Interaction of Integrin β4 With S1P Receptors in S1P- and HGF-Induced Endothelial Barrier Enhancement

Xiuqin Ni,[1,2] Yulia Epshtein,[2] Weiguo Chen,[2] Tingting Zhou,[2] Lishi Xie,[2] Joe G.N. Garcia,[3] and Jeffrey R. Jacobson[2]*

[1]*Department of Anatomy, Harbin Medical University-Daqing, Daqing, Heilongjiang Province, China*

[2]*Division of Pulmonary, Critical Care, Sleep and Allergy, University of Illinois at Chicago, Chicago, Illinois, USA*

[3]*Arizona Health Sciences Center, University of Arizona, Tucson, Arizona, USA*

ABSTRACT

We previously reported sphingosine 1-phosphate (S1P) and hepatocyte growth factor (HGF) augment endothelial cell (EC) barrier function and attenuate murine acute lung inury (ALI). While the mechanisms underlying these effects are not fully understood, S1P and HGF both transactivate the S1P receptor, S1PR1 and integrin β4 (ITGB4) at membrane caveolin-enriched microdomains (CEMs). In the current study, we investigated the roles of S1PR2 and S1PR3 in S1P/HGF-mediated EC signaling and their associations with ITGB4. Our studies confirmed ITGB4 and S1PR2/3 are recruited to CEMs in human lung EC in response to either S1P (1 μM, 5 min) or HGF (25 ng/ml, 5 min). Co-immunoprecipitation experiments identified an S1P/HGF-mediated interaction of ITGB4 with both S1PR2 and S1PR3. We then employed an in situ proximity ligation assay (PLA) to confirm a direct ITGB4–S1PR3 association induced by S1P/HGF although a direct association was not detectable between S1PR2 and ITGB4. S1PR1 knockdown (siRNA), however, abrogated S1P/HGF-induced ITGB4–S1PR2 associations while there was no effect on ITGB4–S1PR3 associations. Moreover, PLA confirmed a direct association between S1PR1 and S1PR2 induced by S1P and HGF. Finally, silencing of S1PR2 significantly attenuated S1P/HGF-induced EC barrier enhancement as measured by transendothelial resistance while silencing of S1PR3 significantly augmented S1P/HGF-induced barrier enhancement. These results confirm an important role for S1PR2 and S1PR3 in S1P/HGF-mediated EC barrier responses that are associated with their complex formation with ITGB4. Our findings elucidate novel mechanisms of EC barrier regulation that may ultimately lead to new therapeutic targets for disorders characterized by increased vascular permeability including ALI. J. Cell. Biochem. 115: 1187–1195, 2014.   © 2014 Wiley Periodicals, Inc.

KEY WORDS: INTEGRINS; RECEPTORS; SPHINGOSINE 1-PHOSPHATE; HEPATOCYTE GROWTH FACTOR; ENDOTHELIAL CELL

Lung endothelial cell (EC) barrier disruption is a key feature of syndromes associated with inflammation and increased vascular permeability including acute lung injury (ALI). We previously reported that sphingosine 1-phosphate (S1P) and hepatocyte growth factor (HGF) are both robust EC barrier-enhancing agonists that confer protection in murine models of ALI [Liu et al., 2002; Dudek et al., 2004; Peng et al., 2004; Singleton et al., 2007]. S1P is a phospholipid angiogenic factor released from activated platelets that, upon ligation of specific S1P receptors, enhances EC barrier integrity through activation of the small GTPase, Rac1, with subsequent actin and junctional protein rearrangement [English et al., 2000; Dudek et al., 2004; Belvitch et al., 2012]. HGF promotes EC barrier enhancement via ligation of c-Met, a cell surface receptor tyrosine kinase, also with downstream effects on Rac1

activation and actin cytoskeletal rearrangement [Liu et al., 2002; Singleton et al., 2007]. Most recently, we reported that HGF/c-Met-mediated EC barrier enhancement is dependent on the dynamic regulation of a signaling complex comprised of the S1P receptor, S1PR1, and integrin β4 (ITGB4) that are both recruited to membrane caveolin-enriched microdomains (CEMs) [Ephstein et al., 2013].

EC signaling by S1P is dependent on ligation of specific receptor subtypes. While five S1P receptors (S1PR1–5) have been identified EC primarily express the highly homologous S1PR1, S1PR2, and S1PR3 receptors [Hla et al., 2001]. Differential G-protein coupling of these receptors, however, affects distinct downstream signaling events as S1PR1 is coupled to G_i while S1PR2 and S1PR3 are coupled to G_i, Gq, and $G_{12/13}$. Moreover, the functional roles of these receptors in vascular barrier regulation are somewhat unclear and

appear to be variable depending on the experimental conditions. For example, in separate murine models, our lab has reported that decreased expression of either S1P2 or S1P3 is associated with decreased susceptibility to LPS-induced lung injury [Sammani et al., 2010] but increased susceptibility to radiation-induced lung injury [Mathew et al., 2011].

Integrins form transmembrane heterodimers consisting of α and β subunits that mediate both inside-out and outside-in signaling. In EC, integrins are known to regulate a variety of functions including cytoskeletal rearrangement [Giusti et al., 2013], barrier regulation [Eliceiri et al., 2002; Su et al., 2007], angiogenesis [Hood et al., 2003; Nikolopoulos et al., 2004], and inflammatory responses [Chen et al., 2012; Luu et al., 2013]. While eight integrin β subunits have been identified, ITGB4 is uniquely characterized by its long cytoplasmic tail comprised of 1,088 amino acids [Hogervorst et al., 1990]. Prior evidence suggests that integrins are involved in the regulation of CEM trafficking [Echarri and Del Pozo, 2006] and we have previously identified ITGB4 as an important mediator of EC protection by simvastatin [Jacobson et al., 2005; Chen et al., 2012]. We subsequently confirmed that ITGB4 is also a significant mediator of EC barrier enhancement induced by both S1P and HGF via effects on S1PR1 [Ephstein et al., 2013]. In this study, we investigated the role of S1PR2 and S1PR3 in EC barrier enhancement by S1P and HGF with specific focus on the role of ITGB4 in this context.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Human pulmonary artery EC obtained from Lonza (Walkersville, MD) were cultured as previously described in ECM-2 complete medium (Lonza) at 37 °C in 5% CO_2 and 95% air, with cell passages 6–10 used for experimentation [Garcia et al., 2001]. Reagents for SDS–PAGE electrophoresis and immobilon-P transfer membrane were purchased from Bio-Rad (Richmond, CA), and gold microelectrodes from Applied Biophysics (Troy, NY). Sphingosine 1-phosphate (S1P) was purchased from Biomol (Farmingdale, NY). Recombinant human HGF was commercially obtained from PeproTech (Rocky Hill, NJ). Rabbit anti-caveolin-1, rabbit anti-S1PR1, mouse anti-S1PR2, goat anti-S1PR2, rabbit anti-S1PR3, goat anti-S1PR3 and rabbit anti-ITGB4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-S1PR2 antibody was purchased from GeneTex (Irvine, CA). Horseradish peroxidase-labeled secondary antibodies were obtained from Cell Signaling (Danvers, MA). Non-specific siRNA (nsRNA) and siRNA specific for S1PR1, S1PR2, and S1PR3 were purchased from Dharmacon (Layfayette, CO). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

CAVEOLIN–ENRICHED MICRODOMAIN (CEM) ISOLATION

CEMs were isolated from EC as previously described [Ephstein et al., 2013]. Briefly, EC were scraped in PBS, centrifuged at 2000 rpm at 4 °C and lysed with 0.2 ml of TN solution [25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, protease inhibitors, 10% sucrose, 1% Triton X-100] for 30 min on ice. Triton X-100-insoluble materials were mixed with 0.6 ml of cold 60% Optiprep™ and overlaid with 0.6 ml of 40%, 30%, and 20% Optiprep™ in TN solution. The gradients were then centrifuged at 35,000 rpm for 12 h at 4 °C, different fractions were collected, and were analyzed by SDS–PAGE plus immunoblotting.

TRANSFECTION OF SMALL INTERFERING RNA (siRNA)

The siRNA sequences targeting human S1PR1, S1PR2, and S1PR3 were purchased from Dharmacon (Lafayette, CO). EC were transfected with siRNA using siPORT™ Amine (Ambion, Austin, TX) according to the manufacturer's instruction. Cells grown to 60–80% confluence, were treated with 100 nM siRNA in 2% FBS media. After incubating for 72 h, biochemical experiments and functional assays were conducted as described.

IMMUNOPRECIPITATION AND WESTERN BLOTTING

EC were incubated in immunoprecipitation buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 1% Nonidet P-40, 0.4 mM Na_3VO_4, 40 mM NaF, 50 μM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, and Calbiochem protease inhibitor mixture III at 1:250 dilution). The samples were then immunoprecipitated with anti-S1PR2, anti-S1PR3, or anti-ITGB4, followed by SDS–PAGE (4–15%), transferred onto immobilon-P membranes and subjected to immunoblotting using specific primary and HRP-labeled secondary antibodies. Visualization of immunoreactive bands was achieved by enhanced chemiluminescence (Thermo Scientific, Rockford, IL). Densitometric data were obtained with Image J software.

MEASUREMENTS OF TRANSENDOTHELIAL MONOLAYER ELECTRICAL RESISTANCE (TER)

EC were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes and TER measurements were then performed using an electrical cell-substrate impedance sensing system (ECIS) as we have previously described [Garcia et al., 2001]. TER values from each microelectrode were pooled at discrete time points and plotted versus time as the mean ± SEM.

PROXIMITY LIGATION ASSAY (PLA)

EC were used for PLA in situ detection using the Duolink Detection Kit (Olin Bioscience, Uppsala, Sweden) according to the manufacturer's protocol. Briefly, Cells grown on coverslips were starved for 3 h and treated with HGF or S1P for 5 min, then washed with cold PBS and fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton and blocked with 1× blocking solution. Fixed cells were then incubated overnight with anti-ITGB4 and either anti-S1PR3 or anti-S1PR2, or incubated with anti-S1PR1 and anti-S1PR2 antibodies at 4 °C. Proximity ligation was performed with PLA PLUS and MINUS Probes for goat and rabbit. Dapi staining was included in the Duolink Detection Kit while anti-Phalloidin Alexa488 (Invitrogen, Carlsbad, CA) at 1:200 was added during the detection reaction. Lastly, slices were mounted and visualized with an epifluorescence microscope under a 60× oil objective. Texas-red signal was analyzed via BlobFinder Imaging Software, developed and optimized for the analysis of images generated by the in situ PLA (Uppsala Science Park, Sweden) [Ephstein et al., 2013]. Four fields were randomly chosen for analysis and averaged and three separate samples were examined per condition (approximately 60–80 cells total/condition).

STATISTICAL ANALYSIS

Data were analyzed using statistical software (SPSS version 15.0; SPSS, Inc., Chicago, IL) and are expressed as mean ± SEM. Differences between groups were analyzed, as appropriate, using *t*-tests and 1-way analysis of variance followed by the Fisher least significant difference test. $P < 0.05$ was considered significant.

RESULTS

S1P AND HGF–INDUCED TRANSLOCATION OF ITGB4, SP1PR2, AND S1PR3 TO EC CEMS

Western blotting for CEM fractions was conducted to assess ITGB4, S1PR2, and S1PR3 translocalization in response to S1P and HGF. Initially, CEM fractions were confirmed in 30% Optiprep fraction by Western blotting for caveolin-1 as we have previously described [Ephstein et al., 2013] (Fig. 1A). Subsequently, translocation of ITGB4, S1PR2, and S1PR3 to EC CEMs after either S1P (1 μM, 5 min) or HGF (25 ng/ml, 5 min) treatment was also confirmed (Fig. 1B).

INTERACTION OF ITGB4 WITH S1PR2 OR S1PR3 IN RESPONSE TO S1P OR HGF

To investigate the potential associations of ITGB4 with S1PR2 and S1PR3 in both untreated EC and EC treated with either S1P or HGF, co-immunoprecipitation and Western blotting was performed. These experiments confirmed ITGB4–S1PR2 and ITGB4–S1PR3 associations in untreated EC that, in both cases, were signficantly increased after treatment with either S1P (1 μM, 5 min) or HGF (25 ng/ml, 5 min) (Fig. 2).

In complementary experiments, PLA studies were conducted to further explore the association of ITGB4 with S1PR2 and S1PR3. Complex formation was measured by in situ PLA using mouse anti-S1PR2 or anti-S1PR3 antibodies and rabbit anti-ITGB4 antibody, and corresponding secondary reagents. Secondary antibodies
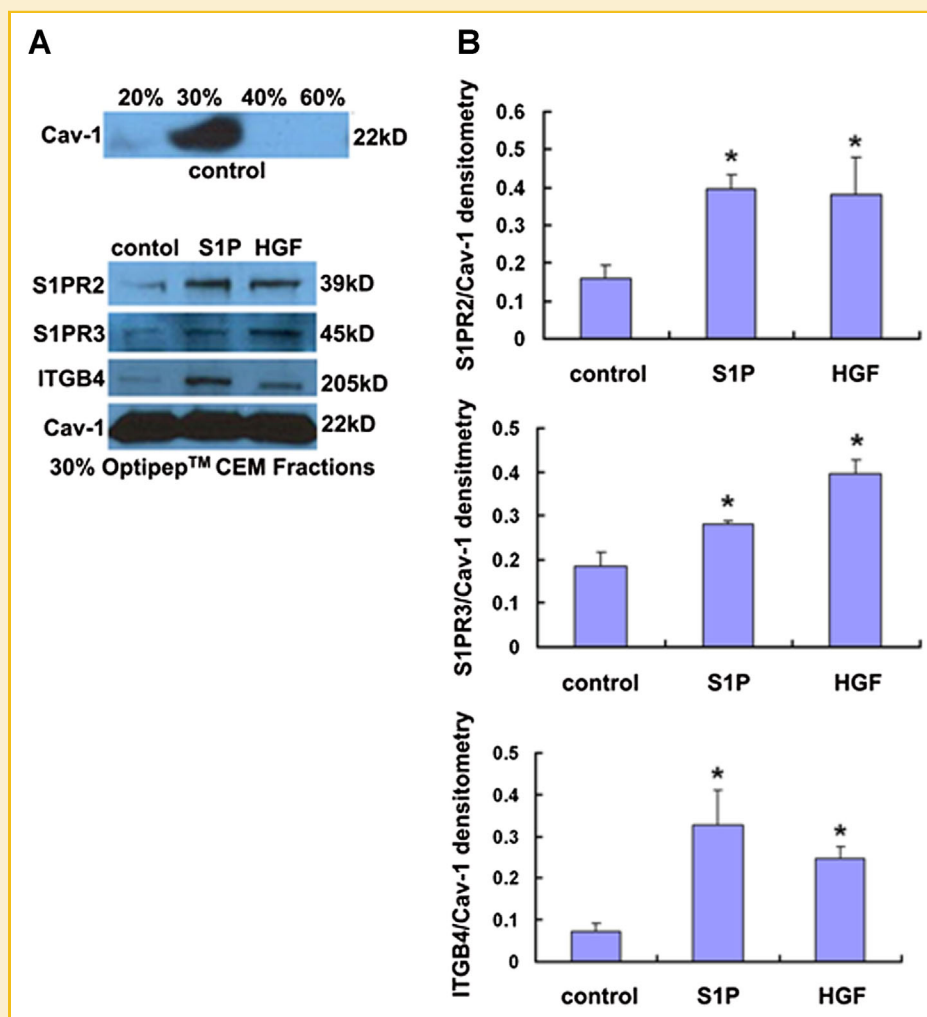

Fig. 1. ITGB4, S1PR2 and S1PR3 translocate to EC CEMs in response to S1P or HGF. (A) Optiprep fractions were isolated and subjected to immunoblotting with an anti–cav–1 antibody. The 30% Optiprep fraction represents the caveolin–enriched CEMs. EC were treated with S1P (1 μM) or HGF (25 ng/ml) for 5 min, and the 30% Optiprep fraction was isolated and subjected to immunoblotting with antibodies specific for ITGB4, S1PR2 or S1PR3. Representative blots are shown. (B) Protein densitometry normalized to caveolin–1 confirms a significant increase in ITGB4, S1PR2, and S1PR3 in CEMs after either S1P or HGF ($n = 3$/condition, *$P < 0.05$ vs. control group).
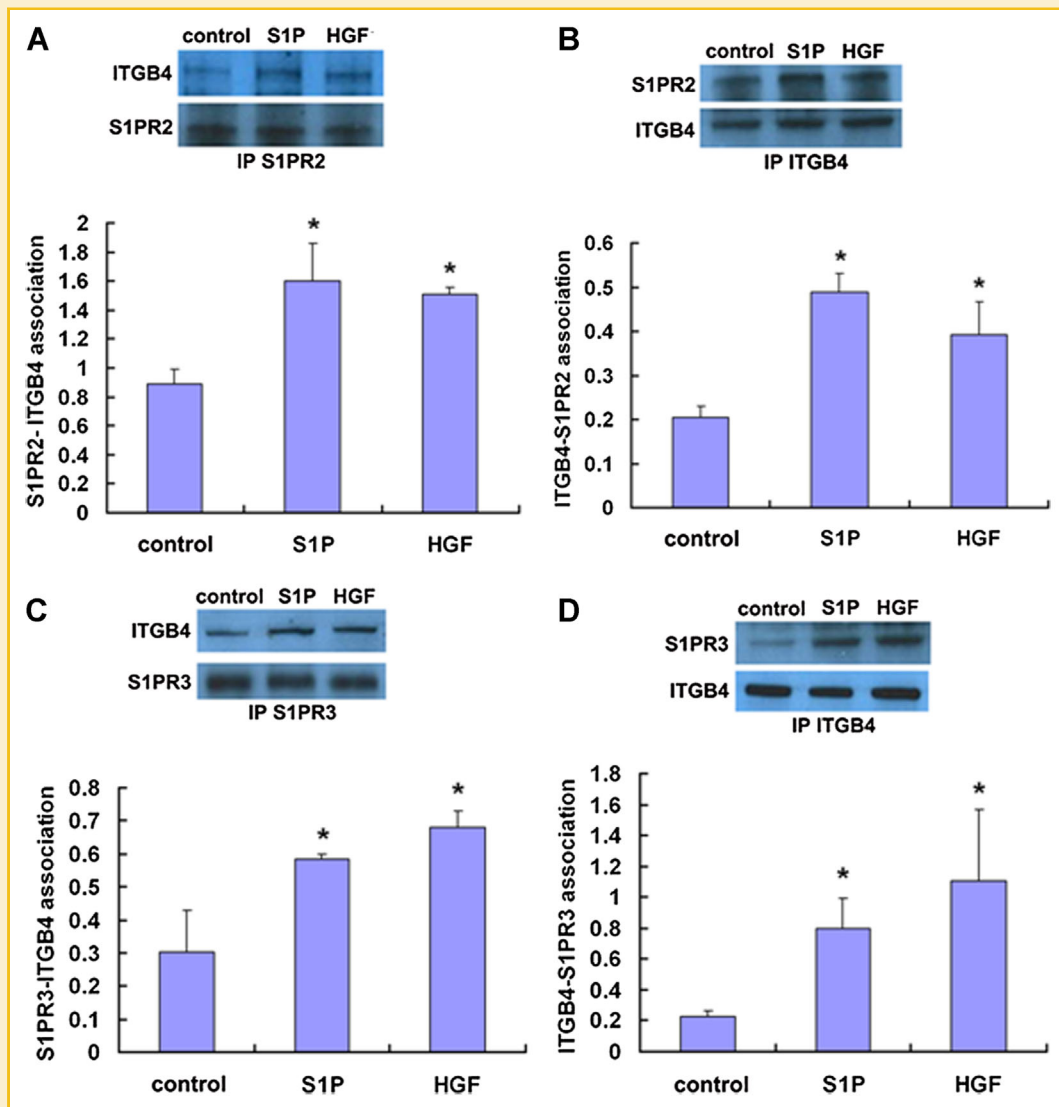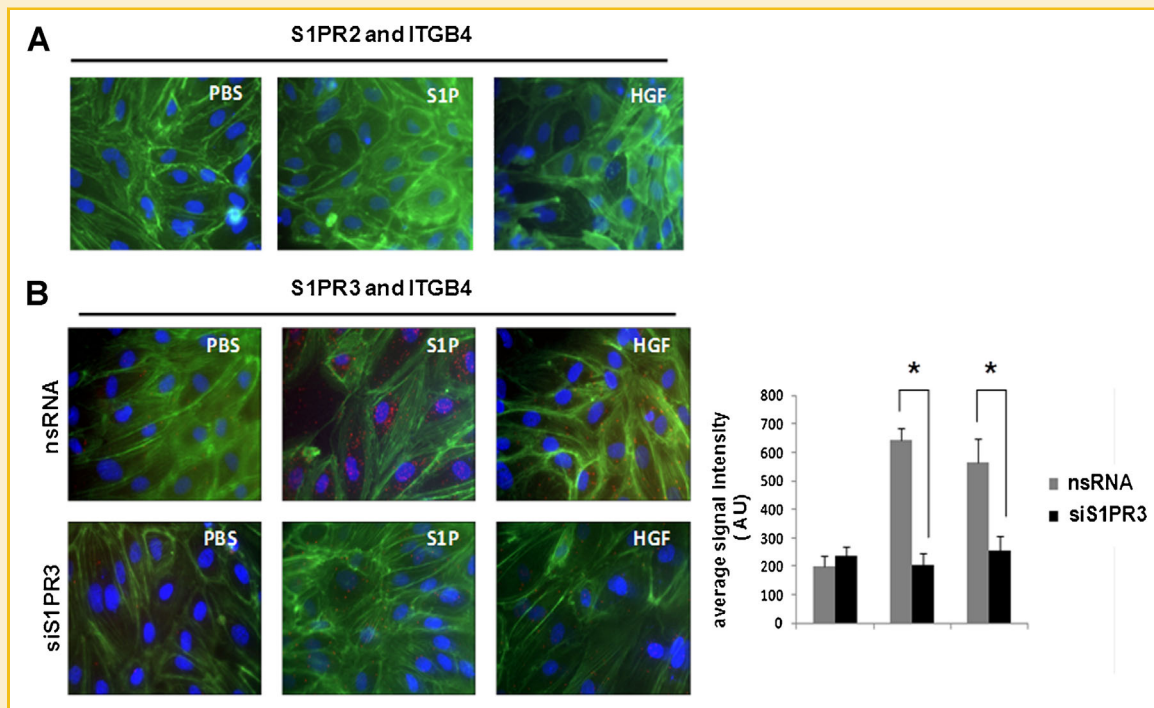
Fig. 2. ITGB4 associates with S1PR2 and S1PR3 after S1P or HGF treatment. (A and B) In co-immunoprecipitation studies, EC were treated with either S1P (1 μM) or HGF (25 ng/ml) for 5 min. Cell lysates were subjected to immunoprecipitation with either an anti–S1PR2 or anti–ITGB4 antibody, and then subjected to immunoblotting with antibody specific for either ITGB4 or S1PR2, respectively. Representative blots are shown. Densitometry confirmed a significant increase in ITGB4–S1PR2 association after treatment with either S1P or HGF ($n = 3$/condition, *$P < 0.05$ vs control group). (C and D) In subsequent experiments with the same conditions, EC treated with either S1P or HGF subjected to immunoprecipitation with an anti–S1PR3 or anti–ITGB4 antibody, and then subjected to immunoblotting with antibody specific for ITGB4 or S1PR3, respectively. Representative blots and corresponding densitometry are shown ($n = 3$/condition, *$P < 0.05$ vs control group).

covalently linked to oligonucleotides were used as proximity probes, forming templates for circularization of two additional oligonucleotides by enzymatic ligation. This ligation requires coincident binding by two affinity reagents and thereby increases the selectivity compared with single recognition assays. Subsequently, one of the oligonucleotides serves as a primer for the RCA reaction, amplifying the circular DNA molecule ~1,000-fold in 1 h using φ29 DNA polymerase. The product represents a bundle of single-stranded DNA composed of tandem repeats of complements of the DNA circle. Individual bundles are easily visualized by hybridization of complementary fluorescence-labeled oligonucleotides. Thus, each red point represents the detection of a protein–protein interaction. Interestingly, these experiments did not identify a direct association between ITGB4 and S1PR2 (Fig. 3). However, there was a direct association between ITGB4 and S1PR3 that was further increased in response to either S1P (1 μM, 5 min) or HGF (25 ng/ml, 5 min), and was markedly attenuated in EC transfected with siRNA specific for S1PR3 (siS1PR3).

ROLE OF S1PR1 IN ITGB4–S1PR2 AND ITGB4–S1PR3 ASSOCIATIONS

To investigate the potential role of S1PR1 in ITGB4 associations with either S1PR2 or S1PR3, EC were transfected with siRNA specific for S1PR1 (siS1PR1) or non-specific siRNA (nsRNA) prior treatment with either S1P (1 μM, 5 min) or HGF (25 ng/ml, 5 min). Relative to control EC transfected with nsRNA, silencing of S1PR1 had no effect on the ITGB4–S1PR2 association at baseline but significantly

Fig. 3. ITGB4 associates directly with S1PR3 but not S1PR2 after treatment with S1P or HGF. The direct association of ITGB4 with either S1PR2 or S1PR3 after treatment with either S1P (1 μM, 5 min) or HGF (25 ng/ml, 5 min) was assessed by PLA. (A) There was no evidence of a direct association between ITGB4 and S1PR2 under any conditions. (B) However, a direct ITGB4–S1PR3 association was detectable after either S1P or HGF (red dots). This effect was abrogated in cells transfected with siRNA specific for S1PR3 (siS1PR3) compared to control cells transfected with non-specific siRNA (nsRNA) ($n = 3$/condition, $*P < 0.05$). Representative images shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

attenuated S1P-induced increases in this association although the reduced association of ITGB4 and S1PR2 in response to HGF was not significant (Fig. 4). Conversely, there was no effect on the interaction of ITGB4 and S1PR3 after silencing of S1PR1 under any condition.

INTERACTION OF S1PR1 WITH S1PR2 INDUCED BY S1P AND HGF

As our results did not identify a direct association between ITGB4 and S1PR2 but did implicate S1PR1 as important determinant of an indirect ITGB4–S1PR2 association, we next explored a direct association between S1PR1 and S1PR2 in response to HGF and S1P by PLA. While there was little S1PR1–S1PR2 association in EC under basal conditions, both S1P and HGF treatment resulted in a significantly increased association (Fig. 5). Together, these results suggest S1PR2 translocation to CEMs in response to S1P- or HGF results in a complex formation characterized by an indirect association of S1PR2 with ITGB4 through S1PR1.

### ROLE OF S1PR2 AND S1PR3 IN S1P– AND HGF–INDUCED EC BARRIER ENHANCEMENT

Finally, as we recently reported that ITGB4 and S1PR1 contribute to HGF- and S1P-mediated EC barrier enhancement, we next investigated the roles of S1PR2 and S1PR3 in this context as measured by TER. Reductions in the expression of S1PR2 by specific siRNA significantly attenuated S1P- and HGF-induced TER enhancement compared to control cells transfected with nsRNA (Fig. 6). Conversely, reductions in the expression of S1PR3 by siRNA knockdown resulted in augmented HGF- and S1P-induced barrier enhancement as measured by TER compared to control cells transfected with nsRNA.

DISCUSSION

The sphingolipid signaling pathway has emerged as an important mediator of EC barrier regulation particularly in the clinical context of ALI [Natarajan et al., 2013]. However, the relative roles of specific S1P receptors in mediating EC barrier responses and the mechanisms underlying these effects remain to be fully characterized. Having recently identified a critical role for ITGB4–S1PR1 complex formation localized to membrane CEMs in S1P- and HGF-induced EC barrier enhancement [Ephstein et al., 2013], we examined the potential association of ITGB4 with S1PR2 and S1PR3 in this same context. Our results indicate that both S1P and HGF induce translocation of ITGB4, S1PR2, and S1PR3 to EC CEMs and promote an indirect association of ITGB4 with S1PR2 through S1PR1 as well as a direct ITGB4–S1PR3 association. Moreover, knockdown of S1PR2 or S1PR3 (siRNA) significantly affected EC barrier enhancement by S1P and HGF. These studies further support growing evidence for ITGB4 as a prominent mediator of EC barrier function and inflammatory responses [Chen et al., 2010, 2012] and implicate for the first time a functional link between ITGB4 and S1PR2/S1PR3 in EC barrier regulation.

The complexities associated with the study of S1P receptor signaling are due in part to variable receptor expression profiles across cell types [Hla et al., 2001; Mazurais et al., 2002] including,
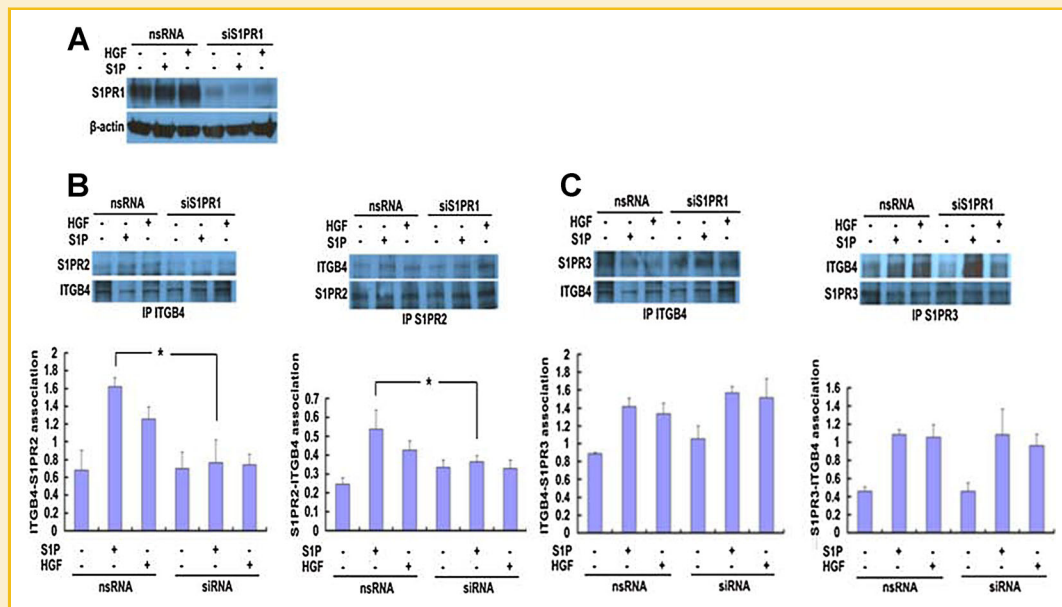
Fig. 4. S1PR1 mediates the ITGB4–S1PR2 association but not ITGB4–S1PR3 association induced by S1P or HGF. EC were transfected with either non–specific siRNA (nsRNA) or siRNA specific for S1PR1 (siS1PR1) prior to treatment with S1P (1 μM, 5 min) or HGF (25 ng/ml, 5 min). (A) Initially, cell lysates were immunoblotted with anti–S1PR1 antibodies to confirm silencing. (B) In separate experiments, cell lysates were subjected to immunoprecipitation with anti–ITGB4 or anti–S1PR2 antibodies, and then subjected to immunoblotting with antibodies specific for S1PR2 or ITGB4, respectively. (C) These experiments were then repeated and immunoprecipitation performed with anti–ITGB4 or anti–S1PR3 antibodies, prior to immunoblotting with antibody specific for S1PR3 or ITGB4, respectively. Representative blots and corresponding densitometry are shown ($n = 3$/condition, $*P < 0.05$).

potentially, different EC phenotypes, as well as evidence of variable functional roles for individual S1P receptors in different in vitro and in vivo models. For example, we have previously reported that both S1PR2−/− and S1PR3−/− mice demonstrate increased susceptibility to inflammatory lung injury induced by radiation [Mathew et al., 2011] while, in contrast, both S1PR2−/− mice and S1PR3-depleted mice (in vivo siRNA) were found to have decreased injury in an LPS-induced ALI model [Sammani et al., 2010]. Additionally, variable concentrations of the same agonist may induce highly discrepant S1P receptor signaling profiles in the same cell type. For example, activation of S1PR1 in response to physiologic concentrations of S1P (0.5 μM) results in EC barrier enhancement mediated by Rac1 while S1PR3 ligation in response to higher dose S1P (>5 μM) evokes EC barrier disruption predominantly through RhoA activation [Shikata et al., 2003]. Accordingly, to claim that S1PR2 mediates EC barrier enhancement and S1PR3 mediates barrier disruption on the basis of our findings now would likely be an inaccurate oversimplification, particularly given conflicting evidence in the literature in both cases. While our findings firmly support the idea that both receptors are important determinants of agonist-mediated EC barrier responses, an idea that is consistent with this growing literature, a full understanding of the relative effects of S1P receptors on EC barrier regulation in variable contexts is an important area of ongoing investigation.
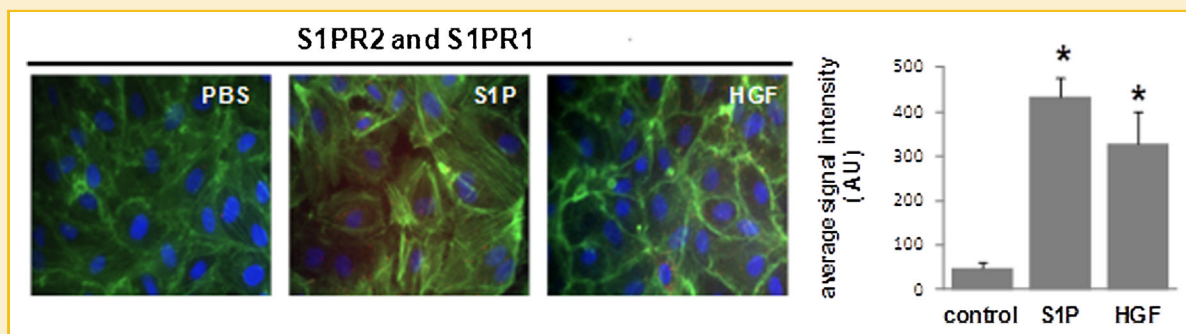
Fig. 5. Increased S1PR1–S1PR2 association in response to S1P or HGF. The direct interaction of S1PR1 and S1PR2 in response to S1P (1 μM, 5 min) or HGF (25 ng/ml, 5 min) was assessed by PLA. Compared to untreated cells, both S1P and HGF induced a significant increase in S1PR1–S1PR2 association (red dots) ($n = 3$/condition, $*P < 0.05$ vs. control group). Representative images shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
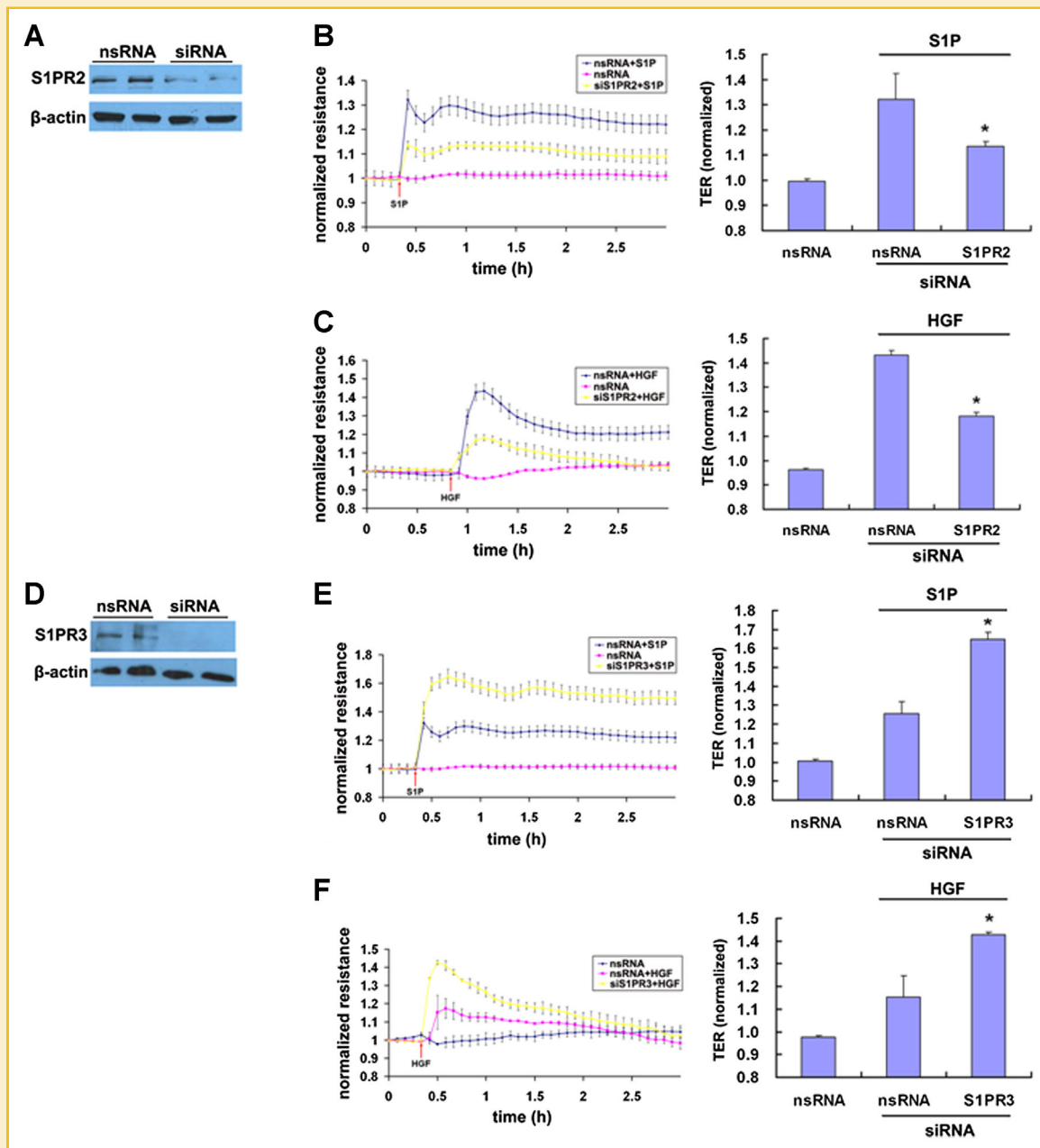
Fig. 6. Role of S1PR2 and S1PR3 in EC barrier enhancement by S1P or HGF. (A) EC were transfected with either non-specific siRNA (nsRNA) or siRNA specific for S1PR2 (siS1PR2) prior to treatment with S1P (1 μM, 5 min) or HGF (25 ng/ml, 5 min). (A) Cell lysates were immunoblotted with anti-S1PR2 antibodies to confirm silencing. (B and C) TER measurements after either S1P or HGF are shown as well as corresponding bar graphs representing maximal TER responses after either S1P or HGF ($n = 4$/condition, $*P < 0.05$ vs controls treated with either S1P or HGF). (D–F) Identical experiments were then performed with siRNA specific for S1PR3 (siS1PR3). Silencing was confirmed by immunoblotting and changes in TER both over time and with respect to maximal responses are shown ($n = 4$/condition, $*P < 0.05$ vs controls treated with either S1P or HGF).

Our identification of ITGB4 as a key component of a dynamically activated EC signaling complex involving S1PR1–3 as well as c-Met, a receptor tyrosine kinase, represents an entirely novel finding. As such, little is known about other potential complex components and it is likely that EC signaling induced by both S1P and HGF involves other proteins that have yet to be characterized. One particularly promising candidate in this regard is Gab-1, a docking protein that mediates a number of signaling pathways including MAPK signaling

via binding to SHP-2, a protein tyrosine phosphatase [Cunnick et al., 2001; Cai et al., 2002]. This is notable as we previously reported that EC inflammatory responses mediated by ITGB4 are dependent on SHP-2 phosphorylation [Chen et al., 2010]. Moreover, separate reports have also characterized Gab-1 as an essential mediator of c-Met signaling [Sachs et al., 2000]. Finally, although there are no reports with respect to ITGB4 specifically, Gab-1 is also a known mediator of integrin signaling [Kuwano et al., 2007]. Indeed, our
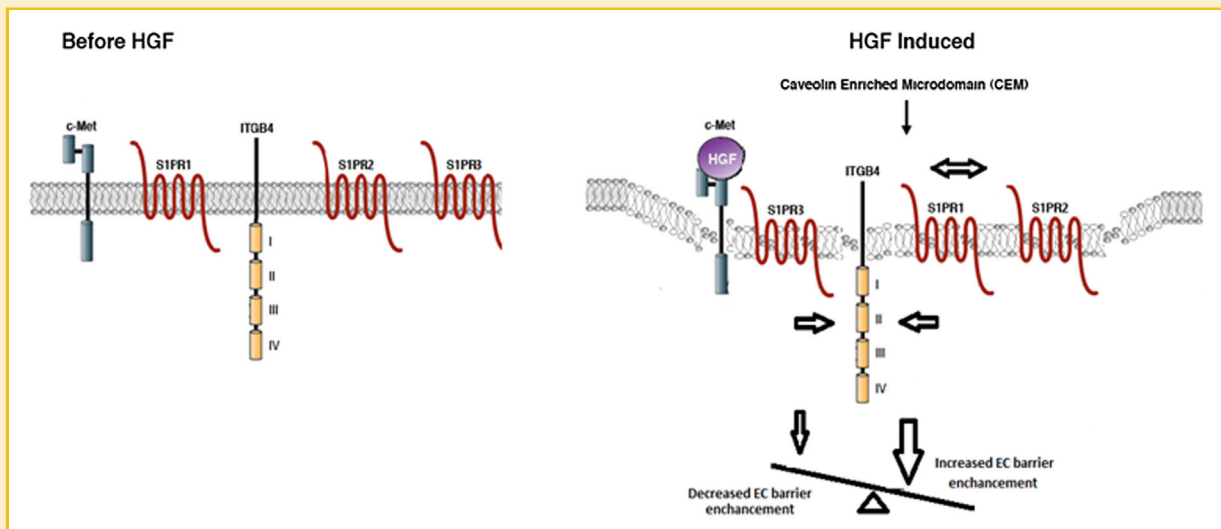
Fig. 7. Proposed mechanisms of agnoist–induced EC barrier enhancement. A model of barrier enhancement by HGF is depicted. Upon ligation of the HGF receptor (c–MET) S1PR1–3 and ITGB4 translocate to CEMs where S1PR3 and S1PR1 associate directly with ITGB4 while S1PR2 associates with S1PR1 (and indirectly with ITGB4). Although barrier enhancement by HGF is attenuated by S1PR3, the overriding effects of S1PR1–S1PR2–mediated signaling result in a net increase in barrier function. Of note, similar effects are induced by S1P also resulting in EC barrier enhancement.

initial studies indicate that partial knockdown of Gab-1 via siRNA transfection significantly attenuates both S1P- and HGF-induced increases in EC barrier function [Ephstein et al., 2011]. Further study is ongoing as are efforts to identify additional complex components.

The potential clinical implications of our findings are significant as evidenced by the protective effects of both HGF and S1P in animal models of ALI [McVerry et al., 2004; Peng et al., 2004; Singleton et al., 2007]. Unfortunately, extending these findings to humans has been limited by potential adverse effects identified in pre-clinical studies including hypotension associated with HGF [Ido et al., 2011] and bradycardia induced by S1P [Forrest et al., 2004]. However, a number of pharmacologic agents that directly target sphingolipid signaling and that have potentially more favorable safety profiles are currently under investigation including a structural analog of S1P, FTY720 (2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol) [Strader et al., 2011], as well as various analogs of phosporylated FTY720 [Hale et al., 2004; Foss et al., 2005; Clemens et al., 2005]. These agents have shown some early promise in relevant in vitro and in vivo models [Camp et al., 2009; Wang et al., 2013]. As these agonists are characterized by variable S1P receptor affinities, a full understanding of the functional roles of S1P receptors in vivo and the underlying mechanisms of downstream signaling events is imperative.

In summary, our results confirm for the first time a functional interaction of ITGB4 with both S1PR2 and S1PR3 that mediates EC barrier regulation induced by either S1P or HGF. Our study suggests that EC barrier function is regulated by the balance between signaling pathways mediated by S1PR1 and S1PR2/3 with induced recruitment of ITGB4, S1PR1, S1PR2, and S1PR3 to CEMs as key events in this setting (Fig.7247707). Finally, our results implicate individual complex components as potential therapeutic targets for diseases characterized by alterations in EC barrier integrity including ALI.

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REFERENCES

Belvitch P, Dudek, SM. 2012. Role of FAK in S1P-regulated endothelial permeability. Microvasc Res 83:22–30.

Cai T, Nishida K, Hirano T, Khavari PA. 2002. Gab1 and SHP-2 promote Ras/MAPK regulation of epidermal growth and differentiation. J Cell Biol 159:103–112.

Camp SM, Bittman R, Chiang ET, Moreno-Vinasco L, Mirzapoiazova T, Sammani S, Lu X, Sun C, Harbeck M, Roe M, Natarajan V, Garcia JG, Dudek SM. 2009. Synthetic analogs of FTY720 [2-amino-2-(2-[4-octylphenyl] ethyl)-1,3-propanediol] differentially regulate pulmonary vascular permeability in vivo and in vitro. J Pharmacol Exp Ther 331:54–64.

Chen W, Garcia JG, Jacobson JR. 2010. Integrin beta4 attenuates SHP-2 and MAPK signaling and reduces human lung endothelial inflammatory responses. J Cell Biochem 110:718–724.

Chen W, Sammani S, Mitra S, Ma SF, Garcia JG, Jacobson JR. 2012. Critical role for integrin-beta4 in the attenuation of murine acute lung injury by simvastatin. Am J Physiol Lung Cell Mol Physiol 303:L279–L285.

Clemens JJ, Davis MD, Lynch KR, Macdonald, TL. 2005. Synthesis of 4(5)-phenylimidazole-based analogues of sphingosine-1-phosphate and FTY720: discovery of potent S1P1 receptor agonists. Bioorg Med Chem Lett 15:3568–3572.

Cunnick JM. Mei L, Wu Doupnik CA, J.. 2001. Phosphotyrosines 627 and 659 of Gab1 constitute a bisphosphoryl tyrosine-based activation motif (BTAM) conferring binding and activation of SHP2. J Biol Chem 276:24380–24387.

del Pozo MA, Alderson NB, Kiosses WB, Chiang HH, Anderson RG, Schwartz MA. 2004. Integrins regulate Rac targeting by internalization of membrane domains. Science 303:839–842.

Dudek SM, Jacobson JR, Chiang ET, Birukov KG, Wang P, Zhan X, Garcia JG. 2004. Pulmonary endothelial cell barrier enhancement by sphingosine

1-phosphate: roles for cortactin and myosin light chain kinase. J Biol Chem 279:24692–24700.

Echarri A, Del Pozo MA. 2006. Caveolae internalization regulates integrin-dependent signaling pathways. Cell Cycle 5:2179–2182.

Eliceiri BP, Puente XS, Hood JD, Stupack DG, Schlaepfer DD, Huang XZ, Sheppard D, Cheresh DA. 2002. Src-mediated coupling of focal adhesion kinase to integrin alpha(v)beta5 in vascular endothelial growth factor signaling. J Cell Biol 157:149–160.

English D, Welch Z, Kovala AT, Harvey K, Volpert OV, Brindley DN, Garcia JG. 2000. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. FASEB J 14:2255–2265.

Ephstein Y, Singleton PA, Chen W, Wang L, Salgia R, Kanteti P, Dudek SM, Garcia JG, Jacobson JR. 2013. Critical role of S1PR1 and integrin beta4 in HGF/c-Met-mediated increases in vascular integrity. J Biol Chem 288:2191–2200.

Ephstein Y, Singleton PA, Jacobson JR, Chen W, Dudek SM, Garcia JG. 2011. HGF-Mediate Lung Endothelial Barrier Regulation: Role of S1PR1 Trans-activation, Integrin Beta 4 And Gab-1. Am J Respir Crit Care Med 183:A4183.

Forrest M, Sun SY, Hajdu R, Bergstrom J, Card D, Doherty G, Hale J, Keohane C, Meyers C, Milligan J, Mills S, Nomura N, Rosen H, Rosenbach M, Shei GJ, Singer II, Tian M, West S, White V, Xie J, Proia RL, Mandala, S. 2004. Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. J Pharmacol Exp Ther 309:758–768.

Foss FW, Clemens JJ, Davis MD, Snyder AH, Zigler MA, Lynch KR, Macdonald, TL. 2005. Synthesis, stability, and implications of phospho-thioate agonists of sphingosine-1-phosphate receptors. Bioorg Med Chem Lett 15:4470–4474.

Garcia JG, Liu F, Verin AD, Birukova A, Dechert MA, Gerthoffer WT, Bamberg JR, English D. 2001. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. J Clin Invest 108:689–701.

Giusti B, Margheri F, Rossi L, Lapini I, Magi A, Serrati S, Chilla A, Laurenzana A, Magnelli L, Calorini L, Bianchini F, Fibbi G, Abbate R, Rosso MD. 2013. Desmoglein-2-integrin Beta-8 interaction regulates actin assembly in endothelial cells: deregulation in systemic sclerosis. PLoS One 8:e68117.

Hale JJ, Neway W, Mills SG, Hajdu R, Ann Keohane C, Rosenbach M, Milligan J, Shei GJ, Chrebet G, Bergstrom J, Card D, Koo GC, Koprak SL, Jackson JJ, Rosen H, Mandala, S. 2004. Potent S1P receptor agonists replicate the pharmacologic actions of the novel immune modulator FTY720. Bioorg Med Chem Lett 14:3351–3355.

Hla T, Lee MJ, Ancellin N, Paik JH, Kluk, MJ. 2001. Lysophospholipidsre-ceptor revelations. Science 294:1875–1878.

Hogervorst F, Kuikman I, von dem Borne AE, Sonnenberg A. 1990. Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain. EMBO J 9:765–770.

Hood JD, Frausto R, Kiosses WB, Schwartz MA, Cheresh DA. 2003. Differential alphav integrin-mediated Ras-ERK signaling during two path-ways of angiogenesis. J Cell Biol 162:933–943.

Ido A, Moriuchi A, Numata M, Murayama T, Teramukai S, Marusawa H, Yamaji N, Setoyama H, Kim ID, Chiba T, Higuchi S, Yokode M, Fukushima M, Shimizu A, Tsubouchi H. 2011. Safety and pharmacokinetics of recombinant human hepatocyte growth factor (rh-HGF) in patients with fulminant hepatitis: a phase I/II clinical trial, following preclinical studies to ensure safety. J Trans Med 9:55.

Jacobson JR, Barnard JW, Grigoryev DN, Ma SF, Tuder RM, Garcia JG. 2005. Simvastatin attenuates vascular leak and inflammation in murine inflam-matory lung injury. Am J Physiol Lung Cell Mol Physiol 288:L1026–L1032.

Kuwano Y, Fujimoto M, Watanabe R, Ishiura N, Nakashima H, Komine M, Hamazaki TS, Tamaki K, Okochi, H. 2007. The involvement of Gab1 and PI 3-kinase in beta1 integrin signaling in keratinocytes. Biochem Biophys Res Commun 361:224–229.

Liu F, Schaphorst KL, Verin AD, Jacobs K, Birukova A, Day RM, Bogatcheva N, Bottaro DP, Garcia JG. 2002. Hepatocyte growth factor enhances endothelial cell barrier function and cortical cytoskeletal rearrangement: potential role of glycogen synthase kinase-3beta. FASEB J 16:950–962.

Luu NT, Glen KE, Egginton S, Rainger GE, Nash GB. 2013. Integrin-substrate interactions underlying shear-induced inhibition of the inflammatory response of endothelial cells. Thromb Haemost 109:298–308.

Mathew B, Jacobson JR, Berdyshev E, HuangY., Sun X, Zhao Y, Gerhold LM, Siegler J, Evenoski C, Wang T, Zhou T, Zaidi R, Moreno-Vinasco L, Bittman R, Chen CT, LaRiviere PJ, Sammani S, Lussier YA, Dudek SM, Natarajan V, Weichselbaum RR. 2011. Garcia JG.. Role of sphingolipids in murine radiation-induced lung injury: protection by sphingosine 1-phosphate analogs. FASEB J 25:3388–3400.

Mazurais D, Robert P, Gout B, Berrebi-Bertrand I, Laville MP, Calmels T. 2002. Cell type-specific localization of human cardiac S1P receptors. J Histochem Cytochem 50:661–670.

McVerry BJ, Peng X, Hassoun PM, Sammani S, Simon BA, Garcia JG, 2004. Sphingosine 1-phosphate reduces vascular leak in murine and canine models of acute lung injury. Am. J. Respir. Crit. Care Med. 170:987–993.

Natarajan V. Dudek SM, Jacobson JR. Moreno-Vinasco L, Huang LS, Abassi T. Mathew B, Zhao Y. Wang L, Bittman R. Weichselbaum R, Berdyshev E. Garcia JG. 2013. Sphingosine-1-phosphate, FTY720, and sphingosine-1-phosphate receptors in the pathobiology of acute lung injury. Am J Respir Cell Mol Biol 49:6–17.

Nikolopoulos SN, Blaikie P, Yoshioka T, Guo W, Giancotti FG. 2004. Integrin beta4 signaling promotes tumor angiogenesis. Cancer Cell 6:471–483.

Peng X, Hassoun PM, Sammani S, McVerry BJ, Burne MJ, Rabb H, Pearse D, Tuder RM, Garcia JG. 2004. Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury. Am J Respir Crit Care Med 169:1245–1251.

Sachs M, Brohmann H, Zechner D, Muller T, Hulsken J, Walther I, Schaeper U, Birchmeier C, Birchmeier W. 2000. Essential role of Gab1 for signaling by the c-Met receptor in vivo. J Cell Biol 150:1375–1384.

Sammani S, Moreno-Vinasco L, Mirzapoiazova T, Singleton PA, Chiang ET, Evenoski CL, Wang T, Mathew B, Husain A, Moitra J, Sun X, Nunez L, Jacobson JR, Dudek SM, Natarajan V, Garcia, JG. 2010. Differential effects of sphingosine 1-phosphate receptors on airway and vascular barrier function in the murine lung. Am J Respir Cell Mol Biol 43:394–402.

Shikata Y, Birukov KG, Garcia JG. 2003. S1P induces FA remodeling in human pulmonary endothelial cells: role of Rac, GIT1, FAK, and paxillin. J Appl Physiol 94:1193–1203.

Singleton PA, Salgia R, Moreno-Vinasco L, Moitra J, Sammani S, Mirzapoiazova T, Garcia JG. 2007. CD44 regulates hepatocyte growth factor-mediated vascular integrity. Role of c-Met, Tiam1/Rac1, dynamin 2, and cortactin. J Biol Chem 282:30643–30657.

Strader CR, Pearce CJ, Oberlies NH. 2011. Fingolimod (FTY720): a recently approved multiple sclerosis drug based on a fungal secondary metabolite. J Nat Prod 74:900–907.

Su G, Hodnett M, Wu N, Atakilit A, Kosinski C, Godzich M, Huang XZ, Kim JK, Frank JA, Matthay MA, Sheppard D, Pittet JF. 2007. Integrin alphavbeta5 regulates lung vascular permeability and pulmonary endothelial barrier function. Am J Respir Cell Mol Biol 36:377–386.

Wang L, Camp SM, Bittman R, Garcia JG, Dudek SM. 2013. Cytoskeletal and junctional complex rearrangement mediates pulmonary endothelial barrier enhancement by FTY720 S-phosphonate. Am J Respir Crit Care Med 187: A3705.