The Andes Virus Nucleocapsid Protein Directs Basal Endothelial Cell Permeability by Activating RhoA

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ABSTRACT Andes virus (ANDV) predominantly infects microvascular endothelial cells (MECs) and nonlytically causes an acute pulmonary edema termed hantavirus pulmonary syndrome (HPS). In HPS patients, virtually every pulmonary MEC is infected, MECs are enlarged, and infection results in vascular leakage and highly lethal pulmonary edema. We observed that MECs infected with the ANDV hantavirus or expressing the ANDV nucleocapsid (N) protein showed increased size and permeability by activating the Rheb and RhoA GTPases. Expression of ANDV N in MECs increased cell size by preventing tuberous sclerosis complex (TSC) repression of Rheb-mTOR-pS6K. N selectively bound the TSC2 N terminus (1 to 1403) within a complex containing TSC2/TSC1/TBC1D7, and endogenous TSC2 reciprocally coprecipitated N protein from ANDV-infected MECs. TSCs normally restrict RhoA-induced MEC permeability, and we found that ANDV infection or N protein expression constitutively activated RhoA. This suggests that the ANDV N protein alone is sufficient to activate signaling pathways that control MEC size and permeability. Further, RhoA small interfering RNA, dominant-negative RhoA(N19), and the RhoA/Rho kinase inhibitors fasudil and Y27632 dramatically reduced the permeability of ANDV-infected MECs by 80 to 90%. Fasudil also reduced vasodilation by activating RhoA. This suggests that the ANDV N protein alone is sufficient to activate signaling pathways that control MEC size and permeability. Further, RhoA small interfering RNA, dominant-negative RhoA(N19), and the RhoA/Rho kinase inhibitors fasudil and Y27632 dramatically reduced the permeability of ANDV-infected MECs by 80 to 90%. Fasudil also reduced the bradykinin-directed permeability of ANDV and Hantaan virus-infected MECs to control levels. These findings demonstrate that ANDV activation of RhoA causes MEC permeability and reveal a potential edemagenic mechanism for ANDV to constitutively inhibit the basal barrier integrity of infected MECs. The central importance of RhoA activation in MEC permeability further suggests therapeutically targeting RhoA, TSCs, and Rac1 as potential means of resolving capillary leakage during hantavirus infections.

IMPORTANCE HPS is hallmarked by acute pulmonary edema, hypoxia, respiratory distress, and the ubiquitous infection of pulmonary MECs that occurs without disrupting the endothelium. Mechanisms of MEC permeability and targets for resolving lethal pulmonary edema during HPS remain enigmatic. Our findings suggest a novel underlying mechanism of MEC dysfunction resulting from ANDV activation of the Rheb and RhoA GTPases that, respectively, control MEC size and permeability. Our studies show that inhibition of RhoA blocks ANDV-directed permeability and implicate RhoA as a potential therapeutic target for restoring capillary barrier function to the ANDV-infected endothelium. Since RhoA activation forms a downstream nexus for factors that cause capillary leakage, blocking RhoA activation is likely to restore basal capillary integrity and prevent edema amplified by tissue hypoxia and respiratory distress. Targeting the endothelium has the potential to resolve disease during symptomatic stages, when replication inhibitors lack efficacy, and to be broadly applicable to other hemorrhagic and edematous viral diseases.
HPS in macaques indicates that pulmonary edema is observed from 6 to 13 days postinfection (dpi) without concurrent T cell or cytokine responses (22). Studies of ANDV-infected Syrian hamsters, which closely mimic human HPS (13–15), indicate that dexamethasone or cyclophosphamide treatment or depletion of macrophages or CD4⁺ or CD8⁺ T cells failed to alter the timing, onset, or severity of HPS (15, 23). In fact, immunosuppression permits SNV to cause lethal edema in Syrian hamsters (24).

Additional findings support roles for hantavirus dysregulation of infected pulmonary MECs in HPS-directed capillary permeability. Pathogenic hantaviruses engage innate, β1, β2, or integrin conformers in order to infect MECs (25–28), and hantaviruses remain cell associated (29, 30), inhibiting conformational changes in order to infect MECs (25–28), and hantaviruses mediate MEC migration days after infection (29, 31, 32). Activated αvβ3 integrins normally restrict the permeabilizing effects of vascular endothelial growth factor (VEGF) by forming a complex with VEGF receptor 2 (VEGFR2) (33, 34). Pathogenic, but not nonpathogenic, hantaviruses uniquely inhibit αvβ3 functions in human MECs, resulting in the hyperpermeability of MECs to VEGF or hypoxia-induced VEGF (31, 32, 35). Edema causes hypoxia, and HPS patients become acutely hypoxic, with elevated VEGF levels in pulmonary edema fluids (36). Secreted VEGF binds to endothelial cell (EC) receptors within 0.5 mm of its release (37), acting locally to disassemble adherens junctions (AJs) and induce EC permeability (34, 38). Bradykinin release following activation of the kallikrein-kinin system was also shown to increase electrical conductance, as a measure of permeability, in ANDV- and Hantaan virus (HTNV)-infected ECs (39). However, the mechanisms by which hantaviruses constitutively cause basal capillary permeability and edema that evolves into later tissue hypoxia remain to be resolved.

AJs are composed of homophilic interendothelial vascular endothelial (VE)-cadherin complexes that form the primary fluid barrier of capillaries (38, 40). Intracellularly, VE-cadherin engages the actin cytoskeleton and is dynamically regulated by extracellular and intracellular signaling pathways that control cell morphology, motility, and leukocyte extravasation (38, 40, 41). Rac1 and RhoA are cytoplasmic cellular GTPases that opposingly control the density of VE-cadherin within AJs, pore formation during diapedesis, EC barrier integrity, and capillary permeability (40, 42–46). Activation of αvβ3 or focal adhesion kinase (FAK) activates Rac1, increasing the density of VE-cadherin between ECs, and FAK also engages and stabilizes actin/VE-cadherin complexes (33, 40, 47, 48). In contrast, inhibition of αvβ3 prevents FAK and Rac1 activation and instead directs RhoA activation (44, 48, 49). In ECs, the conditional knockout of FAK or the RhoA inhibitor Y27632 is sufficient to increase EC permeability and cause pulmonary edema in mice (48, 50, 51).

In HPS patients, hantavirus-infected MECs are reportedly enlarged (1, 7), providing a visible correlate of MEC dysfunction. In vitro, we also found that ANDV-infected MECs were enlarged (3- to 5-fold), with hypoxia increasing both the number of enlarged infected MECs and MEC permeability (52, 53). In contrast, infection of MECs with nonpathogenic Tula virus (TULV) or mock infection resulted in 2 to 10% enlarged MECs under hypoxic conditions and failed to enhance MEC permeability (31, 32, 53). Cell size is controlled by mTOR-directed phosphorylation of S6 kinase (S6K) (54) and normally inhibited by TSC repression of the mTOR GTPase Rheb (54, 55). ANDV-induced increased MEC size was directed by activating the Rheb-mTOR-pS6K signaling pathway (53). Tuberous sclerosis complexes (TSCs) normally inhibit Rheb-directed mTOR activation (54, 56), and mutations in TSC proteins (TSC1-hamartin, TSC2-tuberin) constitutively activate Rheb-mTOR-pS6K and increase cell size (54, 56). Intriguingly, TSCs also regulate Rac1 and RhoA GTPases that play fundamental antagonistic roles in the control of EC permeability (40, 45, 57–59). This suggested that ANDV regulation of TSCs may increase both MEC size and capillary leakage in HPS.

In this study, we evaluated ANDV infection and N protein regulation of TSCs that result in Rheb and RhoA activation in MECs. Our results indicate that expression of the ANDV N protein alone in MECs increases cell size and activates Rheb-mTOR-pS6K by binding to TSCs. Our studies revealed that ANDV N protein coprecipitates TSC2, assembled TSC complexes, and the TSC inhibitor 14-3-3 (60–62). Consistent with this, we found that ANDV infection or N protein expression in MECs activated RhoA and reduced levels of the RhoA inhibitor p190RhoGAP and the Rac1 activator Tiam1. Small interfering RNA (siRNA) knockdown of RhoA, expression of dominant-negative RhoA, or inhibition of RhoA/ROCK with fasudil or Y27632 was found to reduce ANDV-directed MEC permeability by 80 to 90%. These findings demonstrate that ANDV activation of RhoA causes MEC permeability and suggest an underlying edemagenic mechanism that may constitutively decrease the barrier integrity of ANDV-infected MECs. These findings implicate RhoA, TSCs, and Rac1 as potential therapeutic targets for resolving capillary leakage during ANDV infection and a potential means of resolving edema during symptomatic HPS stages.

**RESULTS**

**ANDV N protein expression in human endothelial cells increases cell size.** The mechanism by which ANDV activates mTOR, increases MEC size, and causes MEC permeability remains to be defined. Hantavirus N proteins are highly expressed during infection (63, 64), yet roles for hantavirus proteins in MEC dysfunction and permeability have not been studied. Here we analyzed the constitutive expression of ANDV N protein in early-passage primary human pulmonary MECs. MECs were lentivirus transduced to express ANDV N protein and puromycin selected. ECs persistently expressed N protein in >95% of MECs without notable effects on cell viability or loss of N protein expression in the absence of puromycin selection (Fig. 1A). Similar to ANDV infection (53), we noted that ~15% of N-protein-expressing MECs were enlarged (three to five times normal size) (Fig. 1A and B). Hypoxic conditions increased the number of enlarged MECs (40 to 50%) and the permeability of N-protein-expressing MECs (~3-fold) (Fig. 1B). In comparison, ~5% of mock-transduced, hypoxia-treated control MECs were enlarged (Fig. 1C) (53). Under hypoxic conditions, the percentage of enlarged N-protein-expressing MECs was dramatically reduced by addition of the mTOR inhibitor rapamycin (Fig. 1C).

**ANDV N protein induces mTOR-directed phosphorylation of S6K.** TSCs regulate cell size by inhibiting the mTOR-specific GTPase Rheb (54, 55). Mutations in the TSC1 or TSC2 protein result in increased cell size by derepressing Rheb and constitutively activating mTOR-directed phosphorylation of S6K (54). Analysis of N-protein-transduced MECs revealed that N protein expression directed the phosphorylation of S6K under hypoxic conditions (Fig. 2A). In contrast, S6K was not phosphorylated by hypoxia treatment of MECs alone (Fig. 2A) and pS6K responses of
N-expressing MECs was blocked by rapamycin (Fig. 2A). Consis-
tent with this, expression of N protein in HEK293 cells in the
presence of Rheb dose dependently increased S6K phosphoryla-
tion, while expression of Rheb alone failed to increase pS6K
(Fig. 2B). Interestingly, expression of increasing amounts of TSC2
resulted in a concomitant decrease in N-protein-directed S6K
phosphorylation (Fig. 2C), suggesting that N-directed mTOR ac-
tivation is TSC2 mediated. Collectively, these findings indicate
that ANDV N protein increases the size of MECs by activating the
Rheb-mTOR-pS6K signaling pathway.

ANDV N protein binds TSCs via interactions with N-
terminal domains of TSC2. The findings described above suggest
that N protein may alter normal TSC repression of Rheb. TSC1
and TSC2 form a complex that inhibits Rheb-directed mTOR ac-
tivation through a GTPase-activating protein (GAP) domain in
the TSC2 C terminus (55). We previously reported that ANDV,
but not nonpathogenic TULV, activates mTOR-pS6K and in-
creases cell size (53). In order to determine if the TULV and
ANDV N proteins differ in the ability to interact with TSCs, we
coexpressed TSC2 with ANDV or TULV N protein and assayed N
protein interactions with TSC2. We immunoprecipitated TSC2
from cell lysates and found that TSC2 selectively coprecipitated
ANDV, but not TULV, N protein (Fig. 3A). These findings are
consistent with ANDV activation of mTOR and prompted the
evaluation of ANDV N protein interactions with additional TSC components that normally repress Rheb (56, 58). HEK293 cells were transfected with plasmids expressing N protein, Flag-tagged TSC2, or a C-terminal truncation of TSC2 containing residues 1 to 1403 lacking the GAP domain (Fig. 3B). In contrast, N protein failed to coimmunoprecipitate TSC1, TSC1 truncations, or actin. These findings suggest that ANDV N interacts with assembled TSC1-TSC2 complexes through interactions with the N terminus of TSC2 that are independent of the TSC2 GAP domain.

**ANDV N protein binds TSCs in the presence or absence of the TSC inhibitor 14-3-3.** In addition to TSC1 and TSC2 components, TSCs are present as ternary complexes containing TBC1D7 (62, 65), and TSC regulation of Rheb is inhibited by recruitment of the scaffold protein 14-3-3 (60, 61). Here we immunoprecipitated TSC2 and analyzed N protein interactions with TSCs containing TBC1D7 and 14-3-3. We found that TBC1D7, TSC1, and N protein were coimmunoprecipitated by TSC2 and that N protein formed a complex with TSCs in the presence or absence of 14-3-3 (Fig. 4A). These findings suggest that, instead of disrupting TSCs, N protein binding to TSC2 mediates its association with assembled TSCs and that N protein binding to TSCs is discrete from the binding of inhibitory 14-3-3 proteins (Fig. 4A) (60–62).

We further evaluated endogenous TSC2 interactions with N protein following ANDV infection of MECs. We found that immunoprecipitation of endogenous TSC2 from ANDV-infected MECs resulted in coprecipitation of the ANDV N protein (Fig. 4B). These findings validate coexpression studies by demonstrating endogenous interactions of the Rheb inhibitor TSC2 with N protein during ANDV infection. Together, these findings indicate that ANDV N protein binding to TSCs and TSC–14-3-3 complexes prevents TSC repression of Rheb and results in the activation of mTOR-pS6K signaling pathways.

**ANDV infection and N protein expression activate RhoA in MECs.** Collectively, our findings suggest that N protein binds TSCs and inhibits TSC repression of Rheb. However, TSCs also regulate signaling responses directed by Rac1 and RhoA GTPases (57–59, 66) that antagonistically regulate EC permeability (Fig. 5A) (40, 43, 50). A wide range of factors activate RhoA to cause EC permeability (38, 42, 49, 51, 67), and this prompted us to determine if RhoA was activated by ANDV infection of MECs (40, 46). We assayed acti-
activated (GTP-bound) RhoA by using Rhotekin binding domain assays and found that ANDV infection of MECs constitutively activated RhoA and that RhoA activation was independent of hypoxic conditions (Fig. 5B). We similarly analyzed MECs expressing ANDV N protein and found that RhoA was constitutively activated (Fig. 5C). In contrast, lentivirus expression of GnGc alone in MECs did not activate RhoA and coexpression of N and GnGc in MECs resulted in RhoA activation similarly to N protein expression by WB. WB of endogenous TSC2 and ANDV N protein in infected MEC lysates was analyzed for total input TSC2, N protein, and actin levels.

GTPase-specific GAPs and GEFs (guanine nucleotide exchange factors), respectively, inhibit or activate Rac1 and RhoA (50, 69–71). We analyzed N-protein-expressing MECs for changes in p190RhoGAP, TIAM1, and IQGAP, which are respective regulators of RhoA, Rac1, and cdc42 GTPases. We found that MECs expressing the ANDV N protein had dramatically reduced levels of the RhoA inhibitor p190RhoGAP and the Rac1 activator TIAM1, while levels of IQGAP remained unchanged in N-protein-expressing cells (Fig. 5D). These findings are consistent with the idea that N protein expression prevents p190RhoGAP repression of RhoA. Since TSCs normally inhibit RhoA and activate Rac1, our findings are consistent with N protein activation of RhoA by coordinated inhibition of TSCs, GAPs, and GEFs, which determine the balance of Rac1 and RhoA activation and MEC barrier integrity (70, 71). However, it remains to be determined if these changes are a cause of RhoA activation or whether additional factors that control RhoA (i.e., RhoGDI, FAK,
Syr, Vav2, and p115RhoGEF) (48–50, 69, 71) are engaged by ANDV infection or N expression to constitutively activate RhoA.

ANDV-induced MEC permeability is blocked by inhibition of RhoA. Activation of RhoA directs actin contraction and the disassembly of VE-cadherin within AJs that controls capillary permeability (38, 41, 43, 46, 50, 70). We previously reported that ANDV and hemorrhagic fever with renal syndrome (HFRS)-causing HTNV, but not TULV, infections of MECs induce VE-cadherin disassembly and enhance MEC permeability in response to hypoxia or VEGF (31, 32, 35, 72). The findings described above suggest that RhoA activation may be an underlying edemagenic mechanism that causes capillary leakage and basal pulmonary edema during ANDV infection. Here we determined if ANDV-induced permeability is RhoA mediated by analyzing responses of MECs to discrete RhoA inhibitors. We transfected MECs with RhoA siRNAs or transduced MECs to express dominant-negative RhoA(T19N) (73) and found that RhoA expression levels were specifically reduced in siRNA-treated MECs and increased in RhoA(T19N)-expressing cells (Fig. 6A and B). Using a gold standard fluorescein isothiocyanate (FITC) Transwell permeability assay (31, 32, 53), we found that RhoA-specific siRNA resulted in a 90% reduction of ANDV-induced permeability (Fig. 6A). Similarly, transduction of MECs with a lentivirus expressing an inactive RhoA(T19N) mutant protein resulted in an 80% reduction in ANDV-induced MEC permeability (Fig. 6B).

We further analyzed the effects of the RhoA and Rho kinase (ROCK) inhibitors fasudil (HA-1077) and Y27632 (70, 74) for the ability to reduce ANDV-induced EC permeability. At 3 days after ANDV infection, we added RhoA inhibitors to cells 6 h prior to analysis of MEC permeability. We found that the addition of fasudil or Y27632 dramatically reduced ANDV-induced MEC permeability 80 to 90% (Fig. 6C). A prior study showed that ANDV and HTNV enhanced bradykinin-directed permeability (39). As a result, we determined whether ANDV- and HTNV-directed MEC permeability responses induced by bradykinin were inhibited by the ROCK inhibitor fasudil. We found that the addition of bradykinin to ANDV- and HTNV-infected MECs increased permeability ~3-fold and that coadministration of fasudil inhibited permeability to control levels (Fig. 6D).

These findings indicate that ANDV-induced MEC permeability is RhoA directed and blocked by inhibition of RhoA activation. Collectively, our findings suggest a mechanism by which ANDV induces basal changes in MEC permeability and cell size through N protein interactions with TSC that derepress Rheb and RhoA GTPases. Our findings suggest the potential for RhoA to be a conserved downstream target for hantavirus therapeutics, which may reduce or resolve basal ANDV-induced edema by inhibiting RhoA activation or activating pathways that restore Rac1 activation and TSC function (Fig. 7).

**DISCUSSION**

ECs contain unique receptors, junctions, and signaling pathway effectors that regulate immune cell and platelet binding and activation, transcytosis, vascular tone, and the activation of complement and clotting cascades that collectively regulate hemostasis (75). ECs regulate vascular barrier functions through a series of failsafe mechanisms that are in place to prevent a lethal breach of barrier integrity (33, 34, 38, 75, 76). As a result, it is likely that several EC functions need to be inhibited to cause hemorrhagic or edematous diseases.

ECs are the primary cellular targets of hantavirus infection (1, 6, 7), and this focuses studies of pathogenesis on mechanisms by which hantaviruses dysregulate MEC functions (31, 72, 77) in order to increase vascular permeability and cause the diseases HPS and HFRS (2, 3, 16). Mutation or knocking out of β3 integrins causes vascular leakage (33, 78), and pathogenic hantaviruses bind and inhibit the function of β3 integrins present on platelets and ECs (25–28). HPS patients are acutely thrombocytopenic (7), and on MECs, α3β1 integrins play a fundamental role in cell migration, the formation of focal adhesions, Rac1 activation, and the regula-
Hypoxia, observed at late stages of HPS (4, 7), induces the permeability factor VEGF (34, 53), as well as bradykinin receptors that direct permeability in response to activation of the kallikrein-kinin system (39, 67, 80, 81). In fact, VEGF levels are increased in HPS pulmonary edema fluids (31, 32, 36), and activation of the kallikrein system in HV-infected cells releases bradykinin and increases EC permeability (39). Capillary permeability is commonly mediated by downstream RhoA activation, and findings presented here demonstrate that the ANDV N protein activates RhoA.

In HPS patients, nearly every pulmonary MEC is infected and enlarged (1, 7); similarly, ANDV infection of MECs in vitro results in the generation of enlarged cells (52, 53). Here we show that expression of the ANDV N protein in MECs is sufficient to cause MEC enlargement and activate Rheb-mTOR-pS6K and RhoA signaling responses. TSCs normally repress Rheb (56, 82), and expression of ANDV N protein dose dependently increased S6K phosphorylation that was inhibited by expression of TSC2. We observed that ANDV N protein binds to endogenous or expressed TSC2 and that, instead of displacing TSC components, N bound to an assembled TSC complex with or without the TSC inhibitor 14-3-3 (60, 62, 83, 84). This suggests a novel mechanism by which ANDV N protein inactivates TSCs to control cell size and potentially enhance mTOR-directed increases in HIF1α that may contribute to hypoxia-induced responses of ANDV-infected MECs (60, 62).

TSCs also control the activity of Rac1 and RhoA GTPases that antagonistically control barrier integrity and capillary permeability (42, 46, 51, 58, 60, 85, 86). RhoA activation directs stress fiber organization and contraction, inhibits Rac1 activation, impairs VE-cadherin assembly, and increases vascular permeability (43, 48, 50). In contrast, Rac1 activation directs the formation of filopodia, increases the assembly of VE-cadherin homodimers between MECs, increases capillary barrier integrity, and inhibits RhoA (40, 46, 58, 86, 87). Thus, the balance between the activation of Rac1 and that of RhoA critically regulates AJ barrier function and vascular permeability (38, 40, 41, 70). We found that ANDV infection or N protein expression in MECs constitutively activates RhoA. This suggests a program by which the ANDV N protein inhibits TSC regulation, activates RhoA, and tips the balance from MEC integrity to one of basal MEC leakage (Fig. 7).

Correlates of basal EC permeability during HV infection have not previously been found, in part because vessels, but not EC monolayers, are under pressure and even small changes in barrier integrity are exacerbated in capillaries. In fact even inapparent cellular stresses like breathing-directed cyclic stretching of the pulmonary endothelium (88, 89) may contribute to capillary leakage when uncoupled from normal MEC integrity. The cause of vascular leakage during hantavirus diseases has been speculated to stem from a wide range of effectors, including growth factors, kinins, immune responses, cytokines, T cells, and permeability factors (18–20, 29, 31, 32, 35, 36, 39, 77, 90). Although several
factors are likely to contribute to permeability, immunosuppres-
sion of HV patients has no effect on the disease (21) and recent
findings suggest that immune responses are not determinants of
vascular leakage in animal models of ANDV infection (13, 22, 23).
Prior studies have shown that ECs infected by pathogenic, but
nonpathogenic, hantaviruses are hyperpermeabilized by VEGF
addition or by hypoxic conditions observed at late stages of HPS
(31, 32, 35, 53, 91, 92). In addition, HV-infected ECs are hyperper-
sponsive to bradykinin-directed EC permeability (39). Hypoxia
or VEGF addition directed the nondegradative internalization of
VE-cadherin within HV-infected MECs (31, 32, 35, 53), although
another study suggested that VE-cadherin was transiently de-
graded after VEGF addition (91).

Activated RhoA is linked to EC permeability directed by
thrombin, tumor necrosis factor alpha, and histamine, as well as
bradykinin and VEGF (38, 42, 49, 67, 80, 85). Findings presented
here demonstrate a role for RhoA activation in MEC permeability
during ANDV infection (Fig. 6A to C) and also show that inhibi-
tion of RhoA blocks bradykinin-directed permeability in ANDV
and HTNV-infected ECs (Fig. 6D). This implicates RhoA acti-
vation as a cause of basal changes in MEC integrity that con-
tribute to vascular leakage and edema (31, 32, 36, 39). However,
hypoxic conditions also induce bradykinin receptors and VEGF
(46, 67, 93), and this further suggests a mechanism for ANDV to
amplify RhoA-directed permeability under hypoxic conditions
(38, 41, 43, 46, 49, 60). Given the fundamental role of RhoA acti-
vation in basal and hypoxia-directed EC permeability (42, 43),
these findings suggest RhoA as a central downstream target of
eDMA during HPS.

Additional MEC functions dysregulated by hantavirus infec-
tion may also exacerbate N-protein-directed RhoA activation.
Both α,β3 integrins and FAK normally activate Rac1 (27, 48, 79),
yet pathogenic hantaviruses block α,β3 integrin and FAK activa-
tion during infection (27, 29, 30). This suggests a role for ANDV
inhibition of extracellular α,β3 integrin responses as a means of
reducing Rac1-directed barrier integrity and enhancing RhoA ac-
tivation during ANDV infection. Another potential way for β3
integrins and RhoA to contribute to pulmonary edema is provided
by neutrophil recruitment to pulmonary compartments during
HPS (7, 94–96). As neutrophils traverse the endothelium to enter
tissues, pores are formed in ECs and pore assembly and closure
are regulated by Rac1, RhoA, and β3 integrins (44, 95, 96). In ANDV-
infected MECs, inhibition of β3 and Rac1 and activation of RhoA
may increase the duration of pore opening and thereby diapedesis
alone may trigger pulmonary edema in HPS patients. As a result,
activating α,β3, and Rac1 may be investigated as synergistic targets
for enhancement of EC barrier function and for inhibition of
RhoA activation (Fig. 7). Whether extracellular integrin blockade
(48, 79, 97) or neutrophil extravasation contributes to RhoA acti-
vation and ANDV-directed MEC permeability remains to be in-
vestigated.

There are currently no therapeutic approaches for treating
hantavirus-induced diseases or reducing lethal outcomes of HPS
infections (21). Interferon and replication inhibitors are efficacious
prophylactically but not in viremic or symptomatic patients
(5, 21, 98). However, one Puumala virus patient recovered after
being given a dose of the bradykinin antagonist icatibant (90) and
this supports a role for bradykinin in HFRS pathogenesis (39).
However, further studies are needed to determine if icatibant or
several additional therapeutics provided to the patient played a
key role in recovery (90).

Our findings provide a mechanism for basal capillary permea-
bility during ANDV infection of MECs and uniquely reveal RhoA
as a potential therapeutic target for restoring MEC integrity and
resolving HPS (43, 49, 80, 94). Since RhoA is a central downstream
signaling effector (42, 43, 46, 68, 94, 99, 100), blocking of RhoA
activation may commonly inhibit constitutive and hypoxia-
directed EC permeability responses that are dysregulated by
ANDV infection. ANDV-directed permeability was dramatically
reduced by the pharmacological RhoA/ROCK inhibitors fasudil
and Y27632 (74, 101, 102), and the approval of fasudil for use in
humans (102, 103) suggests its immediate therapeutic potential.
Findings presented here rationalize studying these and other
RhoA inhibitors for their efficacy in resolving lethal HPS disease in
a biosafety level 4 (BSL4) Syrian hamster model (15, 24).

On the basis of our findings, additional inhibitors that protect
endothelial barrier function by activating Rac1 and TSCs or indi-
directly impact Rac1/RhoA also have the potential to inhibit capil-
lar leakage and therapeutically resolve or reduce HPS disease.
Prostaglandin E2 promotes Rac1 activation, and forskolin and
rolipram protect EC barrier function by activating TSCs and pre-
venting Rac1 inhibition (104, 105). Statins were previously noted
to stabilize the endothelium by targeting 3-hydroxy-3-methyl-
glutaryl-coenzyme A reductase, resulting in reduced RhoA gera-
nylgeranylation required for RhoA activation (99, 106, 107).
Activation of α,β3 integrins (108) or use of compounds that lead to
Rac1 activation (i.e., SEW2871 [109], angiopoietin 1 [87, 110],
and FTY720 [31, 111]) may similarly inhibit edemagenic RhoA-
directed responses of ANDV-infected ECs. Although the res-
donses described here were studied in an ANDV-specific context,
they appear to be applicable to HFRS-causing HTNV (Fig. 6D),
and the ubiquitous role of RhoA in vascular permeability (38, 42,
46, 49, 51, 67, 81) suggests that this approach may be germane to
other hemorrhagic and edematous viruses.

MATERIALS AND METHODS

Cells and virus. VeroE6 (ATCC CRL 1586) and HEK239T (ATCC CRL
1573) cells were grown in Dulbecco’s modified Eagle’s medium, 10%
fetal calf serum, and antibiotics as previously described (31). Human
pulmonary MECs were purchased from Cambrex Inc., grown in endo-
thelial growth medium 2MV (Lonza), and supplemented as previously de-
dscribed (31). ANDV (CHI-7913) was cultivated in BSL3 facilities (31).
Viral titers were determined in VeroE6 cells, MECs were ANDV infected
at a multiplicity of infection (MOI) of 0.5 or mock infected, and cells were
>90% infected at 3 dpi, as determined by focus assay of infected MECs
with anti-N-protein antibodies and immunoperoxidase staining with
3-amino-9-ethylcarbazole (25, 26). MECs infected with pathogenic
ANDV (MOI, 0.5) or persistently expressing ANDV N protein were incu-
bated for 18 h under hypoxic conditions (1% O2 by N2 displacement, 5%
CO2 in a multigas incubator [MCO-19M Sanyo Scientific], or cobalt-
chloride [100 μM] treated [Sigma]) to induce hypoxia in basal EBM-2
with 0.5% bovine serum albumin (BSA) for 6 h (52, 53). Cells more than
three times normal MEC size were considered to be enlarged and were
quantitated by microscopy (10 fields, 1,500 cells in duplicate wells
with NIH Image).

Plasmids and constructs. Plasmids expressing TSC2, TSC1, TBC1D7,
14-3-3, S6K, and Rheb were obtained from Addgene (14129, 19911,
32047, 13270, 26610, and 19996). ANDV nucleocapsid open reading
frames were PCR amplified and inserted into the pLenti-CMV-GFP-Puro
vector at the BamHI and XbaI sites, and HEK293T cells were cotrans-
formed (31). ANDV nucleocapsid open reading frames were PCR ampli-
fied and inserted into the pLenti-CMV-GFP-Puro vector at the BamHI and
XbaI sites, and HEK293T cells were cotransfected with third-generation lentiviral packaging plasmids p-RSV-Rev,
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REFERENCES

1. Bustamante EA, Levy H, Simpson SQ. 1997. Pleural fluid characteristics in hantavirus pulmonary syndrome. Chest 112:1133–1136. http://dx.doi.org/10.1016/S0168-1702(97)00053-1
2. Ladhavijitra V. 1982. Clinical features of HFRS in Scandinavia as compared with East Asia. Scand J Infect Dis Suppl 36:93–95.
3. Lee HW. 1982. Hemorrhagic fever with renal syndrome (HFRS). Scand J Infect Dis Suppl 36:82–85.
4. Duchin JS, Koster FT, Peters CJ, Simpson GL, Tempest B, Zaki SR, Ksiazek TG, Rollin PE, Nichol S, Umland ET, Moolenaar RL, Reef SE, Tolte KB, Gallaher MM, Butler JC, Breiman RF, Hantavirus Study Group. 1994. Hantavirus pulmonary syndrome: a clinical description of 17 patients with a newly recognized disease. The Hantavirus Study Group, N Engl J Med 330:949–955.
5. Schmaljohn C, Hoover JW. 2001. Bunyaviridae: the viruses and their replication. p 1581–1602. In Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Strauss SE (ed), Fields virology, 4th ed, vol 2. Lippincott-Raven, Philadelphia, PA.
6. Yanagihara R, Silverman DJ. 1990. Experimental infection of human vascular endothelial cells by pathogenic and nonpathogenic hantaviruses. Arch Virol 111:281–286. http://dx.doi.org/10.1007/BF01311063.
7. Zaki SR, Greer PW, Cowfish LM, Goldsmith CS, Tolte KB, Focur K, Feddersen RM, Zummwall RE, Miller GL, Khan AS, Rollin P, Ksiazek T, Nichol S, C. 1995. Hantavirus pulmonary syndrome: pathogenesis of an emerging infectious disease. Am J Pathol 146:552–579.
8. Nichol ST, Spireopoulos CF, Morunov S, Rollin PE, Ksiazek TG, Feldmann H, Sanchez A, Childs J, Zaki S, Peters CJ. 1993. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. Science 262:914–917. http://dx.doi.org/10.1126/science.8235615.
9. Padula PJ, Edelstein A, Miguel SD, Lopez RM, Ross CI, Rubinovich RD. 1998. Hantavirus pulmonary syndrome outbreak in Argentina: molecular evidence for person-to-person transmission of Andes virus. Virol 241:323–330. http://dx.doi.org/10.1007/00684-8976
10. Enria D, Padula P, Segura EI, Pini N, Edelstein A, Rosco CR, Weissenbacher MC. 1996. Hantavirus pulmonary syndrome in Argentina. Possibility of person to person transmission. Medicina (B Aires) 56:709–711.
11. Lopez N, Padula P, Ross CI, Lázaro ME, Franche-Fernández MT. 1996. Genetic identification of a new hantavirus causing severe pulmonary syndrome in Argentina. Virology 220:223–226. http://dx.doi.org/10.1016/0042-6822(96)00305-1.
12. Lopez N, Padula PA, Ross CI, Miguel SD, Edelstein A, Ramírez E, Franche-Fernández MT. 1997. Genetic characterization and phylogeny of Andes virus and variants from Argentina and Chile. Chile. Virus Res 50:77–84. http://dx.doi.org/10.1016/S0168-1702(97)00053-1.
13. Hammerbeck CD, Hoover JW. 2011. T cells are not required for pathogenesis in the Syrian hamster model of hantavirus pulmonary syndrome. J Virol 85:9929–9944. http://dx.doi.org/10.1128/JVI.05356-11.
14. Hoover JW, Larsen T, Custer DM, Schmaljohn CS. 2001. A lethal disease model for hantavirus pulmonary syndrome. Virology 289:6–14. http://dx.doi.org/10.1006/0042-6822(96)00305-1.
15. Wahl-Jensen V, Chapman J, Asher L, Fisher R, Zimmerman M, Larsen T, Hoover JW. 2007. Temporal analysis of Andes virus and Sin Nombre virus infections in transplanted mice. J Virol 81:10634–10641. http://dx.doi.org/10.1128/JVI.00465-10.
16. Hui C, Loesche WR, Gifford DR. 1989. A gold standard Transwell permeability assay. J Biol Chem 264:16933–16939.
17. WHO. 1995. WHO laboratory manual for the diagnosis of human hantavirus infections. World Health Organization, Geneva.
18. Wahl-Jensen V, Chapman J, Asher L, Fisher R, Zimmerman M, Larsen T, Hoover JW. 2007. Temporal analysis of Andes virus and Sin Nombre virus infections in transplanted mice. J Virol 81:10634–10641. http://dx.doi.org/10.1128/JVI.00465-10.
19. Wahl-Jensen V, Chapman J, Asher L, Fisher R, Zimmerman M, Larsen T, Hoover JW. 2007. Temporal analysis of Andes virus and Sin Nombre
Gorbunova et al.

virus infections of Syrian hamsters. J Virol 81:7449–7462. http://dx.doi.org/10.1128/JVI.00238-07.

16. Koster F, Mackow E. 2012. Pathogenesis of the hantavirus pulmonary syndrome. Future Virol 7:41–51. http://dx.doi.org/10.2217/fv.11.138.

17. Galeno H, Mora J, Villagrá E, Fernandez J, Hernandez J, Mertz GJ, Gavrilovskaya IN, Mora J, Villagrá E, Fernandez J, Hernandez J, Mertz GJ, Gavrilovskaya IN, Pepini T, Mackow E. 2011. VEGF-R2 and Src kinase inhibitors suppress Andes virus-induced endothelial cell permeability. J Virol 85:2296–2303. http://dx.doi.org/10.1128/JVI.02319-10.

18. Gavrilovskaya I, Gorbunova E, Koster F, Mackow E. 2012. Elevated VEGF levels in pulmonary edema fluid and PBMCs from patients with acute hantavirus pulmonary syndrome. Adv Virol 2012:674360. http://dx.doi.org/10.1155/2012/674360.

19. Tuberin activates and controls the distribution of Rac1 via association with p62 and ubiquitin through the RhoGDI-1 modulation of the activity of monomeric RhoGTPase RhoA. Stabilization of RhoA and Rac1 activities. Am J Physiol Lung Cell Mol Physiol 299:L948–L957. http://dx.doi.org/10.1152/ajplung.00309.2005.

20. Galeno H, Mora J, Villagrá E, Fernandez J, Hernandez J, Mertz GJ, Gavrilovskaya IN, Pepini T, Mackow E. 2011. VEGF-R2 and Src kinase inhibitors suppress Andes virus-induced endothelial cell permeability. J Virol 85:2296–2303. http://dx.doi.org/10.1128/JVI.02319-10.

21. Taylor SL, Wahl-Jensen V, Copeland AM, Schmaljohn CS. 2013. Endothelial cell permeability during hantavirus infection involves factor XII-dependent increased activation of the kalikrein-kinin system. PLoS Pathog 9:e1003470. http://dx.doi.org/10.1371/journal.ppat.1003470.

22. Daneshjou N, Sieracki N, van Nieuw Amerongen GP, Schwartz MA, Komarova YA, Malik AB, Conway DE. 2015. Rac functions as a reversible tension modulator to stabilize VE-cadherin trans-interaction. J Cell Biol 208:23–32. http://dx.doi.org/10.1083/jcb.201409108.

23. Gavrilovskaya IN, Brown EJ, Ginsberg MH, Mackow ER. 1999. Cellular entry of hantaviruses which cause hemorrhagic fever with renal syndrome is mediated by beta3 integrins. J Virol 73:3951–3959.

24. Hjelle B, Feldmann H. 2004. Role of alphavbeta3 integrin in the activation of vascular endothelial cadherin internalization in endothelial cells. J Cell Sci 117:4832–4839. http://dx.doi.org/10.1242/jcs.00617.

25. Giannotta M, Taylor SL, Wahl-Jensen V, Copeland AM, Schmaljohn CS. 2013. Endothelial cell permeability during hantavirus infection involves factor XII-dependent increased activation of the kalikrein-kinin system. PLoS Pathog 9:e1003470. http://dx.doi.org/10.1371/journal.ppat.1003470.

26. Zhu X, Becker EC, Handschumacher ME, Mraz SR, Galeno H, Mora J, Villagrá E, Fernandez J, Hernandez J, Mertz GJ, Gavrilovskaya IN, Pepini T, Mackow E. 2011. VEGF-R2 and Src kinase inhibitors suppress Andes virus-induced endothelial cell permeability. J Virol 85:2296–2303. http://dx.doi.org/10.1128/JVI.02319-10.

27. Galen H, Mora J, Villagrá E, Fernandez J, Hernandez J, Mertz GJ, Gavrilovskaya IN, Pepini T, Mackow E. 2011. VEGF-R2 and Src kinase inhibitors suppress Andes virus-induced endothelial cell permeability. J Virol 85:2296–2303. http://dx.doi.org/10.1128/JVI.02319-10.

28. Taylor SL, Wahl-Jensen V, Copeland AM, Schmaljohn CS. 2013. Endothelial cell permeability during hantavirus infection involves factor XII-dependent increased activation of the kalikrein-kinin system. PLoS Pathog 9:e1003470. http://dx.doi.org/10.1371/journal.ppat.1003470.

29. Okura H, Kobayashi T, Koike M, Ohsawa M, Zhang D, Ariai H, Uchiyama Y, Hino O. 2013. Tuberin activates and controls the distribution of Rac1 via association with p62 and ubiquitin through the mTORC1 signaling pathway. Int J Oncol 43:447–456. http://dx.doi.org/10.3892/ijo.2013.1982.

30. Vojvodic A, Brining D, Dahlstrom E, Porcella SF, Ebihara H, Scott DP, Hjelle B, Feldmann H. 2014. Pathophysiology of hantavirus pulmonary syndrome in rhesus macaques. Proc Natl Acad Sci U S A 111:714–719. http://dx.doi.org/10.1073/pnas.1401986111.

31. Schmaljohn CS. 2012. Pathogenesis of the hantavirus pulmonary syndrome. Future Virol 7:41–51. http://dx.doi.org/10.2217/fv.11.138.

32. Giannotta M, Taylor SL, Wahl-Jensen V, Copeland AM, Schmaljohn CS. 2013. Endothelial cell permeability during hantavirus infection involves factor XII-dependent increased activation of the kalikrein-kinin system. PLoS Pathog 9:e1003470. http://dx.doi.org/10.1371/journal.ppat.1003470.

33. Giannotta M, Taylor SL, Wahl-Jensen V, Copeland AM, Schmaljohn CS. 2013. Endothelial cell permeability during hantavirus infection involves factor XII-dependent increased activation of the kalikrein-kinin system. PLoS Pathog 9:e1003470. http://dx.doi.org/10.1371/journal.ppat.1003470.

34. Sukriti S, Tauseef M, Yazbeck P, Mehta D, Malik AB, Voyno-Yasenetskaya T. 2007. RhodGDI-1 modulation of the activity of monomeric RhodGTPase RhoA.

35. Sukriti S, Tauseef M, Yazbeck P, Mehta D, Malik AB, Voyno-Yasenetskaya T. 2007. RhodGDI-1 modulation of the activity of monomeric RhodGTPase RhoA.
regulates endothelial barrier function in mouse lungs. Circ Res 101: 50–58.  
51. Knezevic N, Roy A, Timblin B, Konstantoulaki M, Sharma T, Malik AB, Mehta D. 2007. GDI-1 phosphorylation switch at serine 96 induces RhoA activation and increased endothelial permeability. Mol Cell Biol 27: 6323–6333.  
52. Gavrilovskaya IN, Gorbunova EE, Mackow ER. 2012. Andes virus infection of lymphatic endothelial cells causes giant cell and enhanced permeability responses that are rapamycin and vascular endothelial growth factor C sensitive. J Virol 86:8765–8772.  
53. Gavrilovskaya IN, Gorbunova EE, Mackow ER. 2013. Hypoxia induces permeability and giant cell responses of Andes virus-infected pulmonary endothelial cells by activating the mTOR-S6K signaling pathway. J Virol 87:12999–13008.  
54. Laplante M, Sabatini DM. 2012. MTOR signaling in growth control and disease. Cell 149:274–293.  
55. Inoki K, Li Y, Xu T, Guan KL. 2003. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev 17: 1829–1834.  
56. Goncharova EA, Goncharov DA, Eszterhas A, Hunter DS, Glassberg MK, Yeung RS, Walker CI, Noonan D, Kwiatkowski DJ, Chou MM, Panettieri RA, Jr., Krymskaya VP. 2002. Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary lymphangiomiomatosis (LAM). J Biol Chem 277:39058–39067.  
57. Astrinidis A, Cash TP, Hunter DS, Walker CL, Chernoff J, Henske EP. 2002. Tuberin, the tuberous sclerosis complex 2 tumor suppressor gene product, regulates Rho activation, cell adhesion and migration. Oncogene 21:8470–8476.  
58. DeYoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW. 2006. Tumor suppressors TSC1 and TSC2 differentially modulate actin cytoskeleton and motility of mouse embryonic fibroblasts. J Cell Biol 176:1171–1182.  
59. Ohsawa M, Kobayashi T, Okura H, Igarashi T, Mizuguchi M, Hino O. 2013. TSC1 controls distribution of actin fibers through its effect on function of Rho family of small GTPases and regulates cell migration and polarity. PLoS One 8:e54503.  
60. DeYoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW. 2008. Hypoxia regulates TSC1-2/mTOR signaling and tumor suppression through REDD1-mediated 14–3–3 shuttling. Genes Dev 22:239–251.  
61. Li Y, Inoki K, Yeung R, Guan KL. 2002. Regulation of TSC2 by 14–3-3 and the Rac1 GTPase. J Cell Biol 167:1171–1182.  
62. Dibble CC, Elis W, Menon S, Qin W, Klekota J, Asara JM, Finan PM, Li Y, Inoki K, Yeung R, Guan KL. 2004. TSC2 medically regulatory TSC1-binding domain and the Rac1 GTPase. J Cell Biol 167:1171–1182.  
63. Hejpojok J, Strandin T, Lakenin H, Vaheri A. 2012. Hantavirus structure—molecular interactions behind the scene. J Gen Virol 93: 1631–1644.  
64. Nakashima A, Yoshino K, Miyamoto T, Eguchi S, Oshiro N, Kikkawa U, Yonezawa K. 2007. Identification of TBC7 having TBC domain as a novel binding protein to TSC1-TSC2 complex. Biochem Biophys Res Commun 361:218–223.  
65. Goncharova EA, James ML, Kudryashova TV, Goncharov DA, Krymskaya VP. 2014. Tumor suppressors TSC1 and TSC2 differentially modulate actin cytoskeleton and motility of mouse embryonic fibroblasts. PLoS One 9:e911476.  
66. Lindqvist K, I. Ledskog, TSC1-TSC2 complex upstream of mTORC1. Mol Cell 47:535–546.  
67. Kovalak I, Ledskog K,精子, Kohnken PT, Lindstedt KA. 2009. Hypoxia-induced expression of bradykinin type-2 receptors in endothelial cells triggers NO production, cell migration, and angiogenesis. J Cell Physiol 221:359–366.  
68. van Nieuw Amerongen GP, van Delft S, Vermeer MA, Collard JG, van Hinsberg VV. 2000. Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases. Circ Res 87:335–340.  
69. Beckers CM, van Hinsbergh VW, van Nieuw Amerongen GP. 2010. Driving Rho GTPase activity in endothelial cells regulates barrier integrity. Thromb Haemost 103:40–55.  
70. Woljciak-Sothard B, Tsang LY, Haworth SG. 2005. Rac and Rho play opposing roles in the regulation of hypoxia/reoxygenation-induced permeability changes in pulmonary artery endothelial cells. Am J Physiol Lung Cell Mol Physiol 288:L749–L760.  
71. Komarova Y, Malik AB. 2010. Regulation of endothelial permeability via paracellular and transcellular transport pathways. Annu Rev Physiol 72:463–493.  
72. Gavrilovskaya I, Gorbunova E, Matsysh, D, Dalrymple N, Mackow ER. 2012. The role of the endothelium in HPS pathogenesis and potential therapeutic approaches. Adv Virol 2012:467059.  
73. Ridley A. 2000. Rho GTPases. Integrating integrin signaling. J Cell Biol 150:F107–F109.  
74. Suzuki K, Nemoto K, Nominou N, Kuno M, Kubota M, Yokota H. 2012. Fusadil, a Rho-kinase inhibitor, attenuates lipopolysaccharide-induced vascular hyperpermeability and colonic muscle relaxation in guinea pigs. J Surg Res 178:352–357.  
75. Aird WC. 2006. Endothelium in health and disease. Pharmacol Rep 60:139–143.  
76. Hillgruber C, Pöppelmann B, Weishepp C, Steingräber A, Wessel F, Berdel WE, Gessner JF, Hein-Nöe B, Westeweber D, Goerge T. 2015. Blocking neutrophil diapedesis prevents hemorrhage during thromboembolism. J Exp Med 212:1255–1286.  
77. Geimonde N, Neff S, Raymond T, Kocer SS, Gavrilovskaya IN, Bryan BA, Dennstedt E, Mitchell DC, Walshe TE, Noma K, Loureiro J. 2002. Rapid transactivation of the direct alphaVbeta3-dependent growth-inhibitory action of activin fragment of tumstatin in glioma cells in vitro and in vivo. Cancer Res 62:8765–8772.  
78. Reynolds LE, Wyler L, Lively JC, Taverna D, Robinson SD, Huang X, Sheppard D, Hynes RO, Hodivala-Dilke KM. 2002. Enhanced patholog-ical angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat Med 8:237–34.  
79. Kawaguchi T, Yamashita Y, Kamaniore M, Endersby R, Bankiewicz KD, Baker SJ, Bergers G, Pieper RO. 2006. The PTK7/Akt pathway dictates the direct alphaVbeta3-dependent growth-inhibitory action of an activin fragment of tumstatin in glioma cells in vivo and in vitro. Cancer Res 66:1331–1340.  
80. Menon S, Xue Y. 2010. TSC2 upregulated potential regulation of blood-tumor barrier permeability by bradykinin. J Mol Neurosci 42:67–73.  
81. Thuringer D, Maulon L, Frelin C. 2002. Rapid transactivation of the vascular endothelial growth factor receptor KDR/Flik-1 by the bradykinin B2 receptor contributes to endothelial nitric-oxide synthase activation in cardiac capillary endothelial cells. J Biol Chem 277:2028–2032.  
82. Brugarolas J, Kaelin WG, Jr. 2004. Dysregulation of HIF and VEGF is a unifying feature of the familial hamartoma syndromes. Cancer Cell 6:17–10.  
83. Legate KR, Fässler R. 2009. Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. J Cell Sci 122:187–198.  
84. Boneet V, Kovanakis I, Campbell ID. 2013. Characterization of 14–3–3 beta integrin interactions with integrin tails. J Mol Biol 425:3060–3072.  
85. Bryan BA, Dennstedt E, Mitchell DC, Walshe TE, Noma K, Loureiro J, d'Amore PA. 2010. RhoA/ROCK signaling is essential for multiple aspects of VEGF-mediated angiogenesis. FASEB J 24:3186–3195.  
86. Larson Y, Liu J, Stevens PD, Li X, Li J, Evers BM, Gao T. 2010. Tuberous sclerosis complex 2 (TSC2) regulates cell migration and polarity through activation of CDC42 and RAC1. J Biol Chem 285:24987–24998.  
87. David S, Ghosh CC, Mukherjee A, Parikh SM. 2011. Angiopoietin-1
requires IQ domain GTPase-activating protein 1 to activate Rac1 and promote endothelial barrier defense. Arterioscler Thromb Vasc Biol 31: 2643–2652. 
http://dx.doi.org/10.1161/ATVBAHA.111.233189.

88. Abiko H, Fujiwara S, Ohashi K, Hiatura R, Mashiko T, Sakamoto N, Sato M, Mizuno K. 2015. Rho guanine nucleotide exchange factors involved in cyclic-stretch-induced reorientation of vascular endothelial cells. J Cell Sci 128:1683–1695. 
http://dx.doi.org/10.1242/jcs.157093.

89. Tian Y, Gawlak G, O’Donnell JJ III, Birukova AA, Birukov KG. 2016. Activation of vascular endothelial growth factor (VEGF) receptor 2 mediates endothelial permeability caused by cyclic stretch. J Biol Chem 291:10032–10045. 
http://dx.doi.org/10.1074/jbc.M115.690487.

90. Antonen J, Leppänen I, Tenhenen J, Arvola P, Mäkelä S, Vehari A, Mustonen J. 2013. A severe case of Puumala hantavirus infection successfully treated with bradykinin receptor antagonist icatibant. Scand J Infect Dis 45:494–496. 
http://dx.doi.org/10.3109/00365548.2012.752568.

91. Shrivastava-Ranjan P, Rollin PE, Spirioulou CF. 2010. Andes virus disrupts the endothelial cell barrier by induction of vascular endothelial growth factor and downregulation of VE-cadherin. J Virol 84: 11227–11234. 
http://dx.doi.org/10.1128/JVI.01405-10.

92. Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini D. 2000. A versatile viral vector for efficient transduction of nondividing cells. Nat Biotechnol 18:132–137. 
http://dx.doi.org/10.1038/74725.

93. Shibuya M. 1999. Fasudil, a protein kinase inhibitor, prevents the development of proteinuria in early-stage diabetic nephropathy by inhibition of RhoA/ROCK1. PLoS 1:e6529. 
http://dx.doi.org/10.1371/journal.pone.0006529.

94. Fabry B, Klemm AH, Kienle S, Schaffer TE, Goldmann WH. 2011. Focal adhesion kinase stabilizes the cytoskeleton. Biophys J 101:2313–2318. 
http://dx.doi.org/10.1016/j.bpj.2011.09.043.

95. Alph J, Sen N, Gorbonova E, Gavrilovskaya IN, Mackow ER. 2008. The immunosuppressant FTI720 inhibits tumor angiogenesis via the sphingosine 1-phosphate receptor 1. J Cell Biochem 101:259–270. 
http://dx.doi.org/10.1002/jcb.21181.

96. Campeau E, Ruhl VE, Rodier F, Smith CL, Rahmberg BL, Fuss JO, Campisi J, Yaswen P, Cooper PK, Kaufman PD. 2009. A versatile viral system for expression and depletion of proteins in mammalian cells. PLoS One 4:e6529. 
http://dx.doi.org/10.1371/journal.pone.0006529.

97. Shiue I, Green J, Green OM, Karsa IL, Morgenstern JP, Ram MK, Taylor MK, Zoller MJ, Zydowsky LD, Bolen JB, et al. 1995. Interaction of p75kDa with the gamma and beta subunits of the high-affinity receptor for immunoglobulin E, Fc epsilon RI. Mol Cell Biol 15:227–231. 
http://dx.doi.org/10.1128/MCB.15.1.272.

98. Pepini T, Gorbonova EE, Gavrilovskaya IN, Mackow JE, Mackow ER. 2010. Andes virus regulation of cellular microRNAs contributes to hantavirus-induced endothelial cell permeability. J Virol 84: 11929–11936. 
http://dx.doi.org/10.1128/JVI.01685-10.