RhoC maintains vascular homeostasis by regulating VEGF-induced signaling in endothelial cells

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ABSTRACT

Vasculogenesis and angiogenesis are controlled by vascular permeability factor/vascular endothelial growth factor A (VEGF). Dysregulation of these physiological processes contributes to the pathologies of heart disease, cancer and stroke. Rho GTPase proteins play an integral role in VEGF-mediated formation and maintenance of blood vessels. The regulatory functions of RhoA and RhoB in vasculogenesis and angiogenesis are well defined, whereas the purpose of RhoC remains poorly understood. Here, we describe how RhoC promotes vascular homeostasis by modulating endothelial cell migration, proliferation, and permeability. RhoC stimulates proliferation of human umbilical vein endothelial cells (HUVEC) by stabilizing nuclear β-catenin, which promotes transcription of cyclin D1 and subsequently drives cell cycle progression. RhoC negatively regulates endothelial cell migration through MAPK and downstream MLC-2 signaling and decreases vascular permeability through downregulation of the phospholipase Cγ (PLCγ)/Ca^{2+}/eNOS cascade in HUVEC. Using a VEGF-inducible zebrafish (Danio rerio) model, we observed significantly less vascular permeability in RhoC morpholino (MO)-injected zebrafish than control MO-injected zebrafish. Taken together, our findings suggest RhoC is a key regulator of vascular homeostasis in endothelial cells.
INTRODUCTION

The Rho family of small GTPases regulates diverse signaling effectors and cellular functions to control cellular adhesion, gene transcription, and cell cycle progression by acting as a molecular switch that oscillates between an active GTP-bound state and an inactive GDP-bound form (Van Aelst and D'Souza-Schorey, 1997). For instance, RhoA activates at least 11 different effector molecules to modulate cell motility, cell morphology, and cell-cell adhesion through control of actin cytoskeletal organization (Bishop and Hall, 2000; Paterson et al., 1990; Takaishi et al., 1994). Rho proteins also function in membrane ruffling, smooth muscle contraction, development of stress fibers and focal adhesions, neurite retraction in neuronal cells, and cytokinesis (Hirata et al., 1992; Jalink et al., 1994; Nishiki et al., 1990; Nishiyama et al., 1994; Piekny et al., 2005; Ridley and Hall, 1992). Among Rho subfamily members (RhoA, RhoB, RhoC, RhoE, and RhoG), the most well studied are RhoA, RhoB and RhoC. RhoC shares about 93% amino acid identity with RhoA and 86% homology with RhoB (Ridley, 1997; Wheeler and Ridley, 2004). Although these three Rho proteins are structurally similar, they are functionally distinct.

Rho proteins regulate nascent blood vessel formation, termed vasculogenesis, as well as the development of new capillaries from pre-existing ones, a process named angiogenesis (Carmeliet, 2000; Risau, 1997; Risau and Flamme, 1995). Vascular endothelial growth factor (VEGF) is a multifunctional cytokine, endothelial mitogen, and permeability factor that plays a critical role in both vasculogenesis and angiogenesis. The function of VEGF in EC is mediated primarily by two tyrosine kinase receptors, VEGFR-1 and VEGFR-2. Previously, we have shown that VEGFR-2 regulates endothelial cell migration through activation of RhoA (Zeng et al., 2002). Others have demonstrated that RhoA signaling is essential for various aspects VEGF-induced vasculogenesis and angiogenesis, including endothelial cell motility, proliferation, survival and permeability (Bryan et al., 2010; van Nieuw Amerongen et al., 2003). Global knockout of RhoA in mice has not been reported suggesting embryonic lethality, whereas fibroblast-specific knockout of RhoA inhibits mitosis in mouse embryos (Melendez et al., 2011). RhoB- and RhoC-deficient mice are viable and have no significant developmental impairments (Hakem et al., 2005; Liu et al., 2001). Despite the structural similarity of the Rho isoforms, they likely function distinctly in EC due to varied tissue-specific and context-dependent expression, unique epigenetic and posttranslational modifications, and differential molecular mechanisms of regulation. RhoB regulates stage-specific survival of EC during vascular development by controlling Akt trafficking, and RhoB knockout mice exhibit retarded vascular development in the retina classified by disrupted sprout morphology (Adini et al., 2003). Thus, RhoB regulates endothelial cell migration, vessel assembly, and tube formation (Howe and Addison, 2012), cellular processes required for sprouting angiogenesis. One report has shown VE-cadherin signals
through RhoC to regulate Rho-kinase activity, myosin light-chain 2 (MLC2) phosphorylation, and actomyosin contractility during tube formation in EC cocultured with human dermal fibroblasts (Abraham et al., 2009). However, little is known about the role of RhoC in VEGF-mediated signaling in EC and vascular development as most studies of RhoB- and RhoC-deficient cells and mice have concentrated on their regulation of vesicular trafficking and cancer development.

We sought to determine how RhoC regulates the VEGF signaling pathway and assess its role in vascular development as well as angiogenesis. Our studies suggest RhoC is activated upon VEGF stimulation through VEGFR-2 to regulate endothelial cell proliferation, migration and permeability through modulation of diverse signaling cascades. RhoC promotes HUVEC proliferation by protecting β-catenin from proteosomal degradation and thereby stimulating cell cycle progression. Conversely, RhoC negatively regulates endothelial cell migration by decreasing downstream MAPK-42/44, p38 MAPK, and MLC-2 phosphorylation events and vascular permeability through downregulation of VEGF-mediated PLC-γ1 phosphorylation, intracellular calcium mobilization, and eNOS activity. We corroborated the latter finding in vivo using a VEGF-inducible zebrafish model of vascular permeability and observed greater vascular permeability in RhoC morpholino (MO)-injected zebrafish than controls. Taken together, RhoC represents an important molecular modulator of vascular homeostasis, which may have important clinical implications in the treatment of cancer and vascular diseases, including cardiac and cerebral infarctions.

RESULTS
VEGF stimulation activates RhoC
VEGF-A has been described to induce RhoA activity within 1 min post-stimulation in HUVEC (van Nieuw Amerongen et al., 2003; Zeng et al., 2002). VEGF-A induction results in increased expression but not activity of RhoB protein in HUVEC (Howe and Addison, 2012). Therefore, we sought to determine whether RhoC is activated upon VEGF stimulation. Serum starved HUVEC were treated with VEGF-A for 1, 3 or 5 min and active GTP-bound RhoA and RhoC was immunoprecipitated from cell lysates. Like RhoA, RhoC also is activated within 1 min post-stimulation with VEGF (Fig. 1A).

Relative expression of Rho family members and the effects of RhoC depletion
Given that Rho family members regulate endothelial cell function, we aimed to determine their relative expression in EC and the effect of RhoC depletion. By immunoblotting, RhoC ablation did not change RhoB protein levels but increased RhoA protein expression (supplementary material Fig. S1A). Next, we examined RhoA activity in HUVEC by pull down assay after RhoC depletion. RhoC
knockdown completely abrogated RhoC activity but had minimal effect on RhoA activity (Fig. 1B). In summary, our results suggest RhoC has no effect on RhoB protein expression, and while RhoC ablation increases RhoA protein expression, it has little effect on RhoA activity.

RhoC promotes proliferation and negatively regulates migration in a VEGF-dependent manner

The role of RhoA has been well documented in the regulation of VEGF-induced endothelial cell migration, but RhoA has no effect on the proliferation of EC (Bryan et al., 2010; van Nieuw Amerongen et al., 2003; Zeng et al., 2002). Similarly, RhoB is required for endothelial cell migration, yet dispensable for HUVEC viability (Howe and Addison, 2012). Based on these findings, we sought to determine whether RhoC regulates VEGF-stimulated proliferation and migration in EC.

To this end, we ablated RhoC in EC using two unique RhoC siRNAs and confirmed effective RhoC protein knockdown by immunoblotting (Fig. 1B, supplementary material Fig. S1B). To assess proliferation, HUVEC were transfected with control or RhoC siRNA, stimulated with VEGF, and subjected to thymidine incorporation assays. RhoC knockdown significantly inhibited VEGF-induced proliferation of HUVEC (p=0.00018; Fig. 1C). Basal proliferation of HUVEC was also decreased upon RhoC ablation (p=0.032; Fig. 1C), albeit to a lesser magnitude than in the presence of VEGF.

We performed Boyden chamber migration assays and demonstrated VEGF-dependent HUVEC migration was significantly increased upon RhoC knockdown (Fig. 1D). RhoC ablation had no effect on apoptosis (data not shown).

We next sought to determine whether RhoC promotes proliferation and decreases migration in other endothelial cell types or if these effects of RhoC are specific to HUVEC. Thus, we knocked down RhoC in human lymphatic microvascular EC (LyEC) and human brain microvascular EC (HBMVEC) using siRNA (Fig. 2A). Similar to our results in HUVEC, we observed increased migration upon RhoC ablation in HBMVEC (Fig. 2B) and decreased proliferation in RhoC siRNA-treated LyEC (Fig. 2C). Our results suggest RhoC promotes proliferation and negatively regulates migration in distinct populations of human ECs; however, RhoC ablation had no effect on HBMVEC proliferation (Fig. 2D) or LyEC migration (data not shown).

Interestingly, inhibition of RhoC by siRNA in MDA-MB-231 breast cancer cells led to decreased proliferation and less invasion (supplementary material Fig. S2A-C). Taken together, our findings suggest RhoC promotes endothelial cell proliferation and negatively regulates endothelial cell migration, whereas RhoC promotes both proliferation and migration in breast tumor cells.

Involvement of RhoC in VEGF-mediated downstream signaling cascades

We sought to determine the signaling pathways through which RhoC negatively regulates VEGF-mediated endothelial cell migration. The Ras/Raf/MEK/ERK1/2 pathway has a well-documented role...
in endothelial cell function. ERK1/2, which is also known as MAPK-42/44, regulates endothelial cell migration as well as proliferation in vivo and in vitro (Srinivasan et al., 2009). Control or RhoC siRNA-treated serum-starved HUVEC were administered 10 ng/ml VEGF for 5 or 10 min and immunoblotted for phospho-MAPK-42/44. Upon RhoC knockdown, MAPK-42/44 phosphorylation was detectable after 5 minutes of VEGF stimulation compared to 10 minutes in the control siRNA-treated HUVEC (Fig. 3A; supplementary material Fig. S3A). RhoC depletion also led to increased VEGF-induced phosphorylation of stress induced protein kinases like p38 MAPK (Fig. 3A; supplementary material Fig. S3B) and SAPK/JNK (Fig. 3A; supplementary material Fig. S3D). We observed little to no change in pro-survival molecule Akt-1/2/3 at serine 473 (Fig. 3A; supplementary material Fig. S3C). Phosphorylation of Src has been shown to regulate migration of EC in response to VEGF through binding with T-cell-specific adapter (TSAd) (Matsumoto et al., 2005). However, we did not observe any change in Src phosphorylation upon RhoC knockdown in HUVEC (supplementary material Fig. S2D).

**RhoC regulates migration through MAPK-42/44**

MEK1 is upstream of MAPK-42/44 in the Ras/Raf/MEK/ERK1/2 signaling pathway. To confirm the role of MAPK-42/44 in the RhoC-mediated negative regulation of endothelial cell migration, we repeated the migration assay with control or RhoC depleted HUVEC pre-treated with MEK1 inhibitor for 1 hour, seeded into collagen coated transwell chambers and incubated for another 4 hours in the presence or absence of VEGF. We observed a significant increase in VEGF-induced cell migration after RhoC siRNA treatment compared to controls (Fig. 3B). As expected, this RhoC knockdown-mediated increase in endothelial cell migration was blocked in presence of 10 and 20 μM MEK1 inhibitor (Fig. 3B), whereas MEK1 inhibitor had no effect on the migration of control siRNA-treated HUVEC (supplementary material Fig. S1D). This result suggests RhoC signals through MEK1 and downstream MAPK-42/44 in a VEGF-dependent manner to negatively regulate HUVEC migration. Rho family members activate Rho kinases like ROCKI and ROCKII (Riento and Ridley, 2003), which phosphorylate downstream LIM kinases and regulatory myosin light chains (MLC) (Leung et al., 1996; Uehata et al., 1997). Moreover, MAPK itself influences cell motility by regulating MLC kinase activity and finally phosphorylation of MLC (Klemke et al., 1997). Therefore, we examined the phosphorylation status of LIMK1/2 and MLC-2 upon VEGF stimulation and siRNA-mediated RhoC knockdown in HUVEC. RhoC depletion did not affect LIMK1/2 phosphorylation (Fig. 3C; supplementary material Fig. S3E,F) but increased MLC-2 phosphorylation (Fig. 3C; supplementary material Fig. S3G). While focal adhesion kinase (FAK) activity has also been shown to play a role in VEGF-induced endothelial cell migration (Rousseau et al., 2000), RhoC ablation did not affect VEGF-stimulated FAK phosphorylation (supplementary material Fig. S2E). Taken together, our findings suggest RhoC negatively regulates endothelial cell migration through a cascade that includes
VEGF-mediated MAPK phosphorylation and downstream MLC-2 phosphorylation, but excludes signaling through LIMK1/2 and FAK.

**RhoC promotes proliferation through cell cycle progression**

RhoC knockdown decreases VEGF-induced endothelial cell proliferation yet increases the phosphorylation of pro-proliferative molecules, such as MAPK-42/44. Given these seemingly disparate results, we hypothesized RhoC promotes proliferation through regulation of cell cycle progression. Indeed, we observed a significantly greater percentage of cells in the G1 phase and a concomitant lower percentage in S and G2 phases following RhoC knockdown and VEGF stimulation, thus suggesting that RhoC controls VEGF-induced proliferation through cell cycle regulatory mechanisms (Fig. 4A). RhoC knockdown using two distinct siRNAs in HUVEC inhibited the expression of Cyclin D1 (Fig. 4B; supplementary figure 1B). RhoC ablation in LyEC and HBMVEC also led to decreased VEGF-dependent and -independent of expression Cyclin D1 (Fig. 2A). Cyclin D1 is known to promote cell cycle passage through the G0-S phase; therefore, inhibition of cyclin D through RhoC ablation likely blocks cell cycle progression through G0-S and causes accumulation of cells in G1 phase as we observed. Correspondingly, upon RhoC knockdown and VEGF-induction in HUVEC, we saw no upregulation of Cyclin A and B (Fig. 4B), which control mitosis. Finally, we assessed expression of Cip/Kip family members, p21Cip1 (p21) and p27Kip1 (p27), which regulate cell cycle progression by binding to a variety of cyclin/cdk complexes and inhibiting their kinase activity (Johnson and Walker, 1999). Depletion of RhoC in HUVEC increased both VEGF-dependent and -independent expression of cell cycle inhibitor p27 but had no effect on p21 (Fig. 4B). Our results suggest RhoC promotes endothelial cell proliferation by stimulating cycle cell progression through upregulation of Cyclin D1 and negative regulation of cell cycle inhibitor p27.

**RhoC upregulates β-catenin**

We next sought to further investigate the molecular mechanism through which RhoC regulates cell cycle progression. Because active, nuclear β-catenin directly interacts with Lef1/Tcf transcription factors to promote Cyclin D1 expression, we hypothesized that RhoC may upregulate Cyclin D1 and stimulate cell cycle progression through β-catenin. Indeed, RhoC ablation decreased nuclear β-catenin protein expression in HUVEC in the presence and absence of VEGF stimulation (Fig. 4C). To confirm these findings in vivo, we knocked down zebrafish RhoC homologues, Rhoad and Rhoae (supplementary material Fig. S4A,B), by injecting MOs into transgenic fltl:EGFP embroys in which EGFP is expressed in the vasculature. Notably, we did not observe any defects or delays in zebrafish vascular development upon morpholino-mediated knockdown of Rhoad and Rhoae (data not shown).
At 3 days post-fertilization, we sectioned the zebrafish embryos and performed immunofluorescence staining for β-catenin. As expected, we observed less β-catenin in the vessels of zebrafish injected with Rhoad/Rhoae MO compared to siblings administered control MO (supplementary material Fig. S4C). Our data implies a scenario in which RhoC promotes proliferation by upregulating β-catenin, which in turn, promotes Cyclin D1 expression to subsequently drive cell cycle progression.

**VEGF activates RhoC through VEGFR-2**

We have shown that RhoC regulates endothelial cell migration through the MAPK pathway and proliferation through control of the cell cycle; therefore, we sought to investigate the upstream signaling events that initiate VEGF-dependent RhoC signaling in endothelium. VEGFR-1 and VEGFR-2 are expressed on the cell surface of most vascular EC, and VEGF-A binds to both VEGFR-1 and VEGFR-2 to mediate downstream signaling. We aimed to determine which receptor is involved in VEGF-induced RhoC activation. HUVEC were treated with control, VEGFR-1, or VEGFR-2 siRNA and after 48 hours cell lysates were collected and an activated RhoC pull-down assay was performed. RhoC activation was completely blocked upon VEGFR-2 knockdown (Fig. 5A) but not following VEGFR-1 ablation (supplementary material Fig. 1E), which indicates VEGF activates RhoC through VEGFR-2 signaling.

**RhoC depletion affects VEGF-mediated VEGFR-2 phosphorylation**

We next examined the phosphorylation of VEGFR-2 at different tyrosine residues. RhoC depletion led to a significant increase in VEGF-induced VEGFR-2 phosphorylation at tyrosine residues 951, 1059, and 1175 (Fig. 5B) as well as total tyrosine phosphorylation (Fig. 5C). Upon binding VEGF, VEGFR-2 undergoes endocytosis via a classical clathrin-mediated pathway and either is targeted for recycling back to the plasma membrane or for sequential proteasome and lysosomal degradation (Eichmann and Simons, 2012). We also evaluated total VEGFR-2 levels in RhoC depleted cells at 5, 10 and 15 min post-stimulation with VEGF. Interestingly, RhoC knockdown delayed the degradation of VEGFR-2 compared to control siRNA treated cells (Fig. 5B), thus implicating RhoC in VEGFR-2 trafficking.

**RhoC controls phosphorylation of PLC-γ1 and eNOS and induces intracellular Ca^{2+} release**

VEGF was originally described as vascular permeability factor based on its property of increasing vessel wall permeability (Senger et al., 1983). VEGF regulates vascular permeability by binding to VEGFR-2 and stimulating phospholipase C (PLC)γ-dependent IP3 production, increased the cytosolic calcium concentration, and greater eNOS production (Brock et al., 1991; Jho et al., 2005; Wu et al., 1999). In light of our results suggesting VEGF activates RhoC through VEGFR-2, we sought to...
determine whether RhoC regulates vascular permeability through the VEGFR-2/PLCγ/Ca\textsuperscript{2+}/eNOS cascade. VEGF stimulation is known to activate PLC-γ1 through phosphorylation of Y783 (Tahir et al., 2009). Correspondingly, RhoC knockdown in VEGF-stimulated HUVEC significantly increased PLC-γ1 phosphorylation at Y783 (Fig. 6A). RhoC siRNA treatment increased basal and VEGF-stimulated eNOS phosphorylation at serine 1177 (Fig. 6B), the most thoroughly studied activation site of eNOS (Granger et al., 1994). We performed an intracellular calcium-release assay and demonstrated that EC expressing RhoC siRNA exhibited increased calcium flux compared with controls (Fig. 6C), indicating that RhoC does indeed negatively regulate calcium flux. Taken together, our findings suggest RhoC negatively regulates VEGF-induced vascular permeability.

Given our in vitro observations, we sought to determine whether RhoC controls permeability in vivo. We previously described a heat-inducible VEGF zebrafish model used in conjunction with morpholino (MO)-mediated protein knockdown to assess genetic regulation of vascular permeability in real-time (Hoeppner et al., 2012). In Danio rerio, five Rho gene isoforms exist: Rhoa, Rhoab, Rhoac, Rhoad, and Rhoae, and the protein homology of all human and zebrafish Rho subfamily members is 79.1% with 97.4% overall similarity (Salas-Vidal et al., 2005). Phylogenetic studies have suggested that human RhoC is most similar to zebrafish Rhoad and Rhoae (Salas-Vidal et al., 2005). First, we performed in situ hybridization to demonstrate that RhoC is expressed within the vasculature of zebrafish (Fig. 7A,B). We then designed Rhoad- and Rhoae-specific MOs and achieved knockdown of both genes after co-microinjection of the MOs in 1-2 cell stage zebrafish embryos (supplementary material Fig. S4A,B). At 3 days post-fertilization, we performed microangiography in the MO-injected embryos with fluorophore-conjugated dextrans, induced VEGF through heat induction, and immediately performed live imaging of extravasated red tracer as a measure of vascular permeability. In the presence of VEGF-induction, we observed significantly greater vascular permeability in Rhoad/Rhoae MO-injected zebrafish than control MO-injected zebrafish (Fig. 7C,D). Interestingly, RhoC knockdown also increases vascular permeability in the absence of VEGF induction; however, physiological levels of VEGF likely are present in the zebrafish embryos (Fig. 7C,D). Taken together with the findings that RhoC knockdown promotes PLCγ/Ca\textsuperscript{2+}/eNOS signaling, these in vivo data suggest RhoC negatively regulates VEGF-induced vascular permeability.
DISCUSSION

During the initial phases of vascular development, mesodermal precursors differentiate into EC to form nascent blood vessels, a process termed vasculogenesis (Risau and Flamme, 1995). Subsequently, blood vessels become capable of additional expansion through two forms of angiogenesis: enlargement of pre-existing vessels or formation of new vessels from pre-existing ones. Both processes require endothelial cell proliferation, whereas the latter expansion, known as sprouting angiogenesis, also is dependent upon endothelial cell migration, vessel assembly and tube formation (Risau, 1997).

RhoA, RhoB and RhoC are members of the Rho family of small GTPases. Rho proteins act as molecular switches, alternating between an inactive GDP-bound and an active GTP-bound state to relay signals from cell surface receptors associated with growth factors, adhesion molecules, cytokines, and G-protein-coupled receptors (Van Aelst and D'Souza-Schorey, 1997). RhoA regulates endothelial cell proliferation, migration, vessel assembly, and tube formation and thus plays in an indispensable role in vasculogenesis and angiogenesis (Bryan et al., 2010; van Nieuw Amerongen et al., 2003). RhoB deficiency has been shown to result in apoptosis of primary EC during sprouting angiogenesis in vivo and tube formation in vitro through a mechanism in which RhoB regulates the nuclear trafficking of AKT and protein kinase B to control endothelial cell survival (Adini et al., 2003). Correspondingly, the finding that RhoB knockout mice are smaller than wildtype mice may reflect an angiogenesis defect (Liu et al., 2001), as delays in retinal vascular development have been observed (Adini et al., 2003). The function of RhoC in vasculogenesis and angiogenesis is less defined.

Here, we demonstrate VEGF signals through VEGFR-2 to activate RhoC. We show RhoC negatively regulates global VEGFR-2 tyrosine phosphorylation and precisely phosphorylation of VEGFR-2 tyrosine residues Y951, Y1059, and Y1175. Negative feedback inhibition of VEGFR-2 by RhoC is a novel finding that warrants further future investigation. VEGFR-2 has been shown to undergo endocytosis and subsequent recycling or degradation following binding of VEGF ligand (Eichmann and Simons, 2012). VEGF-induced vascular permeability is stimulated in EC through a signaling cascade involving TSAd and c-Src tyrosine kinase, which is activated by tyrosine phosphorylation of VEGFR2 at Y951 (Sun et al., 2012). Phosphorylation at Y1175 of VEGFR-2 also promotes vascular permeability by enabling VEGF binding, phosphorylation, and activation of PLCγ1 (Takahashi et al., 2001), which ultimately leads to Ca^{2+} influx. Correspondingly, our results indicate ablation of RhoC causes phosphorylation of PLCγ1 and increased Ca^{2+} flux in EC as well as increased VEGF-induced
vascular permeability in zebrafish. We previously demonstrated tyrosine residues Y951 and Y1059 are required for VEGF-induced endothelial cell migration and proliferation, respectively (Zeng et al., 2001). Mutational analysis revealed that MAPK activation requires phosphorylation of Y1059, but not Y951 (Zeng et al., 2001). Here, we observed increased endothelial cell migration and greater phosphorylation of VEGFR-2 Y951 and Y1059 upon RhoC knockdown, which suggests RhoC negatively regulates migration through decreased activation of Y951 and inhibition of MAPK signaling mediated by reduced Y1059 phosphorylation. Given that VEGFR-2 Y1059 activation promotes proliferation and MAPK activation also has a pro-proliferative effect, we expected RhoC ablation to stimulate endothelial cell proliferation. However, RhoC knockdown decreased proliferation despite increased Y1059 phosphorylation and MAPK activity. Given these seemingly disparate results, we sought an alternative mechanism through which RhoC promotes proliferation and found RhoC stabilizes nuclear β-catenin, which stimulates transcription of cyclin D1 and subsequently drives cell cycle progression and proliferation. Taken together, our results suggest the anti-proliferative effects mediated by decreased MAPK activity and VEGFR-2 Y1059 phosphorylation are overridden by the ability of active nuclear β-catenin to stimulate cyclin D1, cell cycle progression, and proliferation in EC.

RhoC clearly regulates processes involved in angiogenesis. However, we did not observe any defects or delays in zebrafish vascular development upon MO-mediated knockdown of Rhoad and Rhoae. Previous studies have demonstrated that RhoC null mice are viable and do not exhibit post-natal developmental defects (Hakem et al., 2005), which corresponds with our finding that RhoC ablation does not adversely affect zebrafish blood vessel formation. Although RhoC is dispensable for vascular development, it likely acts a molecular switch to modulate angiogenesis in processes such as wound healing, tumor growth, diabetic retinopathy, and macular degeneration by controlling endothelial cell proliferation, migration, and permeability. Correspondingly, our studies suggest RhoC helps maintain vascular homeostasis, the delicate balance between vascular injury and repair. Vascular injury is usually triggered by cytokines (Nakagawa et al., 2004), hypoxia (Voelkel and Tuder, 2000), shear stress (Dimmeler et al., 1999; Malek and Izumo, 1994), and/or oxidative stress (Treins et al., 2001). The ability of RhoC to sustain homeostasis in pathological settings is exemplified by our observation that RhoC prevents acute endothelial hyperpermeability in zebrafish. VEGF-dependent RhoC signaling promotes endothelial cell proliferation and negatively regulates migration and permeability to repair and compensate for endothelial cell loss from the vascular wall, an effect of vascular injury. Various vascular beds require unique VEGF signaling modulation for survival and normal turnover of blood vessels (Lazarus and Keshet, 2011). As a small GTPase, the ability of RhoC to act as a molecular switch enables it play a pivotal role in the maintenance of vascular homeostasis
in normal adult physiology as well as pathologic conditions. The precise vascular regulation exerted by RhoC is exemplified by its distinct tissue-specific control. For example, Wang and colleagues observed RhoC ablation decreased migration and invasion of human mammary ECs (Wang et al., 2008), whereas our results suggest RhoC knockdown increased HUVEC migration. Previous HUVEC studies demonstrated RhoC deletion promotes vascular sprouting two-fold (Del Galdo et al., 2013), and RhoC knockdown resulted in a loss of directionality of migration phenotype and inefficient migration from the point of origin in single cell tracking experiments (Mitin et al., 2013). The discrepancy in HUVEC migration may stem from the different assays utilized and inherent differences in the migratory properties of a single cell versus numerous cells. To validate our HUVEC findings and further investigate EC with various tissue origins, we evaluated the effect of RhoC knockdown in lymphatic EC (LyEC) and human brain microvascular EC (HBMVEC). Similar to our results in HUVEC, we observed increased migration upon RhoC ablation in HBMVEC and decreased proliferation in RhoC siRNA-treated LyEC. Furthermore, we also saw decreased expression of cyclin D1 in RhoC ablated HUVEC, HBMVEC, and LyEC, suggesting RhoC promotes cyclin D1 expression to drive proliferation in these EC.

While the studies presented here focus on the function of RhoC in EC, we speculate RhoC has a distinct role in cancer cells. We show that RhoC promotes proliferation and invasion of MDA-MB-231 breast cancer cells. Similarly, a recent report demonstrated MDA-MB-231 cells with reduced levels of RhoC exhibited decreased migration (Willmer et al., 2013). Overexpression of RhoC has been shown to increase angiogenic factors in breast epithelial cells (Kawata et al., 2014). The creation of RhoC knockout mice confirms these in vitro findings as loss of RhoC decreases tumor cell motility and metastatic cell survival leading to inhibition of metastasis (Hakem et al., 2005). Furthermore, another in vivo study determined RhoC promotes the ability of melanoma cells to extravasate from blood vessels and invade the lungs (van Golen et al., 2000). Hence, numerous studies support the notion that RhoC stimulates tumor cell migration and invasion. Conversely, in EC RhoC negatively regulates migration. These opposite functions of RhoC likely reflect differences in EC versus cancer cells of epithelial origin. RhoC has been shown to promote epithelial-mesenchymal transition (EMT) in breast, colon, prostate, and ovarian cancer cells (Bellovin et al., 2006; Gou et al., 2014; Kawata et al., 2014; Sequeira et al., 2008). EMT is a process through which epithelial cells transform from cells with tight cell-cell junctions, definitive basal and apical polarity, and sheet-like growth phenotype to spindle-like and motile cells, which have been linked to chemotherapeutic resistance, cancer progression, formation of subpopulations of cancer stem-like cells, and cell invasion (Savagner, 2010). Given the finding that RhoC is required for tumor metastasis, inhibition of RhoC represents an attractive therapeutic approach to prevent cancer metastasis. The finding that RhoC is not required for
embryonic or postnatal development in mice (Hakem et al., 2005), or zebrafish as shown here, bodes well for therapeutically targeting RhoC in cancer cells; however, our studies suggest RhoC inhibition increases endothelial cell migration and permeability while decreasing proliferation, and the clinical significance of these effects on endothelium should be considered upon therapeutic targeting of RhoC.

**MATERIALS AND METHODS**

**Reagents:** VEGF-A protein was obtained from R&D Systems (Minneapolis, MN, USA). The antibodies to VEGFR-2, p-VEGFR-2 (951), pAkt-1/2/3, total-Akt-1/2/3, PLC-γ1, CyclinB1, CyclinD1, p21, p27, total-LIMK2, β-catenin and c-Src were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-tyrosine (clone 4G10) and p-VEGFR-2 (1059) antibodies were purchased from Upstate (Lake Placid, NY, USA). Antibodies against p-VEGFR-2 (1175), p-MAPK-42/44, total-MAPK-42/44, RhoA, RhoB, RhoC, p38MAPK, p-JNK, p-FAK(Tyr397), total-FAK, p-LIMK1/2, total-LIMK1, p-MLC-2, p-PLC-γ1(Tyr783) and Cyclin A were obtained from Cell Signaling (Danvers, MA, USA). Phospho-Src [pTyr418] antibody was from BioSource International, Inc. (Camarillo, CA, USA). Phospho-eNOS, total-eNOS and β-actin were from BD Biosciences (San Jose, CA, USA) and α-Tubulin antibody was from Abcam (Cambridge, UK). siRNAs against VEGFR-1 and VEGFR-2 were from Santa Cruz Biotechnology. RhoC siRNA #1 was from Sigma-Aldrich (St. Louis, MO, USA) and RhoC siRNA #2 was purchased from Qiagen (Venlo, Limburg). Control siRNA was purchased from Dharmacon. Rho activation kit was purchased from Millipore (Lake Placid, NY). E64d was from Sigma-Aldrich and MG132 was from Boston Biochem (Cambridge, MA, USA). MEK1 inhibitor was purchased from Cell Signaling.

**Cell culture:** HUVEC were purchased from Lonza Group (Basel, Switzerland) and passaged in EGM growth media (Lonza). Primary human brain microvascular endothelial cells (HBMVEC) were purchased from Cell Systems (Kirkland, WA, USA) and cultured in CSC Serum-Containing Medium (Cell Systems). Human lymphatic microvascular endothelial cells (LyEC) were purchased from Lonza and cultured in EBM-2 medium with EGM-2 MV Bullet Kit. MDA-MB-231 (human breast adenocarcinoma) cells were purchased from ATCC (Manassas, VA, USA) and were cultured in RPMI 1640 medium from Life Technologies, Inc. (Grand Island, NY, USA) supplemented with 10% fetal bovine serum, and penicillin-streptomycin.
**siRNA Transfection:** 1 x 10⁵ HUVEC were seeded in 60 mm plates and cultured for 24 h in EGM. Cells were washed with OPTI-MEM reduced serum medium and transfected with 100 nM RhoC, VEGFR-1, VEGFR2 siRNA or control siRNA using Oligofectamine (Life Technologies, Inc.). After 4 h, antibiotic-free EGM was added and cell lysates were prepared. MDA-MB-231 cells transfected with 100 nM control or RhoC siRNA using using DharmaFECT 4 purchased from GE Dharmacon (Lafayette, CO, USA).

**Proliferation assay:** Control or RhoC siRNA-treated HUVEC (4 X10⁴/ml) were seeded in 24-well plates, cultured for 24 h in EGM, and subsequently serum starved (0.2%) in the presence of VEGF (10 ng/ml). The following day, 1 μCi of [³H] thymidine was added to each well; 4 h later, cells were washed with chilled PBS, fixed with 100% cold methanol and trichloroacetic acid-precipitable radioactivity was measured. For the thymidine incorporation assay using cancer cells, 6 x 10⁴ MDA-MB-231 cells/ml were used. Proliferation assays using HBMVEC and LyEC cells were performed as described for HUVEC except HBMVEC and LyEC were cultured in their appropriate media.

**Migration Assay:** 5x10⁴ control or RhoC siRNA-treated serum-starved HUVEC were seeded into collagen-coated transwell chambers with a diameter of 6.5 mm and a pore size of 8 μm (Corning CoStar Corporation, Cambridge, MA, USA) and inserted into 24-well plates containing serum-starved EGM. After incubation at 37°C for 1 h, 10 ng/ml VEGF was added to the lower chamber. To determine the effect of MAPK-42/44 on VEGF-induced cell migration, siRNA treated HUVEC were pre-treated with 10 or 20 μM of MEK1 inhibitor for 1 h and then cells were seeded into collagen coated transwell chambers. Following incubation for 4 h ±VEGF at 37°C, cells that remained in the upper chamber were gently removed with a cotton swab. Cells that had invaded through the filter were fixed in 4% paraformaldehyde (PFA) and then stained with 0.2% crystal violet dissolved in 2% ethanol. Migration was quantitated by counting the number of cells on the filter using bright-field optics with a Nikon Diaphot microscope equipped with a 16-square reticule (1 mm²). Four separate fields were counted for each filter. Three separate experiments were analyzed and the mean was reported. Migration assays using HBMVEC and LyEC cells were performed as described for HUVEC except HBMVEC and LyEC were cultured in their appropriate media and cultured in transwell chambers 12 hours prior to initial serum starvation.
**Cell cycle analysis:** DNA content was measured after staining cells with propidium iodide (PI). HUVEC transfected with control or RhoC siRNA were serum starved (0.2%) for 18 h. Following starvation, the cells were treated ±VEGF (10 ng/ml) and collected at 16 h after treatment. The cells were trypsinized, washed in PBS and fixed in 95% ethanol for 1 hour. Cells were rehydrated, washed in PBS and treated with RNaseA (1 mg/ml) followed by staining with PI (100 mg/ml). Flow cytometric quantification of DNA was performed with a FACScan (Becton Dickinson, San Jose, CA, USA) and data analysis was performed using the Modfit software (Verity Software House Inc., Topsham, ME, USA). Experiments were repeated at least three times.

**Immunoprecipitation and Western blot analysis:** Control or RhoC siRNA treated, serum-starved (0.2% serum for 24 h) HUVEC were pre-treated with VEGF (10 ng/ml) for indicated times. Whole cell lysates from HUVEC were prepared in RIPA buffer supplemented with protease inhibitor ±phosphatase inhibitor. Following centrifugation at 14000 g for 10 min at 4°C, 250 μg of protein lysate was incubated with 2 μg respective antibody for 1 h and 50 μl of protein A/G-conjugated agarose beads overnight at 4°C. Beads were washed with RIPA buffer three times, immunoprecipitates were resuspended in SDS sample-buffer, electrophoresis was performed, transferred to polyvinyl difluoride membranes, and immunoblotted. Antibody-reactive bands were detected by enzyme-linked chemiluminescence (Amersham, Piscataway, NJ, USA). These experiments were repeated at least three times.

**Intracellular Ca2+ Release:** HUVEC transfected with control or RhoC siRNA, were serum starved (0.2% serum) overnight, loaded with Fura-2 AM and then stimulated with VEGF (10 ng/ml). Intracellular Ca2+ concentrations were measured with the DeltaScan illumination system using Felix software (Photon Technology International, Edison, NJ, USA).

**Whole mount in situ hybridization:** 536 bases of the open reading frame of zebrafish RhoC (Rhoad) were amplified and cloned into a vector derived from pCR-BluntIIETOPO (Life Technologies, Inc.) using zebrafish cDNA. Rhoad primers used 5'-GGTGATTGTGGAGATGGAG-3', and 5'-TCTTCTTGCCTACGGACT-3'. To generate digoxigenin (DIG)-labeled antisense probes, the DNA plasmid was linearized with Not1 (New England BioLabs, Ipswich, MA, USA), followed by transcription using SP6 polymerase and the 10 DIG RNA labeling mix from Roche (Indianapolis, IN, USA). In situ hybridization was then performed according to a published procedure (Thisse and Thisse, 2008).
Microinjections and assessment of vascular permeability in VEGF-inducible zebrafish: One cell stage transgenic VEGF-inducible zebrafish (Hoeppner et al., 2012) embryos were arrayed in an agarose microinjection template and 1.5 nl of Cre mRNA (12.5 ng/µl) was microinjected into the cell of the embryo. Rhoad (5-TCCACCTGCAGATCATAATTGGGTA-3) and Rhoae (5-TCCACCTGCAGATCATAATTGGGTA-3) MOs were designed, purchased from Gene Tools, LLC (Philomath, OR, USA), and microinjected into embryos at the 1-2 cell stage. 9.25 ng Rhoad/Rhoae (2.2 nl of 500 µM) or 9.25 ng (2.2 nl of 500 µM) of nonspecific control MO were microinjected. Zebrafish expressing the VEGF-inducible transgene were selected by expression of eGFP in their eyes. Microangiography was performed on anaesthetized 3 d.p.f. embryos placed in an agarose microinjection template by inserting a glass microneedle through the pericardium directly into the ventricle. FITC-Dextran with a molecular weight of 2000 KDa and Texas Red-Dextran with a molecular weight of 70 KDa were used (Life Technologies, Inc.). The visualization and real-time imaging was performed using the previously described SCORE methodology (Petzold et al., 2010) on a ZEISS LSM 780 confocal microscope using standard FITC and dsRed filter sets. Zebrafish were maintained according to Institutional Animal Care and Use Committee guidelines at Mayo Clinic.

Small GTPase pull-down assay: RhoA/B/C activation assay kits from Millipore were used to perform these assays. Magnesium lysis buffer (MLB, Mg\textsuperscript{2+} lysis-wash Buffer) was made by diluting 5X MLB by adding sterile distilled water containing 10% glycerol. To the 1X MLB diluted buffer, protease inhibitor cocktail and phosphatase inhibitor were added. The cells were rinsed twice with ice-cold PBS and an appropriate amount of ice-cold 1X MLB was added. The lysates were transferred to microfuge tubes and the manufacturers protocol was followed. Rho immunoblotting was performed with anti-RhoA, anti-RhoB and anti-RhoC antibodies.

Invasion Assay: 100 µl of 3 mg/ml Matrigel solution (BD Bioscience) was overlaid on the upper surface of transwell chambers with a diameter of 6.5 mm and a pore size of 8 µm (Corning CoStar Corporation). The Matrigel was allowed to solidify by incubating the plates for ~1 h at 37°C. Medium (0.6 ml) containing 10% FBS were then added to the bottom chamber of the transwells. MDA-MB-231 cells that had been treated with siRNA were trypsinized and resuspended in corresponding serum-free medium. Subsequently, 2 x 10^5 cells/ml in a volume of 200 µl of medium were added to the upper chamber of each well. Cells were incubated for 8 h at 37°C. Cells that remained in the upper chamber were removed by gently scraping with a cotton swab. Cells that had invaded through the filter were fixed in 100% methanol and stained with 0.2% crystal violet dissolved in 2% ethanol.
Invasion was quantitated by counting the number of cells on the filter using bright-field optics with a Nikon Diaphot microscope equipped with a 16-square reticule (1 mm²). Four separate fields were counted for each filter. The average of three separate experiments has been documented.

**In vitro Apoptosis Assay:** 

1 x 10⁵ HUVEC were seeded in 60 mm plates and cultured for 24 h in EGM. The next day, cells were washed with OPTI-MEM reduced serum medium and transfected with 100 nM control or RhoC siRNA. After 48 h cells were serum starved (0.2%) and the treated with VEGF (10ng /ml) for 16 h. Annexin-V-FITC (Biovision, Mountain View, CA, USA) was used to assess apoptosis through flow cytometry. Additional exposure to propidium iodide (PI) made it possible to differentiate early apoptotic cells (Annexin-positive and PI-negative) from late apoptotic cells (Annexin- and PI-positive). Average of three separate experiments has been documented.

**Nuclear extract preparation:** Control or RhoC siRNA treated, serum-starved (0.2% serum, for 24 h) HUVEC suspension was incubated in a hypotonic buffer [10 mmol/L HEPES (pH 7.8), 10 mmol/L KCl, 2 mmol/L MgCl₂, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 μg/mL aprotinin, 3 mmol/L dithiothreitol (DTT), and 0.2 mmol/L phenylmethylsulfonylfluoride (PMSF)] for 15 minutes on ice. Nonionic detergent IGE-PAL (Sigma Aldrich) (10%) was then added to the cell suspension and mixed vigorously. Thereafter, the whole mixture was centrifuged at 14,000 rpm for 5 minutes. The pellets were again suspended in a hypertonic buffer solution [50 mmol/L HEPES (pH 7.8), 50 mmol/L KCl, 300 mmol/L NaCl, 0.1 mmol/L EDTA, 10 μg/mL aprotinin, 3 mmol/L DTT, and 0.2 mmol/L PMSF] and mixed on a rotating rack for 25 minutes at 4°C. Finally, the sample was centrifuged at 14,000 rpm for 10 minutes, and the supernatant was collected as nuclear extract.

**Statistical analysis:** The independent-sample t-test was used to test the probability of significant differences between two groups. Statistical significance was defined as (*) p ≤ 0.05 and (**) p ≤ 0.001, and (***) p ≤ 0.0001. Error bars are given on the basis of calculated S.D values.
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Competing Interests: None declared.

Author Contributions: S.S., Y.W., R.B. and S.D. performed the in vitro HUVEC experiments. L.H.H. developed the zebrafish permeability model and performed zebrafish experiments and imaging. Y.W. sectioned zebrafish embryos. V.M.B. performed the zebrafish in situ hybridization. S.S. and C.C. validated MO-mediated RhoC knockdown in zebrafish. L.H.H. and S.S. prepared the manuscript. R.R., S.C.E. and D.M. supervised the project. D.M. developed the original hypothesis and directed the entire project.

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References:


Leung, T., Chen, X. Q., Manser, E. and Lim, L. (1996). The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. 

Liu, A. X., Rane, N., Liu, J. P. and Prendergast, G. C. (2001). RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signaling in transformed cells. 
*Mol Cell Biol* 21, 6906-12.

*Journal of hypertension* 12, 989-99.

*Embo J* 24, 2342-53.


*Biochem Biophys Res Commun* 167, 265-72.


*Zebrafish* 7, 149-54.


*Cell* 70, 389-99.

*Nat Rev Mol Cell Biol* 4, 446-56.


*J Biol Chem* 275, 10661-72.

*Genomics* 86, 25-37.


*Science* 219, 983-5.

*Clinical & experimental metastasis* 25, 569-79.


Figure 1: RhoC promotes proliferation and negatively regulates migration through activation of VEGF. (A) Serum starved HUVEC were stimulated with 10 ng/ml VEGF for 1, 3, and 5 min. Lysates were immunoprecipitated with respective substrate GST-beads, and GTP-bound RhoC and GTP-bound RhoA were detected by immunoblotting. (B) HUVEC were serum starved overnight and stimulated with VEGF for 2 or 5 min. Lysates were immunoprecipitated with respective substrate GST-beads, and GTP-bound RhoC and RhoA were detected by immunoblotting. (A-B) Densitometry of the depicted immunoblots was performed using ImageJ software. (C) HUVEC were transfected with control or RhoC siRNA using oligofectamine for 48 h. 4 x 10^4 cells were plated in a 24-well plate, serum (0.2%) starved overnight and treated with 10 ng/ml VEGF. Thymidine incorporation assays were performed. ***, p ≤ 0.0001, Paired t test, 2 tailed (RhoC siRNA +VEGF treated group vs. control siRNA +VEGF treated group); * p ≤ 0.05 (RhoC –VEGF group vs. control siRNA –VEGF group). (D) 5 x 10^4 serum-starved HUVEC treated with control or RhoC siRNA were seeded into
collagen-coated transwell chambers and inserted into 24-well plates containing serum-starved EGM. 10ng/ml VEGF was added in the lower chamber and transwell migration assay was performed for 4 h. ***, *p* ≤ 0.0001, Paired t test, 2 tailed (RhoC siRNA +VEGF treated group vs. control siRNA +VEGF treated group). (A-D): Experiments were repeated at least three times.
Figure 2: RhoC ablation decreases LyEC proliferation and cyclin D expression in LyEC and HBMVEC, while increasing HBMVEC migration. (A) HBMVEC were transfected with control or RhoC siRNA for 48 h, serum-starved overnight, and treated with VEGF for 16 h. Cyclin D1, RhoC, and β-actin (loading control) were detected by western blot of cell lysates. (B) 5 x 10⁴ serum-starved HUVEC treated with control or RhoC siRNA were seeded into collagen-coated transwell chambers overnight and inserted into 24-well plates containing serum-starved EGM. 10 ng/ml VEGF was added in the lower chamber and transwell migration assay was performed for 4 h. ***, p < 0.0001, Paired t test, 2 tailed. (C-D) LyEC (C) or HBMVEC (D) were transfected with control or RhoC siRNA using oligofectamine for 48 h. 4 x 10⁴ cells were plated in a 24-well plate, serum (0.2%) starved overnight and treated with 10 ng/ml VEGF. Thymidine incorporation assays were performed. **, p < 0.05, Paired t test, 2 tailed (RhoC siRNA -VEGF treated group vs. control siRNA -VEGF treated group); *, p < 0.10 (RhoC +VEGF group vs. control siRNA +VEGF group); NS: Not Significant.
Figure 3: RhoC regulates migration through MAPK-42/44. HUVEC were transfected with control or RhoC siRNA for 48 h, serum-starved overnight, and treated with VEGF for the indicated times (A-C). (A) Cell lysates were collected and western blotted with antibodies against pospho-MAPK-42/44, total MAPK-42/44, phospho-P38MAPK, phospho-Akt1/2/3, total Akt1/2/3, phospho-JNK, and α-tubulin (loading control). (B) After serum starvation, cells were treated with 10 or 20 μM of MEK1 inhibitor for 1 h and 5x10^5 cells were seeded into collagen coated transwell chambers and were then inserted into 24-well plates containing serum-starved EGM. VEGF (10ng/ml) was added in the lower chamber and transwell migration assay was performed for 4 h. Experiments were repeated at least three times in triplicates. *, p ≤ 0.05, and **, p ≤ 0.001 (paired t test, 2 tailed). (C) Cell lysates were collected and western blotted with antibodies against pospho-LIMK1/2, total LIMK1, total LIMK2, phospho-MLC-2, RhoC, and β-actin (loading control). (A,C): Please refer to supplementary materials Fig. S3 for densitometry plots.
Figure 4: RhoC promotes proliferation through cell cycle progression. HUVEC were transfected with control or RhoC siRNA for 48 h, serum-starved overnight, and treated with VEGF for 16 h (A-B) or 6 or 12 h (C). Experiments were repeated at least three times. (A) The cells were fixed, stained with PI, and analyzed by FACS. The mean percentage of cells with DNA content in each of the three phases of the cell cycle is shown over three independent determinations. *, p ≤ 0.05, and **, p ≤ 0.001 (paired t test, 2 tailed). (B) Cyclin D1, cyclin A, cyclin B1, p27, p21, RhoC, and β-actin (loading control) were detected by western blot of cell lysates. Densitometry of the indicated immunoblots was performed using ImageJ software. (C) Nuclear fractions were collected and subjected for Western blot using a β-catenin antibody.
Figure 5: VEGFR-2 is required for RhoC signaling, but VEGFR-2 phosphorylation is negatively regulated by RhoC. HUVEC were transfected with control or VEGFR-2 siRNA, serum starved overnight, and treated with 10 ng/ml VEGF for the indicated times. Experiments were repeated at least three times (A-C). (A) Lysates were immunoprecipitated with GST-beads and GTP-bound RhoC was detected by immunoblotting. Levels of total RhoC, RhoA, VEGFR-2 and β-Actin (loading control) are shown. Experiments were repeated at least three times. (B) Lysates were immunoblotted for phospho-VEGFR-2 Y1175, phospho-VEGFR-2Y951, phospho-VEGFR-2Y1059,
total VEGFR-2, and β-Actin (loading control). Corresponding densitometry for depicted immunoblots was generated using ImageJ software. (C) Lysates were immunoprecipitated with anti-tyrosine antibody and immunoblotted with antibody against VEGFR-2.
Figure 6: RhoC controls phosphorylation of PLC-γ1 and eNOS and induces intracellular Ca\(^{2+}\) release. HUVEC were transfected with control or RhoC siRNA, serum starved overnight, and treated with 10 ng/ml VEGF for the indicated amounts of time. (A-B). (A) Cell lysates were immunoblotted with antibodies against phospho-PLC-γ1(Y783) and total PLC-γ1. Corresponding densitometry is shown. (B) Cell lysates were immunoblotted for phospho-eNOS(S1177), total eNOS, and β-actin (loading control). Corresponding densitometry is shown. (C) HUVEC cells transfected with control or RhoC siRNA were serum-starved overnight, loaded with Fura-2 AM and then stimulated with VEGF (10 ng/ml) at 50 sec. All experiments were repeated at least three times (A-C).
**Figure 7: RhoC negatively regulates VEGF-induced vascular permeability in zebrafish.** (A-B) Using zebrafish cDNA, a probe to zebrafish RhoC (Rhoad) was created and in situ hybridization was performed on 24 hpf zebrafish embryos. Multiple images were captured using a Zeiss Axioplan 2 microscope and overlayed in Photoshop, such that areas of focus were unmasked, to generate a composite image. Lateral (A) and superior (B) views are shown. DA: Dorsal aorta, PCV: posterior cardinal vein, ISVs: anterior intersomitic vessels, NT: neural tubes. (C) Microangiography was performed on anaesthetized 3 d.p.f. zebrafish embryos by injecting FITC-Dextran (2000 KDa) and Texas Red-Dextran (70 KDa), VEGF was induced through heat exposure (when applicable), and extravasation of red tracer as a measure of zebrafish vascular permeability was live imaged using a ZEISS LSM 780 confocal microscope. Control: no MO injection and no VEGF induction; Cont MO: Control MO injection; RhoC MO: Rhoad/Rhoae MO injection; Unind: no VEGF induction; VEGF: heat induction of VEGF transgene. (D) Quantitation of extravasated red tracer. **p < 0.05, (RhoC MO, VEGF Induced vs. Control MO, VEGF Induced); *p < 0.05 (RhoC MO, Uninduced vs. Control; Control MO, VEGF Induced vs. Control), (paired t-test, 2 tailed).
Figure 8: RhoC maintains vascular homeostasis through regulation of endothelial VEGF signaling. VEGF signals through VEGFR-2 to activate the GTP-bound form of RhoC. Active RhoC promotes nuclear stabilization of β-catenin, which acts as a transcription factor to increase the expression of Cyclin D1, drive cell cycle progression, and stimulate endothelial cell proliferation. RhoC negatively regulates VEGF-induced vascular permeability by decreasing phosphorylation of PLC-γ1 and phosphorylation of eNOS to reduce intracellular cellular Ca^{2+} release. Stress-induced protein kinase p38 MAPK is also downregulated by RhoC. Endothelial cell migration is negatively controlled by RhoC through a decreased activation of the Ras/Raf/MEK/MAPK signaling pathway. Specifically, RhoC reduces phosphorylation of p38 MAPK and MAPK-42/44 along with downstream MLC-2 to negatively modulate endothelial cell proliferation. Our data suggests RhoC regulates the phosphorylation of VEGFR-2.