associated material was run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Rac1 antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown). Panel B: Graphical quantitation of immunoreactive bands from experiments depicted in Panel A which were analyzed using ImageQuant™ software (see Materials and Methods). % Rac1 Activation on the y-axis refers to (S.A.G.V. activated Rac1 immunoreactive band divided by S.A.G.V. total Rac1 immunoreactive band) multiplied by 100. Panel C: Immunoblot analysis of siRNA-treated or untreated human EC. Cellular lysates from untransfected (control, no siRNA), scramble siRNA
(siRNA that does not target any known human mRNA) or Rac1 siRNA-transfection were analyzed using immunoblotting with anti-Rac1 (C-a) or anti-actin antibody (C-b) as described in Materials and Methods. Experiments were performed in triplicate each with similar results. Representative data is shown. Panel D: Graphical representation of percent maximal HGF-induced change in EC permeability. EC were plated on gold microelectrodes and treated with scramble siRNA (control) or Rac1 siRNA for 48 hours. EC were then serum starved for one hour followed by addition of 25 ng/ml HGF. The bar graphs represent pooled TER data ± S.E. at 30 minutes after addition of agonist from three independent experiments as described in Materials and Methods.

**Figure 8 - The role of CD44 on HGF-induced protection from LPS-induced vascular permeability in vivo.** Panel A: Immunohistochemical fluorescent staining images of control (untreated) mouse lung using either bright field (DIC) imaging (a) or treatment with anti-Factor VIII (vWF) antibody (b), anti-c-Met antibody (c) or FITC-conjugated anti-CD44 antibody (d) and secondary fluorescent antibody (Alexa Fluor™ 610 (for vWF) and 350 (for c-Met), Molecular Probes) as described in Materials and Methods. Images are shown at 100x magnification. Arrows indicate immunostaining of endothelial cells with (e) being an overlay of (b, c and d). Panel A- insets: Negative controls for immunohistochemical analysis which were done by the same method as above but without primary antibody. Panels B and C: Male C57BL/6J and CD44 knockout mice were anesthetized and were either given saline (control) or LPS (2.5 mg/kg) intratracheally. After 4 hours, mice were given intravenously injections (internal jugular vein) with saline (control) or high molecular weight hyaluronan (HMW-HA, 1.5 mg/kg)(B) or HGF (50 μg/kg)(C). The treated mice were allowed to recover for 24 hours, bronchoalveolar lavage (BAL) fluids were obtained and protein concentrations were determined (see Materials and Methods). For Panels B and C, the single asterisk (*) refers to a significant (p < 0.05) difference between control and LPS treatment. There is also a significant difference (p < 0.05) between LPS and HMW-HA + LPS treatment in the wildtype, but not the CD44 knockout, mouse. Panel C: The double asterisk (**) refers to a significant difference (p < 0.05) between LPS treatment and HGF + LPS treatment. There is also a significant difference (p < 0.05) between the wildtype and CD44 knockout mouse HGF + LPS treatment.

**Figure 9 - Proposed model of HGF-induced vascular integrity.** HGF induces CD44v10-regulated c-Met (1) translocation to caveolin-enriched microdomains (CEM) and association of activated (tyrosine phosphorylated) c-Met with activated (serine phosphorylated) CD44v10 and CD44s (2). CD44 isoforms are required for HGF-induced recruitment of the Rac1 GEF, Tiam1, and the vesicular regulator, dynamin 2, to CEM to join pre-existing Tiam1 and dynamin 2 within these structures (3). Both Tiam1 and dynamin 2 contribute to HGF-induced Rac1 activation (4) and recruitment of the cortical actin cytoskeletal regulatory protein, cortactin, to CEM (5). Further, dynamin 2 forms a complex with caveolin-1 and cortactin within these CEM structures. These events contribute to HGF/c-Met-mediated CD44 regulation of EC barrier function (6).
Figure 1 –

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EC Lysate

B. Immunoblot:

1. Anti-Caveolin-1
2. Anti-c-Met
3. Anti-CD44s
4. Anti-CD44v10
5. Anti-VEGF R.

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4°C Triton X-100 Soluble Material

C. % of Total Protein in CEM

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### A.

**Immunoblot:**

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EC Lysates

### B.

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### C.

**Immunoblot:**

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20% Optiprep™ CEM Fractions
A. Figure 3 –

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20% Optiprep™ CEM Fractions

B. Immunoblot:

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ippt: Anti-c-Met (EC lysates)

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ippt: Anti-CD44 (20% Optiprep™ CEM Fractions)
A. **Figure 4** –

**Immunoblot:**

- a. Anti-c-Met
- b. Anti-CD44
- c. Anti-Actin

**Control (no siRNA)**

**Scramble siRNA**

**c-Met siRNA**

**CD44 siRNA**

EC Lysates

120 kDa

85 kDa

B.

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C.

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D.

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E. **% Maximum S1P-induced TER Response**

- Control (no siRNA)
- Scramble siRNA
- CD44 siRNA
- S1P, siRNA
Figure 5 –

A. Immunoblot:

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<td>b.</td>
<td>Anti-c-Met</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.</td>
<td>Anti-pY^{1234/1235} c-Met</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d.</td>
<td>Anti-c-Met</td>
<td></td>
<td></td>
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</tbody>
</table>

Scramble siRNA

CD44 siRNA

EC Lysates

B. Immunoblot:

<table>
<thead>
<tr>
<th></th>
<th>HGF</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5&quot;</td>
<td>15&quot;</td>
<td>30&quot;</td>
</tr>
<tr>
<td>a.</td>
<td>Anti-c-Met</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b.</td>
<td>Anti-Tiam1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.</td>
<td>Anti-Cortactin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d.</td>
<td>Anti-Dynamin 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e.</td>
<td>Anti-Caveolin-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f.</td>
<td>Anti-c-Met</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g.</td>
<td>Anti-Tiam1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h.</td>
<td>Anti-Cortactin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Anti-Dynamin 2</td>
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<tr>
<td>j.</td>
<td>Anti-Caveolin-1</td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>

20% Optiprep™ CEM Fractions

Scramble siRNA

CD44 siRNA
A.

**Immunoblot:**
- a. Anti-Tiam1
- b. Anti-Dynamin 2
- c. Anti-Cortactin
- d. Anti-Actin

Control (no siRNA)  Scramble siRNA  Tiam1 siRNA  Dynamin 2 siRNA  Cortactin siRNA

EC Lysates

B.

**Immunoblot:**
- a. Anti-Cortactin
- b. Anti-Caveolin-1
- c. Anti-Cortactin
- d. Anti-Caveolin-1
- e. Anti-Cortactin
- f. Anti-Caveolin-1

HGF

Scramble siRNA  Dynamin 2 siRNA  Tiam1 siRNA

20% Optiprep™ CEM Fractions

C.

**Immunoblot:**
- a. Anti-Tiam1
- b. Anti-Cortactin
- c. Anti-Caveolin-1
- d. Anti-Dynamin 2

HGF

Control  5 min.  15 min  30 min.

D.

Ippt: Anti-Dynamin 2

<table>
<thead>
<tr>
<th>Tiam1</th>
<th>Cortactin</th>
<th>Caveolin-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Protein Association with Dynamin-2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HGF (minutes)</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0%</td>
<td>20%</td>
<td>40%</td>
<td>60%</td>
</tr>
<tr>
<td>5</td>
<td>20%</td>
<td>40%</td>
<td>60%</td>
<td>80%</td>
</tr>
<tr>
<td>15</td>
<td>40%</td>
<td>60%</td>
<td>80%</td>
<td>100%</td>
</tr>
<tr>
<td>30</td>
<td>60%</td>
<td>80%</td>
<td>100%</td>
<td>120%</td>
</tr>
</tbody>
</table>

E.

% Maximum HGF-induced TER Response

<table>
<thead>
<tr>
<th>Scramble siRNA</th>
<th>Tiam1 siRNA</th>
<th>Dynamin 2 siRNA</th>
<th>Cortactin siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
</tr>
</tbody>
</table>

Graph showing the association of Tiam1, Cortactin, and Caveolin-1 with Dynamin-2 over time.
Figure 7 –

A. Immunoblot:

- **Activated Rac1**
  - a. Anti-Rac1
  - b. Anti-Rac1

- **Total Rac1**

B. HGF

<table>
<thead>
<tr>
<th>Control</th>
<th>Control MβCD</th>
<th>Scramble siRNA</th>
<th>c-Met siRNA</th>
<th>CD44 siRNA</th>
<th>Dynamin 2 siRNA</th>
<th>Tiam1 siRNA</th>
<th>Cortactin siRNA</th>
</tr>
</thead>
</table>

Graph showing % Rac1 Activation:

- Control
- MβCD
- Scramble siRNA
- c-Met siRNA
- CD44 siRNA
- Dynamin 2 siRNA
- Tiam1 siRNA
- Cortactin siRNA

C. Immunoblot:

- a. Anti-Rac1
- b. Anti-Actin

D. % Maximum HGF-induced TER Response:

- Scramble siRNA
- Rac1 siRNA
Figure 8 –

A.

a. DIC
b. vWF
c. c-Met

d. CD44
e. Merge

B.

Concentration of BAL Protein (mg/ml)

Wildtype CD44 Knockout

Control LPS HMW-HA + LPS

P < 0.05

C.

Concentration of BAL Protein (mg/ml)

Control LPS HGF + LPS

Wildtype CD44 Knockout

P < 0.05

* P < 0.05
Figure 9 –

Endothelial Cell Barrier Enhancement

1. HGF
2. pTyr
3. Tiam1
4. Rac1
5. Cortactin
6. Caveolin-1
7. Caveolin-enriched Microdomain (CEM)
8. CD44v10
9. CD44s/v10
10. c-Met
11. pSer
12. HGF
CD44 regulates hepatocyte growth factor-mediated vascular integrity: Role of c-Met, Tiam1/Rac1, dynamin 2 and cortactin
Patrick A. Singleton, Ravi Salgia, Liliana Moreno-Vinasco, Jaideep Moitra, Saad Sammani, Tamara Mirzapoiazova and Joe G.N. Garcia

J. Biol. Chem. published online August 16, 2007

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