Role of c-Met/Phosphatidylinositol 3-Kinase (PI3k)/Akt Signaling in Hepatocyte Growth Factor (HGF)-mediated Lamellipodia Formation, Reactive Oxygen Species (ROS) Generation, and Motility of Lung Endothelial Cells*

Received for publication, October 15, 2013, and in revised form, February 28, 2014. Published, JBC Papers in Press, March 14, 2014, DOI 10.1074/jbc.M113.527556

Peter V. Usatyuk‡, Panfeng Fu‡, Vijay Mohan‡, Yulina Epshtein§, Jeffrey R. Jacobson†, Julian Gomez-Cambronero†, Kishore K. Wary†, Vytas Bindokas**, Steven M. Dudek§, Ravi Salgia®, Joe G. N. Garcia†, and Viswanathan Natarajan*1

From the ‡Department of Pharmacology, the *Section of Pulmonary and Critical Care Medicine, the †Institute for Personalized Respiratory Medicine, University of Illinois at Chicago, Chicago, Illinois 60612, the **Department of Pharmacological and Physiological Sciences, and the ††Department of Medicine, University of Chicago, Chicago, Illinois 60637, and the †Department of Biochemistry and Molecular Biology, Wright State University, Dayton, Ohio 45435

Background: Lamellipodia structures provide a platform for the spatio-temporal localization of key components necessary for cell migration.

Results: HGF activates c-Met/PI3k/Akt signaling axis, which is essential for the recruitment of actin, cortactin, p47phox, and Rac1 and ROS production in lamellipodia.

Conclusion: HGF-induced spatio-temporal localization of cytoskeletal proteins and NADPH oxidase components regulate lamellipodial ROS and cell migration.

Significance: This study identifies a novel role for lamellipodial ROS in cell motility.

Hepatocyte growth factor (HGF) mediated signaling promotes cell proliferation and migration in a variety of cell types and plays a key role in tumorigenesis. As cell migration is important to angiogenesis, we characterized HGF-mediated effects on the formation of lamellipodia, a pre-requisite for migration using human lung microvascular endothelial cells (HLMVECs). HGF, in a dose-dependent manner, induced c-Met phosphorylation (Tyr-1234/1235, Tyr-1349, Ser-985, Tyr-1003, and Tyr-1313), activation of PI3k (phospho-Yp85) and Akt (phospho-Thr-308 and phospho-Ser-473) and potentiated lamellipodia formation and HLMVEC migration. Inhibition of c-Met kinase by SU11274 significantly attenuated c-Met, PI3k, and Akt phosphorylation, suppressed lamellipodia formation and endothelial cell migration. LY294002, an inhibitor of PI3k, abolished HGF-induced PI3k (Tyr-458), and Akt (Thr-308 and Ser-473) phosphorylation and suppressed lamellipodia formation. Furthermore, HGF stimulated p47phox/Cortactin/Rac1 translocation to lamellipodia and ROS generation. Moreover, inhibition of c-Met/PI3k/Akt signaling axis and NADPH oxidase attenuated HGF-induced lamellipodia formation, ROS generation and cell migration. Ex vivo experiments with mouse aortic rings revealed a role for c-Met signaling in HGF-induced sprouting and lamellipodia formation. Taken together, these data provide evidence in support of a significant role for HGF-induced c-Met/PI3k/Akt signaling and NADPH oxidase activation in lamellipodia formation and motility of lung endothelial cells.

Vascular endothelial cells provide a selective structural barrier between circulation and surrounding tissue, and regulate vascular permeability and blood flow (1, 2). In addition, they play a major role in angiogenesis, a process required for the generation of new blood vessels and repair of damaged vasculature (3). Several pathological conditions, including tumor growth, ischemia/reperfusion, diabetes, rheumatoid arthritis, atherosclerosis, and inflammatory processes also require neoangiogenesis. It is therefore not surprising that angiogenesis is therapeutically targeted in several human disorders (4, 5).

Several growth factors, including the hepatocyte growth factor (HGF),2 regulate angiogenesis. HGF, also known as scatter factor, is the natural ligand for the receptor tyrosine kinase, c-Met (6) that is expressed on a variety of cell types including vascular endothelial cells and is up-regulated during angiogenesis (7, 8). HGF interacts with its cognate receptor c-Met and promotes angiogenesis by enhancing the expression of vascular endothelial growth factor, one of the principal mediators of angiogenesis. HGF binding to c-Met triggers a cascade of signal transduction pathways including activation of phosphatidylinositol 3-kinase (PI3k) and Akt, which have been implicated in cell migration (7–11).

Cell migration is essential for embryonic development, wound healing, and angiogenesis (3, 7, 8, 12–14). It is a multistep process facilitated by highly coordinated cytoskeletal changes, including extracellular adhesion, plasma membrane protrusion at the leading edge (lamellipodia formation), formation of new adhesion sites under the protrusion, and disruption

---

2 The abbreviations used are: HGF, hepatocyte growth factor; ECs, endothelial cells; HLMVECs, human lung microvascular endothelial cells; ROS, reactive oxygen species; TER, trans-endothelial electrical resistance; PI3k, phosphatidylinositol 3-kinase; ECIS, Electrical Cell Substrate Impedance Sensing.
of older adhesions at the rear and contraction, thereby drawing the cell forward (12–20). The lamellipodium is characterized by a dense network of short, branched actin and cortactin filaments, with elongated filaments that have barbed-end capping (13, 15, 16, 18–24). At the edge of the lamellipodia are club-like projections referred to as the filopodia that generate the adhesion sites. Recent studies suggested that the cytoskeletal proteins, particularly actin and cortactin, play a role in phagocytic and non-phagocytic activation of NADPH oxidase (25–29). Cortactin redistribution to the cell periphery and its co-localization with p47^phox, a NADPH oxidase subunit, are important in the assembly of NADPH oxidase components with the actin cytoskeleton during agonist-induced generation of reactive oxygen species (ROS) in human lung ECs (26–28). Thus, targeting NADPH oxidase components to focal complexes in lamellipodia may provide a mechanism to specifically generate ROS in localized structures such as lamellipodia, a requirement for stimulus-induced cell migration (10, 30–34).

During our investigations into cytoskeletal regulation of NADPH oxidase activation and endothelial barrier function, we observed that inhibition of HGF-induced NADPH oxidase activation in membrane protrusions attenuated lamellipodia formation and migration of HLMVECs. However, signaling mechanism(s) of HGF-induced, ROS-dependent lamellipodia and cell migration is not well understood. Here, we determined the role of the HGF/c-Met/PI3k signaling axis in ROS-dependent lamellipodia formation and cell migration. Our results demonstrated that HGF-induced c-Met activation as well as phosphorylation of downstream targets such as PI3k/Akt kinases is required for lamellipodia formation and EC motility. In addition, the HGF/c-Met signaling axis stimulated ROS formation at the lamellipodia, and blocking activation of PI3k, Akt, and NADPH oxidase components (p47^phox, Rac1) attenuated HGF-induced lamellipodia formation and cell migration.

**EXPERIMENTAL PROCEDURES**

*Materials*—HLMVECs and endothelial basal media (EBM-2) were obtained from Lonza (San Diego, CA). Phosphate-buffered saline (PBS) was from Biofluids Inc. (Rockville, MD). Glass bottom micro well dishes were purchased from MatTek (Ashland, MA). Gold antifade mounting media, DAPI, Hoechst and precast Tris-glycine PAGE, Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor Phalloidin 568, CellLight Actin-RFP BacMam 2.0 were procured from Invitrogen (Eugene, OR). HGF was from R&D Systems (Minneapolis, MN) and PeproTech (Rocky Hill, NJ). SU11274 was purchased from Selleck Chemicals (Houston, TX). Anti-c-Met, anti-phospho-c-Met (Tyr-1234/1235), anti-phospho-c-Met (Tyr-1349), anti-phospho-c-Met (Ser-985), anti-phospho-c-Met (Tyr-1003), anti-Akt, anti-phospho-Akt (Thr-308), anti-phospho-Akt (Ser-473), LY294002, and cell lysis buffer were from Cell Signaling Technology (Danvers, MA). Scrambled siRNA and siRNA for c-Met, PI3k p85, and p47^phox antibodies for anti-cortactin anti-phospho-c-Met (Tyr-1313), and BSA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin (Sigma), anti-p47^phox (National Institutes of Health, Bethesda), anti-phospho-PI3k (Tyr-458) (Thermo Scientific, Rockford, IL), anti-Rac1 and anti-PI3k p85 (BD Biosciences, San Jose, CA), Fibrin Gel and in vitro Angiogenesis Assay Kit (Millipore, Billerica, MA), IgG (H+L) HRP conjugates (Bio-Rad) were all commercially obtained. Gene Silencer was from Gene Therapy System (San Diego, CA). pHyPer-cyto plasmid was purchased from Evrogen (Moscow, Russia). Actin-RFP and cortactin-RFP plasmids were provided by Dr. Steven Dudek (University of Illinois at Chicago). FuGENE HD transfection reagent was from Promega (Madison, WI), ECIS electrodes 8W1E were procured from Applied Biophysics (Troy, NY). Immunobilon-P, 0.45 μm was procured from Millipore (Bedford, MA).

**Endothelial Cell Culture**—HLMVECs cultured in complete media (EBM-2), were maintained at 37 °C and 5% CO2 and grown to contact-inhibited monolayers that revealed typical cobblestone morphology. Cells were then detached with 0.05% trypsin and resuspended in fresh medium and cultured on gold electrodes for electrical resistance determinations, on glass coverslips for fluorescent microscopy studies, or in 60–100-mm culture dishes for preparation of cell lysates and Western blot analysis.

**Mouse Aortic Ring Sprouting Assay**—Thoracic aortic rings from 2-month-old mice were prepared (20–25 g body weight; The Jackson Laboratory), housed under pathogen-free conditions at the University of Illinois at Chicago (UIC) Animal Care Vivarium and treated humanely in accordance with institutional guidelines (35). Briefly, thoracic aortae were isolated, dissected from connective tissues, and washed in sterile PBS extensively under aseptic conditions and the aortae were cut into rings ~1 mm in thickness. The rings were placed in the middle of the glass bottom 35-mm dishes, overlaid with 100 μl of Fibrinogen/Thrombin solution according to the manufacturer’s protocol, and left to polymerize for 1 h at 37 °C before the addition of EBM-2 media without serum and growth factor, which was replaced each day with fresh media or media containing 20 ng/ml HGF. After 6 days of culture, emergent angiogenic sprouts were examined using Zeiss Axiovert 40 phase contrast microscope (lens ×10). Images were captured with the use of a digital camera. The area of angiogenic sprout outgrowth was quantified by using image acquisition and analysis software (Image J).

**Lamellipodia Formation Ex Vivo in Aortic Sprouts**—To determine lamellipodia formation ex vivo, aortic ring were grown in complete media for 4 days, infected for 24 h with CellLight Actin-RFP BacMam 2.0 according to protocol, and living cells transiently expressed actin-RFP were visualized by laser scanning microscope Zeiss 510 Meta (lens ×40). Several cells were selected from each glass bottom dish, and actin dynamics was monitored before and after HGF (20 ng/ml) stimulation with autofocus mode.

**Preparation of Cell Lysates and Western Blotting**—HLMVECs were cultured in 60-mm dishes to ~95% confluence and starved for 3 h in EBM-2 medium containing 0.1% FBS. Cells were stimulated with HGF (2–30 ng/ml) for 15 min, washed with PBS, and lysed as described (28). Clarified cell lysates were boiled with the sample buffer for 4 min, and the proteins (20–30 μg) were subjected to SDS-PAGE analysis, transferred to PVDF membranes, blocked in TBST containing 1% BSA prior to incubation with primary antibody (1:1000 dilution) overnight. After blocking and washing, membranes were incu-
bated with appropriate secondary antibody (1:10,000 dilution), and blots were developed using an ECL chemiluminescence kit. Western blots were scanned by densitometry and integrated density of pixels in identified areas was quantified using ImageJ.

**Immunofluorescence Microscopy**—HLMVECs grown in chamber slides were starved for 3 h in EBM-2 containing 0.1% FBS, pretreated with inhibitors or siRNA as indicated prior to treatment with HGF for 15 min. Cells were fixed in 3.7% formaldehyde in PBS for 10 min, washed three times with PBS, permeabilized with 0.25% Triton X-100 or methanol for 4 min, blocked with 1% BSA in TBST, incubated for 1 h with appropriate primary antibody (1:200 dilution), washed with TBST, and stained for 1 h with secondary antibody (1:200 dilution) in TBST containing 1% BSA. Cells were visualized using a Nikon Eclipse TE2000-S immunofluorescence microscope and a Hamamatsu digital camera with ×60 oil immersion objective.

**siRNA Transfection of HLMVECs**—Cells grown to ~50% confluence in 6-well plates or chamber slides were transfected with Gene Silencer® transfecting reagent containing scrambled siRNA (50 nM) or siRNA for target proteins (50 nM) in serum-free EGM-2 medium according to the manufacturer’s recommendation.

**cDNA Transfection of HLMVECs**—To determine intracellular hydrogen peroxide levels, HLMVECs grown to ~50% confluence were transiently transfected with 1 μg/ml of pHyPer-cyto plasmid (36) using FuGene HD (3 μg/ml) transfection reagent in serum-free EGM-2 medium according to the manufacturer’s recommendation. Similarly, to estimate ROS production in lamellipodia, ECs were double transfected with 0.5/0.5 μg/ml of pHyPer-cyto/actin-RFP or pHyPer-cyto/cortactin-RFP plasmids. After 3 h, the media of transfected cells were replaced by complete EGM-2 containing 10% fetal bovine serum, and the cells were incubated for 72 h.

**Determination of Intracellular Hydrogen Peroxide in Lamellipodia**—Intracellular hydrogen peroxide levels were determined by a mammalian expression vector encoding a fluorescent hydrogen peroxide sensor pHyPer (36). Cells were grown on glass bottom 35-mm dishes, transfected with 1 μg/ml of pHyPer-cyto plasmid, and treated as described above. Cells were washed twice with Phenol Red free basal EBM-2 and fluorescence of pHyPer-cyto in living cells was examined under a Nikon Eclipse TE 2000-S fluorescence microscope with a Hamamatsu digital CCD camera, using a ×60 objective lens. To calculate pHyPer-cyto fluorescence intensity (pixels) similar rectangular cell periphery regions were selected in control and treated cells using MetaVue software (Universal Imaging Corp.), and values are expressed as % of Control. At least 6–10 cells were evaluated for each treatment in triplicate, averaged to three independent experiments, and background fluorescence from an empty dish was subtracted.

Confocal micrographs of pHyPer images were acquired every minute on a Marianas Yokogawa CSU-X1 spinning disk confocal system (Intelligent Imaging Innovations, Denver) on a Zeiss Observer Z1 platform using a ×40 NA1.3 oil objective under control of SlideBook 5.5 software. pHyPer-cyto emission (525/50 nm) was excited using 405 and 488 nm lasers, mCherry was excited with 561 nm laser, and emission collected at 607/43 nm. Temperature was maintained at 37 °C, and cells were kept in 5% CO₂ (OKO Labs). Reporter response was calculated as the ratio of background-subtracted 488 and 405 signals. pHyPer-cyto/Actin-RFP and pHyPer-cyto/Cortactin-RFP fluorescent intensity profiles (3-pixel-wide average over 10 μm) across lamellipodia were estimated using ImageJ (Wayne Rasband, NIH).

**Quantification of Lamellipodia**—Quantification of lamellipodia was performed as described earlier (37). Briefly, for each image, background signal was subtracted by drawing a region of interest around the cell periphery of individual cells. All areas outside the cell were cleared to best visualize the leading edges including cell periphery, and the fluorescence intensity within the entire cell was summed by MBF ImageJ bundle (Tony Collins, McMaster University and Wayne Rasband, NIH).

**Electrical Cell Substrate Impedance Sensing (ECIS) Assay**—HLMVEC were cultured in 8-well ECIS electrode arrays (8W1E, Applied Biophysics, NY) to ~95% confluence and starved in the EBM-2 medium with 0.1% BSA for 1–3 h. An elevated field (3 V at 40,000 Hz for 10 s) was applied to wound the cells on the electrode. Inhibitors and HGF were reconstituted in EBM-2 medium containing 0.1% BSA, and endothelial wound healing was monitored for 12–16 h by measuring the trans-endothelial electrical resistance (TER) using ECIS equipment (38).

**Statistical Analysis**—ANOVA with Student-Newman-Keul’s test was used to compare means of clearance rates of two or more different treatment groups. The level of significance is p < 0.05 unless otherwise stated. Data are expressed as mean ± S.E.

**RESULTS**

**HGF Stimulates Lamellipodia Formation in Lung ECs**—Cell motility plays a central role in migration, wound healing, and angiogenesis. The driving force for cell migration is lamellipodia formation propelled by the reorganization of the actin and cortactin cytoskeleton at the cell front and the retraction of the cell at the rear (13–15, 18, 19, 21–24, 39). We therefore determined the effect of HGF on lamellipodia formation. HLMVECs were either treated with vehicle or vehicle containing HGF (2, 5, 10, 20, and 30 ng/ml for 15 min). Cells were immunostained for actin and cortactin co-localization, an index of lamellipodia formation (19, 24, 25). The vehicle-treated cells revealed typical F-actin staining with a few stress fibers in the central area of the cell and diffused cortactin staining; however, HGF in a dose-dependent manner induced F-actin stress fiber formation (red) and cortactin (green) redistribution to the cell periphery, which were co-localized in lamellipodia (merge, yellow) (Fig. 1, A and B). Despite extensive actin polymerization at the cell periphery, stress fibers remained stable. These results suggest a key role for HGF in lamellipodia formation in HLMVECs.

**HGF Enhances Hydrogen Peroxide Formation in Lamellipodia of Lung ECs**—The cytoskeletal proteins, actin and cortactin, are known to play a role in NADPH-oxidase assembly, activation, and ROS generation (27–29). HGF activated NADPH oxidase and increased ROS generation (10, 40, 41); however, the role of HGF in lamellipodial ROS generation is unclear. Therefore, we hypothesized that HGF may stimulate ROS accumulation in lamellipodia. HLMVECs transfected with pHyPer-cyto (36) were challenged with varying concentrations of HGF (2–30...
ng/ml), and hydrogen peroxide generated in living cells was analyzed using fluorescent microscopy. As shown in Fig. 2, A and B, HGF, in a dose-dependent manner, induced pHyPer-cyto green fluorescence (a measure of hydrogen peroxide accumulation, in the cytosol, peri-nuclear, and in cell periphery), which was confirmed by confocal microscopy (Fig. 2, C and D). To further elucidate HGF-induced ROS accumulation in lamellipodia, ECs were double transfected with pHyPer-cyto/actin-RFP and pHyPer-cyto/cortactin-RFP, and living cells were analyzed under confocal microscopy. As shown in Fig. 3, A and B, HGF stimulated hydrogen peroxide accumulation in cell periphery, which were co-localized with actin and cortactin in lamellipodia. pHyPer-cyto/actin-RFP, and pHyPer-cyto/cortactin-RFP fluorescent intensity profiles (3-pixel-wide average over 10 μm) across lamellipodia demonstrated colocalization of signals and significant increases in both red and green fluorescence after HGF exposure (Fig. 3, C and D). Taken together, these data support accumulation of hydrogen peroxide in lamellipodia in response to HGF in human lung ECs.

**FIGURE 1. HGF modulates lamellipodia formation in HLMVECs.** HLMVECs grown on slide chambers were treated with HGF for 15 min, probed with anti-actin and anti-cortactin antibodies, and examined by immunofluorescence microscopy. A, actin and cortactin reorganization to lamellipodia was visualized by immunocytochemistry as described in “Experimental Procedures.” Nuclei were stained with DAPI. Shown are representative immunofluorescence images from three independent experiments. B, actin and cortactin co-localization in lamellipodia shown in panel A was quantified using image analysis as described under “Experimental Procedures.” Values are means ± S.D. from three independent experiments. *, p < 0.05 compared with cells without HGF.

**HGF in Endothelial Cells Motility**

---

**Down-regulation of c-Met or Inhibition of c-Met Phosphorylation Attenuates HGF-stimulated Phosphorylation of c-Met, PI3k, and Akt in Lung ECs—Binding of HGF to its receptor, c-Met, induces dimerization and autophosphorylation of specific tyrosine residues resulting in enhanced kinase activity and activation of diverse intracellular signaling pathways including MAPKs, STAT3, Rac1, and Akt (7, 10, 41–46), which promote EC barrier function (47, 48). To further characterize HGF/c-Met signaling axis in EC function, we used SU11274 (49) to inhibit HGF-mediated c-Met phosphorylation. As expected, HGF, in a dose-dependent manner, induced c-Met phosphorylation at residues Tyr-1234/1235, Tyr-1349, Tyr-1003, and Tyr-1313 as well as Ser-985 in HLMVECs (Fig. 4A). Pretreatment of cells with SU11274 (1 μM) for 1 h, prior to HGF treatment (20 ng/ml, 15 min) attenuated HGF-induced c-Met phosphorylation at various tyrosine residues (Fig. 4B). Down-regulation of c-Met with siRNA or SU11274 also inhibited HGF-enhanced phosphorylation of PI3k (Tyr-548) and Akt (Thr-308 and Ser-473) (Fig. 5, A and B). These results show that HGF mediated activation of PI3k/Akt pathway is via c-Met phosphorylation in HLMVECs.

**Inhibition of c-Met Tyrosine Phosphorylation by SU11274 Attenuates HGF-induced Lamellipodia Formation, Accumulation of ROS in Lamellipodia, and Lung EC Migration—**Having established that HGF stimulates lamellipodia formation in HLMVECs via c-Met signaling, we next determined the potential link between c-Met inhibition and HGF-induced lamellipodia formation, ROS localization in lamellipodia and cell migration. As shown in Fig. 6A, SU11274 significantly attenuated HGF-induced actin and cortactin redistribution to cell periphery and their co-localization in lamellipodia. Further, inhibition of c-Met phosphorylation with SU11274 reduced HGF-induced hydrogen peroxide accumulation in lamellipodia of
HLMVECs (Fig. 6B) as measured by pHyPer-cyto fluorescence. We also determined the role of c-Met on EC motility using a wound repair ECIS assay. Local area of cell culture monolayer, grown on gold microelectrodes, was mechanically disrupted (3 V at 40,000 Hz for 10 s) that resulted in a very rapid drop in TER followed by an increase due to the migration of cells from the perimeter of the electrode to replace the wounded cells (Fig. 6C). Restoration of TER in the presence of HGF (20 ng/ml) was more pronounced compared with control cells suggesting that HGF is a potent inducer of wound repair processes in lung ECs, and pretreatment of cells with SU11274 resulted in a dramatic suppression of HGF-mediated wound healing as determined by decreased cell migration (Fig. 6C). Interestingly, SU11274 alone caused a significant reduction in endothelial wound repair in the absence of HGF, suggesting the importance of basal c-Met activation in EC motility. Taken together, these results establish a key role for HGF/c-Met signaling in lamellipodia formation, ROS, and EC motility.

Role of PI3k/Akt Signaling in HGF-mediated Lamellipodia Formation, ROS in Lamellipodia, and Lung EC Migration—To further investigate the role of PI3k/Akt signaling in HGF-mediated lamellipodia formation, we used LY294002, a PI3k specific inhibitor (50) and also down-regulated PI3k using specific siRNA. Both LY294002 and siRNA against PI3k significantly reduced HGF-induced PI3k (Tyr-458) and Akt (Thr-308 and Ser-473) phosphorylation (Fig. 7A and B). Furthermore, HGF (20 ng/ml, 15 min) induced co-localization of actin and cortactin at the lamellipodia was inhibited by pretreatment of cells with the PI3k inhibitor LY294002 (Fig. 7C). Similar experiments carried out in PI3k siRNA-transfected cells revealed comparable results to those obtained with LY 294002 (Fig. 7D).

In parallel experiments, we also demonstrated that down-regulation of PI3k/Akt signaling pathways attenuated HGF-induced formation of hydrogen peroxide accumulation in lamellipodia (Fig. 7E and F) and wound repair in HLMVECs (Fig. 7G and H). The above results demonstrated a key role for HGF-mediated PI3k/Akt activation in lamellipodia formation, ROS accumulation in lamellipodia, and cell migration.

c-Met/PI3k/Akt Signaling Is Essential for HGF-mediated p47phox, Cortactin, and Rac1 Redistribution to Lamellipodia in Lung ECs—Recent studies indicate that PI3k and Akt play an important role in p47phox activation and production of ROS (10, 40, 51–53). Also, we have demonstrated previously that hypoxia-induced activation of lung endothelial NADPH oxidase that results in ROS generation is dependent on p47phox and cortactin translocation to cell periphery (26–28, 54). Further, cortactin acts as a scaffold protein for NADPH oxidase assembly and is essential for agonist-induced p47phox translocation to cell periphery, cortactin/p47phox co-localization, and O2·− production in lung ECs (27, 29). To determine the role of c-Met/
PI3k/Akt signal transduction in translocation of p47phox, cortactin and Rac1 to lamellipodia, HLMVECs were pretreated with c-Met inhibitor SU11274, PI3k inhibitor LY294002, or transfected with PI3k siRNA (50 nM, 72 h) followed by HGF (20 ng/ml) challenge for 15 min. As shown in Fig. 8A, HGF enhanced co-localization of p47phox and cortactin in lamellipodia (merged color yellow) as compared with control cells challenged with vehicle alone. Treatment of cells with either SU11274 or LY294002 or knockdown of PI3k with siRNA blocked the redistribution and co-localization of p47phox and cortactin in lamellipodia (Fig. 8A). Small GTPase Rac1 also plays an important role in lamellipodia formation, cortactin redistribution to cell periphery and in the activation of NADPH oxidase and ROS generation (26, 44, 55–57). We therefore examined the localization of Rac1 in lamellipodia using the above three approaches. Clearly, HGF also induced Rac1 translocation to lamellipodia in HLMVECs, while down-regulation of c-Met or PI3k/Akt signaling attenuated the process (Fig. 8B).

Statistical analyses of the data revealed a strong role for c-Met/PI3k/Akt signaling to redistribute Rac1 to the cell periphery (Fig. 8, C and D).

Inhibition of NADPH Oxidase and Down-regulation of Cortactin Impairs Lamellipodia Formation and ROS Accumulation in Lung ECs—To further evaluate the role of ROS in HGF-mediated cytoskeletal rearrangement and lamellipodia formation, NADPH oxidase-dependent ROS was blocked by pretreatment with apocynin (a general ROS inhibitor) or by silencing p47phox with siRNA. As shown in Fig. 9A, apocynin treatment or p47phox siRNA transfection of HLMVECs resulted in a significant loss in HGF-induced lamellipodia formation compared with control cells. In parallel experiments, a concomitant decrease in ROS generation in apocynin or p47phox siRNA-transfected cells was observed (Fig. 9B). In addition, knockdown of cortactin using specific siRNA significantly attenuated HGF-induced lamellipodia formation, ROS accumulation in lamellipodia, and p47phox and Rac1 localization in lamellipodia (Fig. 9, A–D). Similarly, inhibition of NADPH oxidase by apocynin, or down-regulation of p47phox or cortactin with siRNA attenuated HGF-induced cell migration as measured by wound-repair assay (Fig. 10, A–C). These results suggest a potential role for p47phox and cortactin in ROS formation in lamellipodia by HGF in lung ECs.

HGF-induced Endothelial Sprouting and Lamellipodia Formation Ex Vivo—Having established a role for HGF-stimulated lamellipodia formation via c-Met in HLMVECs, we next determined the role of HGF/c-Met signaling axis in an ex vivo system using aortic ring angiogenesis assay (58). As shown in Fig. 11A, isolated aortic rings in fibrin matrigel formed endothelial sprouts and tube elongation into fibrin matrix in the presence of HGF.
of serum. Addition of HGF stimulated mouse aortic sprouting density (~1.9-fold) compared with control aortic rings without HGF. To further elucidate the role of c-Met in HGF-mediated lamellipodia formation in the sprouts, aortic rings embedded in fibrin matrigels were transfected with an actin-RFP construct, and lamellipodia formation was visualized in living cells under a

FIGURE 4. HGF stimulates c-Met phosphorylation in HLMVECs. HLMVECs grown to ~95% confluence on 100-mm dishes were treated with varying concentrations of HGF for 15 min (A) or pretreated with 1 μM SU11274 (c-Met inhibitor) (B) for 1 h, prior to challenge with HGF (20 ng/ml) for 15 min. Cell lysates (20–40 μg of protein) were subjected to 10% SDS-PAGE, probed with total- or anti-phospho-c-Met (Tyr-1234/1235, Tyr-1349, Tyr-1003, Tyr-1313, Ser-985), and anti-actin antibodies as indicated. Shown are representative blots from three independent experiments. Western blots were scanned and quantified by an automated digitizing system. Values are means ± S.E. *, significantly different from untreated control (p < 0.05).

FIGURE 5. Down-regulation of c-Met phosphorylation attenuates PI3k and Akt activation in HLMVECs. HLMVECs were transfected with c-Met siRNA (50 nM, 72 h) (A) or pretreated with 1 μM SU11274 (c-Met inhibitor) (B) for 1 h, prior to challenge with HGF (20 ng/ml) for 15 min. Cell lysates (20–40 μg of protein) were subjected to 10% SDS-PAGE, and probed with total- and anti-phospho PI3k (p85), total- and anti-phospho Akt (Thr-308, Ser-473), and anti-actin antibodies as described under “Experimental Procedures.” Shown are representative blots from three independent experiments. Western blots were scanned and quantified by an automated digitizing system. Values are means ± S.E. *, significantly different from untreated control cells (p < 0.05); **, significantly different from HGF-treated cells (p < 0.05).
confocal microscope. As shown in Fig. 11, B and C, HGF enhanced actin accumulation in lamellipodial structures of the aortic endothelial sprouts, which was blocked by the c-Met tyrosine kinase inhibitor, SU11274. Thus, our aortic ring endothelial sprout assay further substantiates that HGF stimulates endothelial sprouting and lamellipodia formation ex vivo.

DISCUSSION

Cell migration, a complex process that involves protrusion, adhesion, contraction, and retraction, is essential for a variety of cellular functions such as cell growth, motility, angiogenesis, and morphogenesis (12–15, 19, 21, 59). ECs like many other eukaryotic cells also undergo directed cell migration mediated by lamellipodial protrusions for the formation of adhesions at leading edge of the cells (59 – 61). While the participation of cytoskeleton, mainly composed of actin microfilaments, microtubules, and intermediary filaments, is essential in the polarized cell migration, mechanisms regulating the generation of spatially and temporally restricted signaling molecules in lamellipodial protrusions are yet to be completely defined. Here, we report that in human lung ECs, the HGF-mediated activation of c-Met/PI3k/Akt signaling axis regulates cell migration, which requires formation of lamellipodial protrusions. Further, we demonstrate that the lamellipodia are not only enriched in actin and cortactin but also with components of NAPH oxidase such as p47phox and Rac1 and hydrogen peroxide generated via NADPH oxidase, which require the c-Met/PI3k/Akt signaling axis. All of the above, triggered by HGF/c-Met signaling, are required for lamellipodia formation in ECs and their migration.

HGF is the same as scatter factor that was originally shown to be fibroblast-derived cell motility factor for epithelial cells (6). Through its natural receptor c-Met, HGF is now known to promote matrix invasion, angiogenesis, tumorigenesis, and tissue regeneration (7, 8, 62 – 64). HGF has been previously shown to promote cell motility in several normal and cancer cell lines (8, 10, 44, 48, 63, 65). The driving force for cell migration is pro-
vided by continuous growth of actin filaments and cortactin rearrangement in the lamellipodial protrusions in the front end of the cell (13–15, 18–22, 24, 59, 66). The formation of lamellipodia is controlled by Rho family G proteins, particularly Rac and Cdc42, which regulate the Arp2/3 complex through the Wiskott-Aldrich syndrome protein (WASP) to promote lamellipodia formation (23, 24, 31, 44, 56, 57), while Rho regulates the formation of stress fibers and focal adhesions and induces actomyosin contractility (14, 67). Our results in lung ECs demonstrated that HGF-induced activation of c-Met mimicked the classic c-Met phosphorylation at Tyr-1234/1235, Tyr-1349, Ser-985, Tyr-1003, and 1313 (Fig. 4), which regulate the tyrosine kinase activity and recruit other key signaling molecules (6, 63, 68). Phosphorylation of c-Met at Tyr-1313 is known to recruit PI3k to the cell periphery (63) and inhibition of c-Met tyrosine kinase activity using SU11274 not only suppressed the activation of c-Met and the immediate downstream targets PI3k/Akt, but also the ensuing accumulation and co-localization of actin and cortactin, NADPH oxidase components at the lamellipodia (Figs. 4, 5, 6, and 7). The regulatory p85 subunit of PI3k can bind either directly to c-Met at phosphorylated Tyr-1313 or indirectly through GAB1, which then signals through Akt/protein kinase B (6). Although we have not shown the direct binding of PI3k to c-Met, our studies revealed clear acti-
vation of both PI3k and Akt, as evidenced by specific phosphorylation of Akt (Thr-308, Ser-373), and blocking PI3k with the inhibitor LY294002 or the knockdown of PI3k by siRNA attenuated HGF-mediated EC motility (Fig. 7, G and F). Involvement of PI3k/Akt signaling pathway in HGF-mediated migration of cancer cells has been described. In Uveal melanoma cells, HGF was found to enhance cell migration, and that HGF-induced migration was dependent on PI3k/Akt pathway. The activation of the PI3k/Akt pathway induced by the HGF/c-Met axis is migration was dependent on PI3k/Akt pathway. The activation was found to enhance cell migration, and that HGF-induced cancer cells has been described. In Uveal melanoma cells, HGF of PI3k/Akt signaling pathway in HGF-mediated migration of HGF-treated cells (and values are means ± S.E.).

**FIGURE 7.** Inhibition of HGF-induced PI3k signaling attenuates Akt activation, hydrogen peroxide accumulation in lamellipodia, and cell migration in HLMVECs. A, HLMVECs grown to ~95% confluence in 100-mm dishes were pretreated with 10 μM LY294002 (PI3k inhibitor) for 1 h, prior to challenge with HGF (20 ng/ml). Cell lysates (20–40 μg of protein) were subjected to 10% SDS-PAGE, probed with anti-phospho-PI3k, anti-phospho-Akt (Thr-308, Ser-473), anti-E-cadherin and β-catenin, contributing to the attenuation of cell-cell adhesion and promoting the enhanced motility (69). Although localization of PI3k in membrane ruffles and lamellipodia is unclear, the product of PI3k, namely phosphatidylinositol 3,4,5-trisphosphate (PIP3) is highly localized at the site of cell edges and lamellipodia, and the spatial and temporal generation PIP3 has been implicated in directed cell migration (69–71). Results presented here clearly demonstrate a role for c-Met/PI3k/Akt signaling pathway in the accumulation of ROS at the lamellipodia and cell migration as well as endothelial...
barrier function. In addition to actin and cortactin, Rac1 and p47phox components of NADPH oxidase are targeted to the lamellipodia and blocking Rac1 and p47phox attenuated lamellipodia formation and cell migration (31). These results suggested that targeting of NADPH oxidase to lamellipodia represents a novel mechanism for a cell to promote directed migration to external stimuli such as HGF and other growth factors. Although details on targeting NADPH oxidase components to lamellipodia are largely unknown, targeting p47phox to focal complexes seems to be mediated via adaptor protein.
In a recent study, Martinelli et al. showed that ventral lamellipodia of ECs were enriched in and functionally dependent on cortactin, IQGAP1, and p47phox (17). Additionally, IQGAP1 may also regulate directional migration by binding Rac1 and recruitment of p47phox and other components to leading edge of the cell required for directed cell migration. We previously showed the involvement of phospholipase D2-mediated activation of IQGAP1 through Rac1 in tyrosine phosphorylation of Src and cortactin (26), and a potential role for cytoskeletal proteins and dynamin 2 in the assembly and activation of NADPH oxidase in lipid rafts in response to hyperoxia in human lung endothelial cells (44).

**FIGURE 9.** Down-regulation of p47phox and cortactin or inhibition of NADPH oxidase attenuates lamellipodia formation, and hydrogen peroxide accumulation in lamellipodia of HLMVECs. HLMVECs grown on slide chambers to 70% confluence were transfected with scrambled or siRNA for p47phox and cortactin (50 nM, 72 h). In experiments with apocynin, HLMVECs grown to 95% confluence on slide chambers were pretreated with 30 μM apocynin, a NADPH oxidase inhibitor for 1 h. Cells were challenged with HGF (20 ng/ml) for 15 min. Actin and cortactin co-localization in lamellipodia was visualized by immunocytochemistry and quantified using image analysis as described under “Experimental Procedures.” Nuclei were stained with DAPI. Values are means ± S.E. from three independent experiments. *, significantly different from untreated control (p < 0.05); **, significantly different from HGF-treated cells without apocynin, p47phox siRNA, or cortactin siRNA (p < 0.01).

**FIGURE 10.** Down-regulation of p47phox and cortactin or inhibition of NADPH oxidase attenuates cell migration of HLMVECs. A, in experiments with apocynin, HLMVECs grown on gold electrodes to 90% confluence were pretreated with apocynin (30 μM) for 1 h prior to HGF (20 ng/ml) stimulation. B and C, HLMVECs grown on gold electrodes to 60% confluence were transfected with scrambled, p47phox siRNA (50 nM, 72 h) or cortactin siRNA (50 nM, 72 h) prior to HGF (20 ng/ml) stimulation. TER was recorded using ECIS for 12 h. HGF-mediated wound repair (% control) was calculated from the tracings as described under “Experimental Procedures.” Values are means ± S.E. from three independent experiments. *, significantly different from untreated control (p < 0.05); **, significantly different from HGF-treated cells without apocynin, p47phox siRNA, or cortactin siRNA (p < 0.01).

TRAF4-Hic5 complex (72). In a recent study, Martinelli et al. showed that ventral lamellipodia of ECs were enriched in and functionally dependent on cortactin, IQGAP1, and p47phox (17). Additionally, IQGAP1 may also regulate directional migration by binding Rac1 and recruitment of p47phox and other components to leading edge of the cell required for directed cell migration. We previously showed the involvement of phospholipase D2-mediated activation of IQGAP1 through Rac1 in tyrosine phosphorylation of Src and cortactin (26), and a potential role for cytoskeletal proteins and dynamin 2 in the assembly and activation of NADPH oxidase in lipid rafts in response to hyperoxia in human lung endothelial cells (44).
FIGURE 11. HGF potentiates endothelial sprouting formation in mouse aortic rings. Aortic rings (1 mm) isolated from mouse thoracic aortae were grown in fibrin matrix for 6 days without (Control) and in the presence of 20 ng/ml HGF and sprouts outgrowth (A) was examined using Zeiss Axiovert 40 phase contrast microscope (lens ×10) as described under "Experimental Procedures." B, aortic rings grown in fibrin matrix were infected for 24 h with CellLight Actin-RFP BacMam 2.0 according to the manufacturer’s protocol, pretreated with 1 μM SU11274 (c-Met inhibitor) for 1 h prior to challenge with HGF (20 ng/ml). Living cells transiently expressing actin-RFP were visualized by laser scanning microscope Zeiss 510 Meta (lens ×40). Actin dynamics were monitored before and after HGF stimulation. Inset depicts enhanced Actin-RFP accumulation in lamellipodia due to HGF treatment, which was significantly abrogated by c-Met inhibitor (C). Values are means ± S.E.*, significantly different from untreated control cells (p < 0.05); **, significantly different from HGF-treated cells (p < 0.05). Bars, 20 μm.
Interestingly, the hyperoxia-induced IQGAP1 activation and redistribution to cell periphery was attenuated in Rac1 down-regulated human lung ECs suggesting Rac1 activation up-stream of IQGAP1 (26). Although there is considerable evidence for the localization of NADPH oxidase components and generation of hydrogen peroxide in lamellipodia, the physiological relevance for this spatio-temporal enrichment in cell migration or wound healing is unclear. Blocking NADPH oxidase activation either with apocynin or p47^phox^ siRNA not only significantly reduced lamellipodia formation and hydrogen peroxide production in the lamellipodia (Fig. 9), but also attenuated ventral lamellipodia micro-wound healing (17). Mechanism(s) of regulation of cell migration or wound healing by localized lamellipodial ROS is unclear; however, it may be involved in controlling the cofillin activity (73–75) or in the regulation of temporal and spatial concentrations of PI(3,4,5)P3 (70, 76), a critical component in determining actin polymerization and lamellipodia projections. Other possible mechanisms may involve SeIR/Mical-mediated oxidation of actin to regulate F-actin dynamics (77, 78). Recent studies demonstrate that endogenous hydrogen peroxide play an important role in angiogenesis in vitro and neovascularization in vivo (79–81). Aortic ring ex vivo model also demonstrates impaired vascular formation, vessel sprouting and tube elongation by endothelial-specific catalase overexpression (79), further emphasizing a critical role for endogenous ROS in angiogenesis. In our ex vivo experiments, using mouse aortic rings, HGF enhanced capillary sprouting and tube elongation into fibrin matrix (Fig. 11A). Further, HGF-induced lamellipodia formation in aortic ring sprouts was significantly attenuated by inhibition of c-Met tyrosine phosphorylation (Fig. 11, B and C).

In summary, we have demonstrated a novel role for c-Met/PI3k/Akt signaling in HGF-induced lamellipodia formation in lung endothelium, which is dependent on recruitment of cortactin, Rac1, and p47^phox^ components and localized ROS production (Fig. 12). We also show localized ROS production in lamellipodia to be involved in EC migration, an important component of angio- and vasculo-genesis. Further understanding of lamellipodial ROS accumulation and the role of this localized ROS in regulation of signal transduction pathways in lamellipodia are necessary to define the physiological relevance of localized ROS signal, as in lamellipodia, in directional migration and wound healing.

REFERENCES
1. Komarova, Y., and Malik, A. B. (2010) Regulation of endothelial permeability via paracellular and transcellular transport pathways. Annu. Rev. Physiol. 72, 463–493
2. Dudek, S. M., and Garcia, J. G. (2001) Cytoskeletal regulation of pulmonary vascular permeability. J. Appl. Physiol. 91, 1487–1500
3. Marcelo, K. L., Goldie, L. C., and Hirschi, K. K. (2013) Regulation of endothelial cell differentiation and specification. Circ. Res. 112, 1272–1287
4. You, W. K., and McDonald, D. M. (2008) The hepatocyte growth factor/c-Met signaling pathway as a therapeutic target to inhibit angiogenesis. BMB Rep. 41, 833–839
5. Sala, V., and Crepaldi, T. (2011) Novel therapy for myocardial infarction: can HGF/Met be beneficial? Cell Mol. Life Sci. 68, 1703–1717
6. Nakamura, T., Sakai, K., Nakamura, T., and Matsumoto, K. (2011) Hepatocyte growth factor twenty years on: Much more than a growth factor. Journal of Gastroenterology Hepatology 26, 188–202
7. McKinnon, H., Gherardi, E., Reidy, M., and Bowyer, D. (2006) Hepatocyte growth factor/scatter factor and MET are involved in arterial repair and atherosclerosis. Am. J. Pathol. 168, 340–348
8. Kuhlmann, C. R., Schaef er, C. A., Fehsecke, A., Most, A. K., Tillmanns, H., and Erdogan, A. (2005) A new signaling mechanism of hepatocyte growth factor-induced endothelial proliferation. J. Thromb. Haemost. 3, 2089–2095
9. Graupera, M., and Potente, M. (2013) Regulation of angiogenesis by PI3K signaling networks. Exp. Cell Res. 319, 1348–1355
10. Jagadeeswaran, R., Jagadeeswaran, S., Bindokas, V. P., and Salgia, R. (2007) Activation of HGF/c-Met pathway contributes to the reactive oxygen species generation and motility of small cell lung cancer cells. Am. J. Physiol. Lung Cell Mol. Physiol. 292, L488–L494
11. Schroeder, K., Schutz, S., Schloeffl, I., Bätz, S., Takac, I., Weissmann, N.,
HGF in Endothelial Cells Motility

Michaels, U. R., Koyanagi, M., and Brandes, R. P. (2011) Hepatocyte growth factor induces a proangiogenic phenotype and mobilizes endothelial progenitor cells by activating Nox2. *Antioxid. Redox Signal.* 15, 915–923

12. Hoedle, M. K., and Svitkina, T. (2012) The cytoskeletal mechanisms of cell–cell junction formation in endothelial cells. *J. Mol. Biol. Cell* 23, 310–323

13. Insall, R. H., and Machesky, L. M. (2009) Actin dynamics at the leading edge: from simple machinery to complex networks. *Dev. Cell* 17, 310–322

14. Le Gaigner, C., and Carlier, M. F. (2008) Regulation of actin assembly associated with protrusion and adhesion in cell migration. *Physiol. Rev.* 88, 489–513

15. Burnet, D. T., Manley, S., Sengupta, P., Sougrat, R., Davidson, M. W., Kachar, B., and Lippincott-Schwartz, J. (2011) A role for actin arcs in the leading-edge advance of migrating cells. *Nat. Cell Biol.* 13, 371–381

16. Chichili, G. R., and Rodgers, W. (2009) Cytoskeleton-membrane interactions in membrane raft structure. *Cell Mol. Life Sci.* 66, 2319–2328

17. Martelli, R., Kamei, M., Sage, P. T., Massol, R., Varghese, L., Siccardi, T., Toporsian, M., Dvorak, A. M., Kirchhausen, T., Springer, T. A., and Carlier, M. C. (2013) Release of cellular tension signals self-restorative ventral lamellipodia to heal barrier micro-wounds. *J. Cell Biol.* 201, 449–465

18. Sung, B. H., Zhu, X., Kaverina, I., and Weaver, A. M. (2005) Cortactin controls cell motility and lamellipodial dynamics by regulating ECM secretion. *Curr. Biol.* 21, 1460–1469

19. Vinzenz, M., Nemethova, M., Schur, F., Mueller, J., Narita, A., Urban, E., Chichili, G. R., and Rodgers, W. (2009) Regulation of NADPH oxidase in vascular endothelium: the role of cortactin. *Antioxid. Redox Signal.* 13, 2319–2328

20. Viola, A., and Gupta, N. (2007) Tether and trap: regulation of membrane–raft dynamics by actin-binding proteins. *Nat. Rev. Immunol.* 7, 889–896

21. Bryce, N. S., Clark, E. S., Leysath, J. L., Currie, J. D., Webb, D. J., and Vinzenz, M., Nemethova, M., Acconcia, F., Benesch, S., Auinger, S., Faix, J., Small, J. V., and Machesky, L. M. (2009) F- and G-actin concentrations in lamellipodia of moving cells. *PloS one* 4, e8610

22. Koestler, S. A., Rottner, K., Lai, F., Block, J., Vinzenz, M., and Small, J. V. (2009) F-actin regulates cortical myosin flow in lamellipodium persistence. *Curr. Biol.* 19, 1276–1285

23. Lai, F. P., Szczodrak, M., Oelkers, J. M., Ladwein, M., Acconcia, F., Benesch, S., Auinger, S., Faix, J., Small, J. V., Polo, S., Stradal, T. E., and Rottner, K. (2009) Cortactin promotes migration and platelet-derived growth factor–induced actin reorganization by signaling to Rho-GTPases. *J. Cell Sci.* 122, 4351–4361

24. Weeds, A. S., Karginov, A. V., Chaer, A. W., Kinley, A. W., Kinley, A. W., Kinley, A. W., and Kinley, A. W. (2009) Regulation of NADPH oxidase in vascular endothelial cells: role in NAD(P)H oxidase regulation by p47phox associates with the cytoskeleton through cortactin in human vascular smooth muscle cells. *Biochimie* 91, 512–518

41. Mishina, N. M., Tyurin-Kuzmin, P. A., Markvicheva, K. N., Vorotnikov, A. V., Tkachuk, V. A., Laketa, V., Schultz, C., Lukyanov, S., and Belousov, V. V. (2011) Does cellular hydrogen peroxide diffuse or act locally? *Antioxid. Redox Signal.* 14, 1–7

42. Madonna, R., Bolli, R., Rokosh, G., and De Caterina, R. (2013) Targeting phosphatidylinositol 3-kinase-Akt through hepatocyte growth factor for cardioprotection. *J. Cardiovasc. Med.* 14, 249–253

43. Ruggeri, R. M., Vitarelli, E., Barresi, G., Trimarchi, F., Benvenga, S., and Trovato, M. (2010) The tyrosine kinase receptor c-met, its cognate ligand HGF and the tyrosine kinase receptor transducers STAT3, PI3K and RHO regulates endothelial cell proliferation and angiogenesis. *Blood* 117, 1761–1769

44. Singleton, P. A., Salgia, R., Moreno-Vinasco, L., Moitra, J., and Satter, M. (2011) Biphasic regulation of the NADPH oxidase by HGF/c-Met signaling. *Curr. Biol.* 21, 775–778

45. Brown, M., Adyesh, D., Bindokas, V., Moitra, J., Garcia, J. C., and Dudek, S. M. (2010) Quantitative distribution and colocalization of non-muscle myosin light chain kinase isoforms and cortactin in human lung endothelium. *Microvasc. Res.* 79, 75–88

46. Reese, C. R., Wegener, J., Walker, S. R., and Giaever, I. (2004) Electrical wound-healing assay for cells in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 101, 1554–1559

47. Kelley, L. C., Hayes, K. E., Ammer, A. G., Martin, K. H., and Weed, S. A. (2010) Cortactin phosphorylated by ERK1/2 localizes to sites of dynamic actin regulation and is required for carcinoma lamellipodia persistence. *PloS one* 5, e13847

48. Clavijo-Cornejo, D., Enríquez-Cortina, C., López-Reyes, A., Domínguez-Pérez, M., Nuño, N., Domínguez-Meraz, M., Bucio, L., Souza, V., Factor, V. M., Thorpe, J., Gutiérrez-Ruiz, M. C., and Gómez-Quiroz, L. E. (2013) Biphasic regulation of the NADPH oxidase by HGF/c-Met signaling pathway in primary mouse hepatocytes. *Biochim. Biophys. Acta* 1830, 1177–1184

49. Zhou, Y. J., Wang, H. W., Wang, X. G., and Zhang, H. (2009) Hepatocyte growth factor prevents advanced glycation end products-induced injury and oxidative stress through a PI3K/Akt-dependent pathway in human endothelial cells. *Life Sci.* 85, 670–677

50. Madonna, R., Bolli, R., Koxhik, G., and De Caterina, R. (2013) Targeting phosphatidylinositol 3-kinase-Akt through hepatocyte growth factor for cardioprotection. *J. Cardiovasc. Med.* 14, 249–253

51. Ruggieri, R. M., Vitarelli, E., Barresi, G., Trimarchi, F., Benvenga, S., and Trovato, M. (2010) The tyrosine kinase receptor c-met, its cognate ligand HGF and the tyrosine kinase receptor transducers STAT3, PI3K and RHO in thyroid nodules associated with Hashimoto’s thyroiditis: an immunohistochemical characterization. *Eur. J. Histochem.* 54, e24

52. Singleton, P. A., Salgia, R., Moreno-Vinasco, L., Moitra, J., Samman, S., Mirzapoiazova, T., and Garcia, J. G. (2007) CD44 regulates hepatocyte growth factor-mediated vascular integrity. Role of c-Met, Tiam1/Rac1, and cortactin. *J. Biol. Chem.* 282, 30643–30657

53. Zhao, Y., He, D., Stern, R., Usatyuk, P. V., Spanhake, E. W., Salgia, R., and Nataraajan, V. (2007) Lysophosphatidic acid modulates c-Met redistribution and hepatocyte growth factor/c-Met signaling in human bronchial epithelial cells through PKCδ and E-cadherin. *Cell Signal.* 19, 2329–2338

54. Martinez-Palacios, A., del Castillo, G., Suárez-Causado, A., García-Alvaro, M., de Morena-Frutos, D., Fernández, M., Roncero, C., Fabregat, I., Herrera, B., and Sánchez, A. (2013) Mouse hepatic oval cells require Met-dependent PI3K to impair TGF-β-induced oxidative stress and apoptosis.
null