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The pericyte: Cellular regulator of microvascular blood flow

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Abstract

The vascular system – through its development, response to injury, and remodeling during disease – constitutes one of the key organ systems sustaining normal human physiology; conversely, its dysregulation also underlies multiple pathophysiologic processes. Regulation of vascular endothelial cell function requires the integration of complex signals via multiple cell types, including arterial smooth muscle, capillary and post-capillary pericytes, and other perivascular cells such as glial and immune cells. Here, we focus on the pericyte and its roles in microvascular remodeling, reviewing current concepts in microvascular pathophysiology and offering new insights into the specific roles that pericyte-dependent signaling pathways may play in modulating endothelial growth and microvascular tone during pathologic angiogenesis and essential hypertension.

Overview

A complete understanding of the complex regulation of the vascular system requires both a systemic, structural understanding of vascular function, as well as the focused dissection of multiply-intertwined signaling pathways in both vascular and non-vascular cell types.[1] While tumor growth is known to depend on concomitant angiogenesis,[2] it has been further suggested that angiogenesis may, in fact, be a common template underlying numerous other disparate phenomena including wound healing, diabetic retinopathy, age-related macular degeneration, chronic inflammatory states, capillary permeability, and microvascular tone regulation.[3] A complete understanding of the physiological cross-talk between endothelial cells and the cellular regulators that modulate their (dys)function holds promise for understanding the underpinnings of key physiologic and pathololgic processes, while offering opportunities for innovation in the treatment of life threatening and chronic illness.

Interactions between endothelial cells and surrounding perivascular cells have long been thought to mediate control of the vascular system on a local level. In addition to the endothelial cell, several perivascular cell types play key roles in dynamic regulation of the vascular system, including arterial smooth muscle[4] and capillary and venular pericytes.[5, 6] Regulation of the capillary microenvironment by these perivascular cell types occurs via three principal mechanisms: (1) communication with the underlying endothelium by soluble mediators and cell-cell contact, (2) synthesis, remodeling, and maintenance of the basement membrane, and (3) regulation of microvascular tone. All of these mechanisms involve an overlapping array of

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biochemical and biomechanical signaling pathways,[7–9] with considerable gaps in knowledge prompting extensive current interest and investigation. A complete understanding of the cellular physiology underpinning vascular development, blood-brain barrier function, capillary permeability, and microvascular tone regulation, therefore, may be expected to illuminate the corresponding pathophysiology of tumor angiogenesis,[10] age-related macular degeneration,[11] and diabetic retinopathy,[12] as well as both pulmonary and systemic hypertension.[13, 14]

Physiological and Pathological Angiogenesis: Current Concepts and Challenges

Pericyte control of microvascular remodeling and proliferative status

The pericyte in particular has drawn increased attention as an emerging key mediator in multiple microvascular processes, including: (i) endothelial cell proliferation and differentiation,[15, 16] (ii) contractility and tone,[17, 18] (iii) stabilization and permeability,[19] and (iv) morphogenesis during disease onset.[20] First described in early studies of vascular development by Rouget in 1873,[21] pericytes have subsequently been shown to regulate multiple stages of vascular development and differentiation.[6–8] During angiogenesis, nascent microvessels are heralded by an actively motile and proliferative endothelium with an immature basement membrane. This migratory and proliferative phase yields a primitive capillary tube, followed by a microvascular maturation phase marked by an endothelial FGF-2- and PDGF-dependent recruitment of presumptive pericytes, occurring concomitantly with basement membrane remodeling. Triggered by endothelial cell contact, the presumptive pericyte then assumes a mature contractile status by initiating expression of its smooth muscle contractile protein repertoire.[22]

Pericytes have been postulated to govern the phenotypic change from a proliferative angiogenic sprout to a mature microvascular conduit possessing a quiescent capillary endothelium.[15, 23–26] Through both pericyte/endothelial cell contact-dependent as well as endothelial-independent mechanisms, pericytes suppress endothelial growth[27] and migration.[28] Additionally, *in situ*, there is striking coincidence of pericyte investment and microvessel stabilization,[19, 23] and densely pericyte-invested capillary beds demonstrate reduced rates of endothelial turnover.[29–31] Interestingly, pericyte investment has also been directly implicated in conferring capillary resistance to regression *in vivo*.[32, 33] As well, knockout studies using pericyte-deficient PDGF-receptor-beta mice demonstrate endothelial hyperplasia in the usually densely-pericyte associated capillary beds of the brain.[34, 35] This work suggests that in order for pathological neovascularization to occur, the quiescent endothelium must escape from its growth-arrested phenotype, perhaps actively destabilizing and disengaging from its association with pericytes as it re-enters the cell cycle. Outcomes of this would be both consistent with the observable ‘pericyte dropout’ seen histologically in proliferative diabetic retinopathy[36–38] as well as the notion that pericyte apoptosis need not be required for initiation of pathologic angiogenesis.

Pericyte dysregulation in pathological angiogenesis

There is growing interest in the regulatory roles for pericytes in modulating endothelial phenotype during pathologic angiogenesis, such as tumor-induced angiogenesis, age related macular degeneration and proliferative diabetic retinopathy. Based on early histological observations, frank pericyte loss (‘pericyte dropout’) via apoptosis or de-differentiation was initially proposed to underlie the observed endothelial dysfunction and hyperplasia in these states.[39] Recent models of anti-angiogenic chemotherapy confirm that small molecule- and antibody-mediated disruption of endothelial survival signaling show specific destruction of non- or loosely pericyte-associated vessels, while mature vessels with secure pericyte association may be more refractory to therapy.[19, 32, 40] Other studies predicted that alterations

in basement membrane composition could modulate pericyte-endothelial interactions,[41, 42] and that matrix-associated molecular signaling via endothelial-derived FGF-2 could control pericyte recruitment,[43] suggesting possible mechanisms for dysfunction of pericyte-endothelial growth regulation without actual pericyte loss. As well, El-Bizri *et* al. have shown that pericyte-targeted deletion of bone morphogenetic protein receptor 1A (BMPR1A) is associated with reduced matrix metalloproteinase activity and pericyte resistance to apoptosis *in vitro*, associated with failure of appropriate vascular pruning and impaired organogenesis *in vivo*.[33] Also consistent with a prominent role for matrix as an arbiter of endothelial-pericyte interaction, rapid regrowth of neovascular sprouts into empty basement membrane sleeves was also observed following regression of immature tumor vasculature less than 24 hours following discontinuation of VEGF-targeted monotherapy.[44] As the importance of the platelet-derived growth factor (PDGF) system to the recruitment and differentiation of pericytes at the level of the nascent capillary has also been strongly supported by multiple developmental[34, 35, 45–47] and pharmacological[48] studies, PDGF has become a promising additional target for anti-angiogenic chemotherapy. Several groups have shown augmented efficacy and greater durability of anti-angiogenic therapy by combining small molecule inhibition of both VEGF and PDGF receptor signaling in tumors.[48–55] A similar mechanism has also been shown in models of ocular neovascularization,[56] where microvascular regression is coupled to the 'drop-out' of vessel-associated pericytes, derived either by apoptosis or microenvironmentally-driven 'de-differentiation' without frank loss. Recent reports of pericyte drop-out in mouse models of diabetic retinopathy suggest that one of the local mediators controlling this phenomenon in the diabetic eye may be the hyperglycemia-induced production of Ang-2, leading to a Tie2-mediated increases in pericyte apoptosis and migration *in vitro* that mirrors the dissociation of pericytes from retinal capillaries observed *in vivo*.[57, 58] The identification of such molecular cues for pericyte de-differentiation and dissociation is a promising source of both new drug targets for diabetic retinopathy, as well as a potential source of insight into the pathophysiology of hyperglycemic damage in other organ beds.

Consistent with the theory that dysfunctional pericyte stabilization of tumor endothelium may prove to be a potent additional target for tumor microvascular instability and microvascular regression, one group's careful analysis of baseline tumor vasculature by thick-section confocal microscopy revealed pericyte association with greater than 97% of tumor vessels, but with electron-microscopically evident abnormal associations between endothelial cells, pericytes, and matrix.[59, 60] Interestingly, intravital microscopy performed on glioma vasculature growing in a dorsal skinfold chamber during treatment with a small molecule inhibitor of both VEGF receptor-2 and PDGF receptor-beta revealed that vascular regression occurred despite a surprisingly high index of continued endothelial investment by pericytes.[51] These data imply that pericyte-endothelial destabilization without frank pericyte drop-out or apoptosis may be sufficient to produce marked vascular regression. Interestingly, recent work has revealed that the ability of pericytes to growth-arrest adjacent endothelial cells *in vitro* can be attenuated by manipulation of pericyte Rho GTP status. These results demonstrate that alterations in the mechanochemical coupling of endothelial cells and pericytes may be sufficient to initiate pathological angiogenesis, *without* the requirement for pericyte drop-out or apoptosis.[61]

Soluble and trans-matrix extracellular signaling via TGF-beta

The above-described bridging between intracellular signaling and force transduction pathways in pericytes parallels the emerging role for the transforming growth factor-beta (TGF-beta) family of ligands, receptors, and signal-transducing effectors (recently reviewed by Bertolino[62]). In vascular endothelial cells, human analogues of the Drosophila *mothers against decapentaplegic* (MAD) family of proteins have recently been characterized as primary transducers of TGF-beta signaling through multiple pathways. These include the mitogen-activated protein kinase, MAPK,[63] which may function through the cyclic AMP-responsive

element binding protein CREB.[64] There is also evidence to indicate that a subset of MAD family proteins (Smads 6 and 7) are shear stress-inducible, and therein constitute a mechanism of fluid flow-sensitive mechanochemical regulation of endothelial gene expression.[65] Smad-mediated signaling also plays a role in the regulation of alpha-smooth muscle actin expression and proliferative state in pericytes, which are downstream of TGF-beta and FGF-2[10, 22] signal transduction. Given that both TGF-beta[66] and FGF-2[41] are known to be associated with sub-endothelial matrix (reviewed by Dinbergs *et al*.[67]), these data together raise the possibility that a mechanochemical link exists between pericytes and endothelial cells: in particular, TGF-beta- and SMAD-dependent signaling may mediate a continuous signal originating from the luminal endothelial surface, spanning sub-endothelial matrix, and arriving at a neighboring pericyte. Further, it is likely that this heterocellular mechanochemical coupling is mediated at the pericyte level by a Rho GTP-dependent network of cytoskeletal effectors.

Interestingly, recent evidence from of acute lung injury models also implicate Rho and Rho kinase-dependent signaling pathways in the transduction of TGF-beta signaling in endothelial cells. Increased levels of circulating TGF-beta have been noted in trauma patients and are associated with the development of the acute respiratory distress syndrome (ARDS) and sepsis.[68] In two animal models of ARDS, adenovirus-mediated overexpression of TGF-beta induces[69] and delivery of soluble TGF-beta receptor inhibits[70] the acute increase in vascular permeability as well as the later development of pulmonary fibrosis in animal models of septic lung injury modeling human ARDS patients. One group has recently demonstrated that treatment with TGF-beta induces myosin light chain phosphorylation, stress fiber formation, and endothelial permeability, and that these alterations in cytoskeletal structure are partially dependent on downstream signaling through Rho GTPase and its principle effector, Rho kinase.[71, 72]

The *in vivo* finding that circulating systemic TGF-beta has potent effects on the microvasculature of the lung is consistent with the known role of local TGF-beta signaling in pericyte-mediated endothelial maturation and survival. Recent *in vivo* experiments showing that pericyte-derived TGF-beta enhances retinal endothelial cell survival through upregulation of endothelial VEGF receptor-1[73] complements extensive evidence *in vitro* implicating TGF-beta as a key mediator of pericyte control of endothelial growth state and phenotype.[27, 74–79] Importantly, the biochemical activity of TGF-beta is dependent upon activation requiring the involvement of both alpha-V integrins (such as beta-6 and beta-8)[80] as well as latent TGF-beta binding protein (recently reviewed by Sheppard *et al*.[81]), allowing specific local control of TGF-beta activation even in the face of its systemic presence.

Although TGF-beta is currently the best understood soluble mediator of pericyte-endothelial cell interactions, recent evidence indicates that other as yet unknown molecules may also play a parallel role. Kondo *et al.* recently noted that treatment of cultured retinal endothelial cells by retinal pericyte-conditioned media suppressed endothelial cell proliferation, and that this effect was only partially reversible by a TGF-beta-1 blocking antibody, in contrast to the complete reversibility observed following heat-treatment.[82] As well, our lab has recently demonstrated that signaling through the Rho GTPase and its downstream effector Rho kinase underlies both pericyte contractility[61, 83] as well as pericyte regulation of endothelial growth, which appears to be TGF-beta-independent.[61] Thus, while matrix-associated TGF-beta and FGF2[43] play roles in modulating pericyte recruitment, pericyte-endothelial interactions, and microvascular junctional integrity, there are also likely roles for as yet undescribed soluble mediators, which function to regulate endothelial growth in both contact-dependent and -independent manners.[61, 84, 85]

Intracellular signaling via Rho GTPase – Smooth muscle cell pathways

Evidence is accumulating that vascular morphogenesis may be regulated by members of the Rho family of small GTPases (as recently reviewed by Bryan and D'Amore,[86] as well as Mammoto *et al*.[87]). Rho GTPase-mediated cytoskeletal adaptations modulate normal maintenance of the arterial vasculature, as well as mediate the tone dysregulation and hypertrophic remodeling observed during essential hypertension.[88–91] Here, we will briefly review the Rho signaling in vascular smooth muscle as a template for understanding its role in pericytes.

Biochemical tools

Multiple experimental tools are available for both the *in vitro* and *in vivo* perturbation of signaling through Rho GTPase and its downstream effector, Rho kinase. The first such signaling studies were conducted using GTP-gamma-S-bound Rho;[92] shortly thereafter, an array of Rho mutants were established by site-directed mutagenesis, including two dominant-active (Rho-V14 and RhoL-63) and one dominant-negative (Rho-N19) mutant.[93, 94] As well, microinjection or transfection with either *Clostridium botulinum* exoenzyme C3 or *Clostridium difficile* toxin B produces inactivation of endogenous Rho via ADP-ribosylation and glucosylation, respectively.[92, 95]

Recent focus has also been placed on the development of small molecular pharmacologic inhibitors of Rho kinase (also referred to as ROCK1), one of the principle downstream effectors of Rho GTPase signaling in multiple cell types (recently reviewed by Liao *et al*.[96]). The first of these specific Rho kinase inhibitors was Y-27632, a pyridine derivative which binds to and inhibits the catalytic site of Rho kinase in an ATP-competitive fashion, and is efficiently taken up via carrier-mediated facilitated diffusion.[97] The second major well-characterized inhibitor was fasudil (also referred to as HA1077 and AT877), initially explored for use as a calcium antagonist in models of subarachnoid hemorrhage and smooth muscle-mediated cerebral vasospasm.[98] Unlike other previously described calcium channel blockers investigated for their protective effect in vasospasm, fasudil's mechanism of action was independent of upstream calcium channel blockade.[99] Subsequently, this mechanism was elucidated as dependent on the selective inhibition of Rho kinase in smooth muscle.[100] Of the described Rho kinase inhibitors thus far, fasudil has demonstrated safety and efficacy in multiple clinical trials of cardiovascular disease treatment, beginning with the prevention of subarachnoid hemorrhage-induced smooth muscle-mediated vasospasm in the early 1990's.[101, 102] Since its clinical introduction, it has been explored for further use in cerebral infarction,[103] coronary vasospastic angina and ischemia,[104, 105] ventricular remodeling and myocardial fibrosis following myocardial infarction,[106, 107] intimal hyperplasia,[108] pulmonary and essential hypertension (reviewed by Fukumoto *et al*.[109] and Shimokawa and Takeshita,[110] respectively), pulmonary interstitial fibrosis,[111] and peripheral nerve injury.[112] The growing clinical applications of fasudil and other small-molecule inhibitors of Rho kinase signaling in human disease are beyond the scope of this review, but have been recently and thoroughly reviewed by Olson[113] and others.

Rho GTPase and Rho kinase signaling in vascular smooth muscle

Through application of the above biochemical tools, the importance of Rho GTPase signaling through Rho kinase has been explored in some detail in smooth muscle cells, and has been extensively reviewed elsewhere.[114, 115] While considerable overlap between Rho signaling in smooth muscle cells and pericytes exists, clear evidence for pericyte-specific aspects of the Rho cascade is emerging as well.[61, 116] In order to highlight both parallel and divergent pathways of Rho GTP-dependent signaling in pericytes, we will briefly outline the Rho pathway in vascular smooth muscle here.

Downstream signaling via activation of the Rho GTPase involves three principle pathways: dynamic interactions with Rho GAPs (G-protein activating proteins) and GEFs (guanine nucleotide-binding factors), activation of mDia, and activation of Rho kinase (see Figure 1). Regulators of GAPs and GEFs include such kinases as Src and FAK, with principle downstream regulation of focal adhesion formation. mDia leads to activation of profilin, an actin monomer-binding protein which orchestrates actin filament and stress fiber assembly. Downstream of Rho kinase there appear to be multiple targets, ultimately signaling through focal adhesion kinase and alpha-smooth muscle actin to regulate stress fiber formation.[117] Four Rho kinase-mediated pathways in particular deserve detailed attention as promising candidates for bridging the activation of Rho kinase seen in smooth muscle with direct functional relationships likely to be central in remodeling pericyte cytoskeletal dynamics during contractility: myosin light chain kinase, ADF/cofilin, ERM family proteins, and p38 MAP kinase.

Myosin light chain kinase and myosin phosphatase

In smooth muscle cells, contractility is controlled by the myosin light chain; the phosphorylation state of its myosin-binding subunit is mediated by the dynamic balance between calcium/calmodulin-dependent myosin light chain kinase [MLCK; reviewed by Kamm and Stull[118]) and myosin phosphatase activity (reviewed by Ito *et al*.[119]). While MLCK activity is regulated by intracellular calcium and calmodulin-based signaling, myosin phosphatase activity is chiefly governed by signaling through Rho kinase.[100, 120, 121] Recent work also describes linkage of myosin light chain phosphatase to the cytoskeleton via a Rho GTPase-interacting protein, linking Rho both structurally and functionally to myosin phosphatase regulation.[122–124] The possibility of a similar biomechanical link in pericytes is currently under investigation (Surks and Herman, unpublished data).

ADF/Cofilin

An additional force transduction system known to operate downstream of Rho kinase hinges upon phosphorylation-inhibition of members of the actin depolymerizing factor (ADF)/cofilin family, and their control of actin filament disassembly (reviewed by Bamburg[125]). Phosphorylation of ADF/cofilin family members is controlled by the LIM kinases, LIMK1 and LIMK2, of which LIMK1 operates under the control of Rac[126, 127] while LIMK2 is the principal ADF/cofilin effector downstream of Rho and Cdc42.[128–130] Pericyte-specific aspects of the ADF/cofilin cascade remain to be thoroughly characterized.

ERM family proteins

A third mechanism of force transduction known to interact with the Rho GTPase pathway is the ezrin/radixin/moesin (ERM) family of proteins, which governs crosslinking of actin filaments to the plasma membrane (reviewed by Louvet-Vallee[131]). ERM proteins are activated via both Rho kinase-dependent[132] as well as -independent mechanisms.[133, 134] Recent work in our laboratory further describes the actin-specific regulator betacap-73 as a critical bridge between ezrin and the actin network via its association with Rho GTPase effectors (Durham *et al*., 2008; manuscript in preparation).

p38 MAP Kinase

A fourth downstream effector of Rho GTPase via Rho kinase reported in both smooth muscle and endothelial cells is p38 MAP kinase[135, 136], via the stabilization of polymerized actin.[137, 138] p38 MAP kinase has also been shown to contribute to the production of inflammatory cytokines in response to endotoxin exposure in both the myocardium[139] and pulmonary arteries,[140]. In the pulmonary vasculature, this is particularly of interest, as inflammatory cytokines such as TNF-alpha, IL-1, and IL-6 are known to contribute to hypoxic pulmonary

vasoconstriction, possibly through induction of pro-contractile signaling at the smooth muscle, and perhaps pericyte, level.[141–144]

Intracellular signaling via Rho GTPase – Pericyte-specific pathways

The Rho family of small GTPases has long been known to play a role in control of the actin cytoskeleton in many cell types other than vascular smooth muscle.[145] With reference to the above-described pathways, recent work has also demonstrated that similar mechanisms are operative in pericytes. These Rho GTP-dependent (as well as other Rho GTP-independent) pathways function to coordinate pericyte growth and contractile phenotype while simultaneously modulating capillary endothelial function and microvascular tone.

Pericyte-specific assays of Rho activity

Previous work in our lab had indicated that Rho (but not Rac or Cdc42) GTPase signaling modulates pericyte morphology and contractile phenotype in an isoactin-dependent manner.[83] Using a transient transfection system in which cultured pericyte cytoskeletal elements were analyzed differentially based on Rho phenotype, it was shown that expression of dominant-positive Rho fostered specific remodeling of alpha-smooth muscle actin- and myosin-containing stress fiber arrays, whereas nonmuscle actin-containing stress fibers were sustained. These results suggest that, in pericytes, alterations in Rho GTPase control the isoactin-specific regulation of pericyte shape and resultant contractile phenotype. Using adenoviral-mediated delivery of dominant-active and -negative Rho mutants,[61, 146, 147] we have also described two novel assays to quantify both pericyte contractile phenotype as well as pericyte-mediated juxtacrine modulation of endothelial growth state. Results of these experiments not only reveal central parallels between Rho GTP-dependent signaling in vascular smooth muscle and microvacular pericytes; at the same time, there appears to be distinct divergence in Rho GTP-mediated alterations in pericytes as compared to smooth muscle cells. Clearly, additional work will be needed to continue to define the distinct Rho-mediated pathways existing in vascular smooth muscle cells and pericytes and their differential contributions to vascular remodeling during development and disease.

Rho GTPases in pericyte contractility

Based on morphological differences observed in dominant-active RhoA-transfected pericytes compared to control- and dominant-negative RhoA-transfected pericytes previously described,[83] we hypothesized that this phenotypic change reflected a mechanochemical coupling mechanism both in the pericyte cytoplasmic space as well as between pericytes and endothelial cells in the regulation of microvascular tone. In order to dissect the functional aspects of this pathway, we re-implemented methods pioneered by Harris and others[148] to quantify cellular force transduction as a function of Rho activation. Real-time imaging reveals that pericyte deformation of extracellular matrix-treated silicone substrates can be altered by expression of mutant Rho GTPases and treatment with Rho GTP-specific pharmacologic inhibitors (see Figure 3).[61] These results indicate that pericyte contractility is dependent upon and proportional to signaling through Rho and its downstream effectors, in parallel to the known dependence of smooth muscle contraction on Rho GTP-dependent signaling.[149, 150] These findings raise the possibility that the control of microvascular flow, regulation of endothelial cell migration and growth, and the maintenance of capillary permeability are each orchestrated via mechanochemical signal transduction cascades involving Rho GTPase. Thus, whereas the mechanical functionality of Rho signaling has been explored thoroughly in vascular smooth muscle, the importance of Rho GTP-dependent signaling in pericytes is only now emerging.

Rho GTPases in pericyte-mediated endothelial growth arrest

One of the hallmarks of pericyte function in the microvascular milieu is the ability to induce the growth arrest of nearby endothelial cells via both contact dependent- and independent pathways. [6, 15] Contact-dependent pericyte-mediated arrest of endothelial growth was originally described by comparison of side-by-side and porous membrane-separated models of endothelial cell co-culture, in which pericytes and smooth muscle cells (but not fibroblasts or epithelial cells) were noted to inhibit endothelial proliferation.[27] Subsequent work also identified TGF-beta as a soluble, pericyte-derived regulator of endothelial cell migration and proliferation,[28] requiring activation from its latent form for pro-proliferative activity.[78] In addition to endothelial growth arrest, pro-survival signaling via pericyte-derived juxtacrine vascular endothelial growth factor (VEGF) was demonstrated.[76] Taken together, these studies solidify a model in which pericyte investment constitutes a critical stage in angiogenesis, at which migratory, proliferative endothelium transitions into stable, quiescent endothelium.[151]

Building on these initial co-culture models, we developed an assay in which the ability of pericytes to arrest the growth of nearby endothelial cells could be quantified based on pericyte Rho signaling status. In side-by-side pericyte/endothelial co-cultures in which pericyte Rho GTP signaling was modified using mutant Rho GTPase constructs, both over- and under-expression of Rho were associated with distinct morphological changes but uniform inhibition of pericyte-mediated endothelial growth arrest (see Figure 4).[61] These results indicate that pericyte contact-mediated endothelial growth arrest is sensitively dependent on Rho signaling, and is disrupted by altering cellular steady state levels of Rho GTP, since both over- as well as under-expression of Rho GTP could be shown to modulate pericyte-driven control of endothelial cell proliferation.

Indeed, endothelial cell proliferation rate was noted to be more than 20% higher in endothelial cells co-cultured with either dominant-active or -negative Rho-expressing pericytes compared with controls, raising the possibility of an additional contact-independent soluble mediator that is also Rho GTP-regulated. TGF-beta was initially investigated as a likely candidate, but preliminary experiments indicate that protein levels of both latent and active TGF-beta as well as soluble TGF-beta receptor II are identical in both experimentally manipulated and control cultures (M.E.K., J.T. Durham, and I.M.H., unpublished observations). In fact, a recent study by another group[82] has demonstrated a similar phenomenon in a contact-independent system examining the effects of pericyte-conditioned media on endothelial growth. Here, a TGF-beta-1-specific function-blocking antibody only partially reverses the endothelial growth suppression caused by exposure to pericyte-conditioned media, while heat-treatment of the media completely abrogates the effect. This suggests that additional, currently uncharacterized soluble mediators are likely to exist downstream of pericyte Rho signaling. Intriguingly, the above findings suggest that the Rho GTPase signaling pathway in pericytes involve both an acute, juxtacrine, contact-mediated phase of immediate alteration in contractile tone, as well as a slower-acting, structural phase via regional regulation of angiogenesis by soluble mediators.

Rho signaling in microvascular tone dysregulation

In addition to the role of disrupted endothelial control in vasoproliferative disease, microvascular signaling dysregulation is emerging as a causative factor in the pathogenesis of several non-proliferative vascular pathologies as well. Based on the initial understanding of Rho family GTPase signaling in the regulation of smooth muscle contractility,[92] a key role for Rho signaling through Rho kinase has recently been elucidated in both physiological maintenance[89] and pathological dysregulation[88, 90] of the cardiovascular system. In particular, dysregulated Rho kinase signaling in vascular smooth muscle is being demonstrated and actively explored in animal models of cerebral vasospasm,[152, 153] stroke,[103] coronary

vasospasm,[154, 155] post-myocardial infarction ventricular remodeling[156] and heart failure,[157] atherosclerosis,[158, 159] and oxidative stress.[160, 161] Investigation of Rho kinase signaling in endothelial cells is also producing insight into the processes of atherogenesis,[162] cardiovascular protection,[163] and endothelial damage in diabetes.[164, 165] In the following paragraphs, we offer evidence in support of the notion that, in addition to the tonal dysregulation present at the smooth muscle/arterial level, parallel but distinct regulatory pathways exist at the level of the microvascular pericyte, which are relevant to the pathophysiology underlying hypertension.

Pulmonary hypertension

The pulmonary vasculature represents one of the most dynamically flow-responsive vascular beds known. Physiologically, the lung must accommodate the sudden, wide fluctuations in cardiac output required to balance varying oxygen demand and systemic vascular resistance, while simultaneously protecting alveoli from unpredictable changes in arterial pressure. Intricate mechanisms to control local vasomotor tone and capillary recruitment have evolved in order to translate fluctuating pulmonary arterial pressure into constant, predictable alveolar capillary pressure. Both pulmonary artery vasoconstriction and vascular remodeling are key components of the pathology underlying failure of these mechanisms and resultant pulmonary arterial hypertension; see recent extensive reviews by Mandegar,[166] Morrell,[167] and Pak.[168] Reduced endothelial-derived nitric oxide production has been implicated in this vasomotor dysfunction, and *in vitro* work in human pulmonary artery endothelial cells suggests that the hypoxia-induced downregulation of endothelial nitric oxide synthase is mediated by Rho signaling through Rho kinase.[169] Furthermore, several studies have shown that both the acute vasoconstriction and the chronic vascular remodeling associated with hypoxia are attenuated by Rho kinase inhibition.[170–173] These recent studies inform and reinforce the importance of original ultrastructural studies in human and animal lung, which revealed both increased medial thickness and extent of the muscular wall in the pulmonary arterial tree during chronic hypoxia, involving both smooth muscle cells and pericytes.[174, 175] In fact, recent *in vitro* evidence suggests that part of the morphological vascular change observed during chronic hypoxia may be due to transdifferentiation of endothelial cells into a smooth muscle-like phenotype.[176] Indeed, the role of Rho signaling in the pathology of pulmonary arterial hypertension currently represents an exciting and active research arena, with prospects for the elucidation of pericyte- versus smooth muscle-dependent mechanisms.

Essential hypertension

Rho kinase plays a key role in the pathogenesis of essential hypertension and its attendant microvascular remodeling (recently reviewed by Lee *et al*.[91] and Loirand *et al*.[90]) The calcium-dependent dynamic balance between ligand-activated contractility[177] and nitric oxide-mediated relaxation[178] in the determination of vascular tone appears to be mediated by Rho kinase at the level of smooth muscle-invested arteries.[179–182] In parallel, emerging data from human patients indicates that inhibition of the Rho kinase pathway can correct the peripheral vascular tone dysregulation present in heart failure,[183] coronary artery disease,[184] and even in cigarette smoking,[160] while having minimal effects on tone in normal controls, pointing to the Rho kinase pathway as a promising therapeutic target in vascular disease. Many of these therapeutic uses of Rho antagonists have been shown using *in vitro* assays of smooth muscle function or in whole-animal and human studies. However, while *in vitro* assays specifically demonstrate the role of smooth muscle in arterial and arteriolar tension, whole-animal models (for example, mouse models of hypertension) may incorporate additional microvascular- and, notably, pericyte-specific effects of Rho inhibition at the capillary and post-capillary levels that have so far been attributed solely to smooth muscle. It will be important to further clarify the role of pericytes in the global pathophysiology of tone

dysregulation in order to develop potential new avenues for pericyte-specific therapeutic intervention in systemic hypertension.

Specific roles for pericytes in microvascular tone regulation

Although systemic arterial pressure is the principal parameter used to monitor and study essential hypertension, cellular-level metabolic exchange principally occurs under conditions of microvascular capillary flow. In addition to protective mechanisms at the arterial and pre-capillary arteriolar level shielding the capillary bed from both extreme fluctuations in pressure as well as consistent hypertension, the microvasculature appears to have additional complementary regulatory mechanisms to control tone and flow on a local level. Important components of the real-time, dynamic regulation of such vascular beds as the central nervous system and retinal vasculature, for example, may rest with Rho GTP-dependent signaling in microvascular pericytes. In the central nervous system, control of vascular tone is exquisitely sensitive to metabolic demands.[185–187] Indeed, within the cerebellum, functional imaging studies have shown that *in vivo* moment-to-moment local control of demand-induced functional hyperemia is mediated by neuronal nitric oxide synthase.[188–193] Inhibition of Rho kinase signaling by nitric oxide has been demonstrated in the regulation of extracranial arterial tone as well,[181] and *in situ* hybridization and immunohistochemical evidence indicate the extensive presence of Rho and Rho kinase in cerebellar tissue.[194–196] Altogether, these findings suggest that Rho kinase may regulate cerebellar functional hyperemia at some level. Additionally, a role for Rho kinase has been demonstrated in the hypertensive brainstem, including likely novel vasodilation-independent effects of Rho kinase activation in the regulation of the sympathetic nervous system,[197–199] suggesting that Rho kinase may underlie both vasogenic as well as neurogenic mechanisms of hypertension. Interestingly, early comparative studies on the cerebral microvasculature in spontaneously hypertensive rats versus normotensive Wistar-Kyoto rats revealed two- to five-fold increases in pericyte investment of endothelial cells as well as loss of normal stress fiber distribution in pericytes associated with the hypertensive microvasculature both *in situ* and in cell culture.[200, 201] This work suggests that Rho kinase-mediated pericyte contractility may be a novel means of local tone regulation, particularly in the pericyte-rich cerebral microvasculature.

Parallel evidence from the retinal microvasculature supports and extends the hypothesis that Rho kinase signaling in pericytes may play a principle role in tone regulation in other capillary beds as well. Calcium-dependent chloride channel activation downstream of multiple vasoactive ligands mediates pericyte contractility in the retina,[202–206] in which nitric oxide has again been shown to counterbalance ligand-mediated contraction by promoting pericyte relaxation.[207, 208] The retina is a particularly elegant microvascular bed for the investigation of pericyte-autonomous control of local microvascular tone, as it is one of the most densely pericyte-invested vascular beds in the human body[209] and lacks the smooth muscle precapillary sphincters that play a regulatory role in many other vascular beds.[210] Careful dissection of smooth muscle signaling pathways underlying tone dysregulation in the arterial system of both spontaneously hypertensive as well as angiotensin II-treated rodent models of hypertension implicate activation of Rho kinase downstream of angiotensin receptor-1[211–214] in smooth muscle contraction; this contractile impulse appears to be dynamically balanced by the countervailing Rho kinase-mediated release of nitric oxide from adjacent endothelial cells,[215–217] suggesting cell-type specificity in the downstream signaling of Rho kinase. Recent work from our laboratory highlights the role of signaling via Rho kinase in the regulation of pericyte contractility as well as both pericyte contact-dependent and -independent control of endothelial growth state *in vitro*.[61] Specifically, activation of Rho signaling in pericytes was seen to cause a hypercontractile phenotype concomitant with a failure to growth arrest nearby endothelial cells, demonstrating failure of the mechanochemical feedback which normally regulates microvascular tone (see Figure 3 and 4). Indeed, Rho and the Rho kinase

pathway may play a parallel role to that of smooth muscle regulation at the arteriolar level by controlling pericyte contractility at the level of the capillary bed *in vivo*.

Interestingly, recent results indicate that the pericyte may in fact be of previously unrecognized importance not only at the local capillary bed level, but as a mediator of the systemic effects of microvascular tone dysregulation. In both cerebellar cortex and retina, as well as in contracting striated muscle, increased oxygen demand initiates a vasodilatory response in nearby capillary segments that propagates through a gap junctional network of capillary endothelial cell-associated pericytes, extending far enough to dilate proximally-located precapillary arterioles.[218–222] This work implies that regulation of vascular tone in some capillary beds may in fact originate locally at the level of the capillary-associated pericyte, with subsequent feedback conducted upstream to proximal arteriole-associated smooth muscle cells. This intriguing possibility suggests that some elements of vascular tone regulation previously attributed to smooth muscle alone may, in fact, be pericyte-mediated. If this is so, further investigation into the regulation of microvascular tone and endothelial cell function may elucidate novel capillary-level signaling mechanisms, orchestrated by the activity of multiple perivascular cell types.

Conclusions and future directions

Recent work indicates that Rho GTPase and its downstream signaling cascade, previously thought to be confined to vascular smooth muscle cells, has been functionally extended to include the microvascular pericyte. Although the bulk of current literature has investigated the specific nuances of vascular smooth muscle cell-associated and Rho GTPase-dependent signaling, interest is currently developing in the exploration of parallel but distinct roles for Rho signaling in the microvascular pericyte. This body of work proceeds from the first suggestion of independent mechanisms of regulation between arterial and capillary tone, framed in August Krogh's 1920 Nobel Prize acceptance speech, initially implicating 'Rouget cells' (now known as pericytes) as specific regulators of capillary contractility.[223] Subsequent work has described critical components of microvascular cross-talk between pericytes and endothelial cells involving soluble, matrix-mediated, and direct contact-mediated pathways. These interactions have been further shown to dynamically regulate the physiology of vascular development, capillary permeability, and microvascular tone regulation as well as the pathophysiology accompanying tumor angiogenesis, macular degeneration, and diabetic neuropathy. A fruitful area of recent inquiry has emerged in elucidating the role of Rho signaling in pulmonary and systemic hypertension, with potential therapeutic uses in multiple human pulmonary and cardiovascular disease states. While much of this work has focused on Rho signaling in smooth muscle cells, we postulate that Rho signaling in pericytes plays parallel but unique roles in the regulation of microvascular tone – particularly within such pericyte-rich vascular beds as the central nervous system, the retinal microcirculation, and striated muscle – via capillary-autonomous dynamic fluctuations in vascular tone above and beyond precapillary arteriolar contractility. Indeed, we predict that a deepened understanding of the detailed mechanisms of dynamic capillary-level regulation of tone will likely complement and significantly extend current models of end-organ damage in systemic hypertension. In turn, this would present a new avenue for novel drug discovery and the hopeful creation of innovative therapeutic modalities.

In the pursuit of understanding the dynamics of Rho signaling in pericytes, several challenges exist. Signaling mediators specifically linking Rho and Rho kinase with the pericyte actin cytoskeleton must be carefully elucidated. And, while several *in vitro* models exist for the fine dissection of these pathways in pericyte contractility, cell-to-cell signaling, and pericyte-endothelial interactions, the nature of biochemical cross-talk between the various microvascular cell types involved must be carefully dissected. To this end, the

mechanochemical signaling that links contractile force production with respect to matrix and neighboring cells must also be examined; this will likely require innovative three-dimensional systems and interdisciplinary investigation of the biophysics principles involved governing this dynamic reciprocity. As well, *in vivo* systems for the examination and elucidation of pericyte-specific, Rho GTP-dependent signaling are also needed. In this way, the dynamic and specific contributions of pericytes and pericyte-dependent signal transduction can be dissected and characterized *in situ*. We look forward to the next decades of microvascular research, which should help to unveil the regulatory roles that pericytes play in the global control of vascular dynamics.

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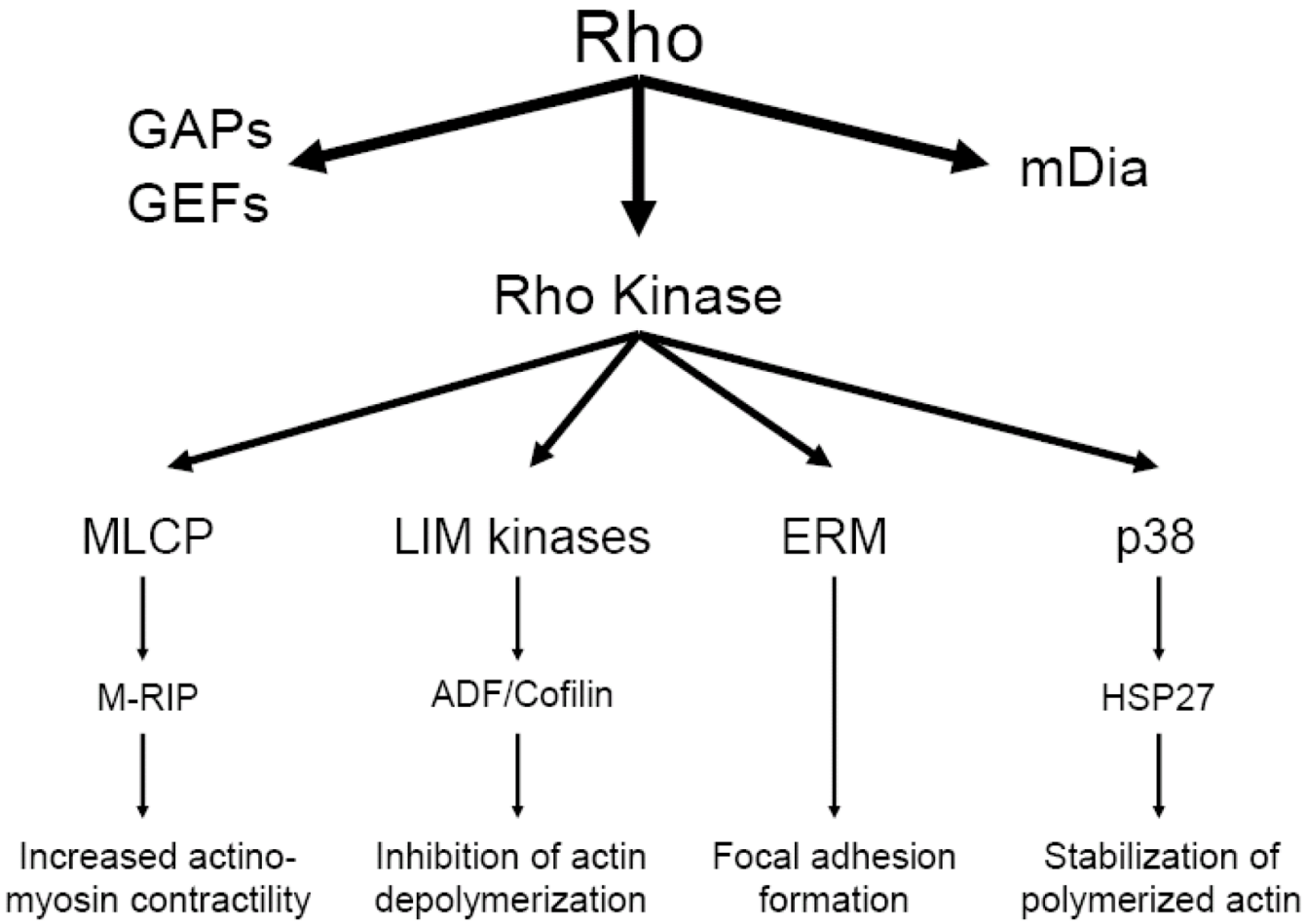

Figure 1. The Rho GTPase – Rho kinase signaling pathway in vascular smooth muscle

The Rho GTPase mechanochemical signaling apparatus links transmembrane and intracellular signaling-associated Rho GTPase with functional alterations in the actin cytoskeleton. Rho kinase, a principal downstream effector of Rho GTPase, mediates linkage to the actin cytoskeleton via four principle pathways discussed in the text: myosin light chain phosphatase (MLCP), ADF/cofilin, ERM proteins, and p38 MAP kinase.

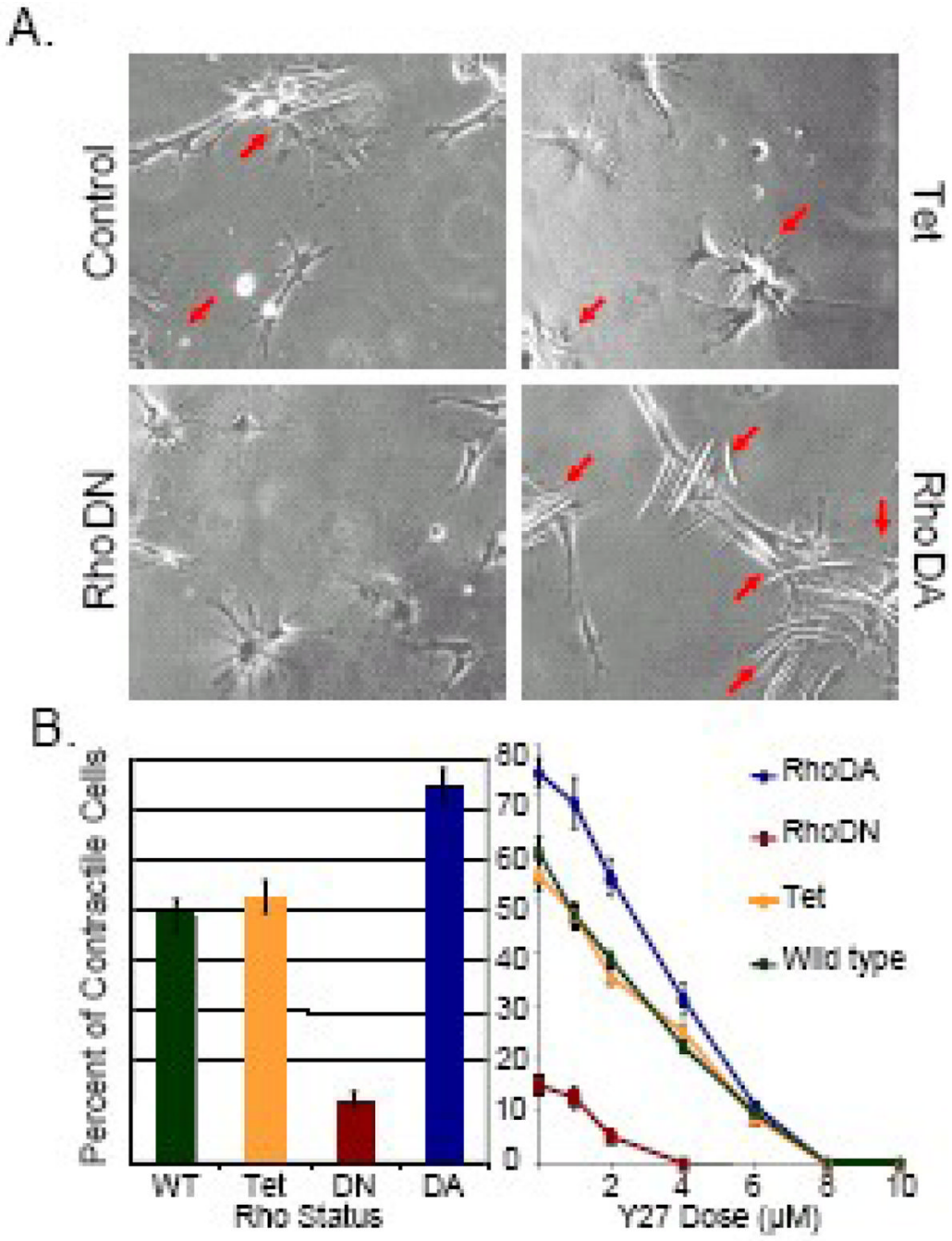

Figure 2. Altered Rho GTPase signaling modulates pericyte contractile phenotype

Bovine retinal pericytes were transduced with dominant-active (RhoDA) or dominant-negative (RhoDN) Rho GTPase-expressing adenovirus, tetracycline-repressible transactivator (Tet)-expressing adenovirus, or were mock-infected (Control). Pericytes were then re-plated onto plasma glow discharge-prepared, Type I collagen-coated silicon substrates containing 0–10 μM of the small molecule Rho kinase inhibitor Y-27632, and monitored by real-time phase contrast imaging. *A:* Representative images are provided in as labeled, where arrows indicate substrate-wrinkling, actively contractile pericytes; images are 400x. *B:* After 24 hours, force transduction was quantified as the percentage of cells capable of producing substrate-

deforming contractile force sufficient to produce substrate wrinkling visible by phase-contrast microscopy.

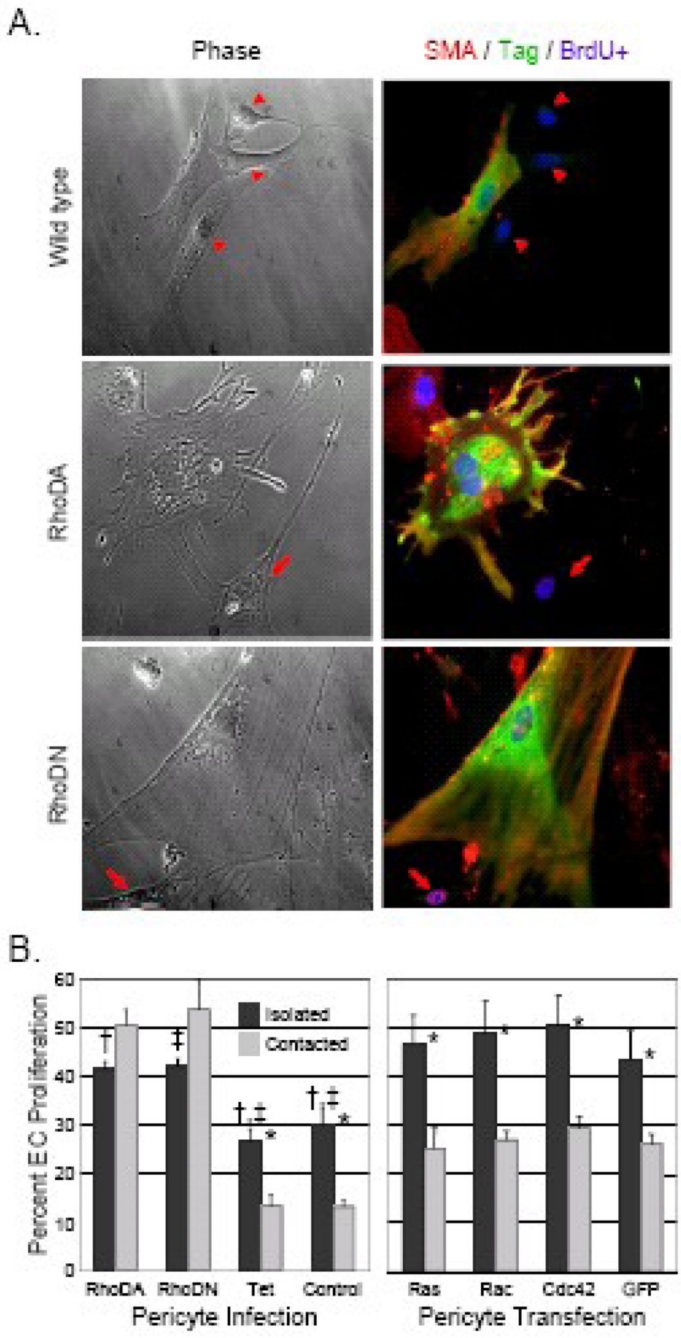

Figure 3. Alteration of pericyte Rho (but not Ras, Rac1, or Cdc42) GTPase signaling impedes pericyte-mediated endothelial cell growth arrest

Bovine retinal pericytes were either transfected with plasmids (*A*) or infected with adenoviruses (*B*) containing dominant-active Rho GTPase (RhoDA) or dominant-negative Rho GTPase (RhoDN). Mock-transfected (Wild type) as well as tetracycline transactivator-infected (Tet) and mock-infected (Control) pericytes were used as controls as labeled. Rho-altered pericytes were then co-cultured with bovine retinal endothelial cells for 24 hours. BrdU was incorporated into the co-culture medium for the last 4 hours. *A*: Altered Rho-transfected pericyte co-cultures were then fixed and stained for the pericyte marker cytoplasmic alpha-smooth muscle actin and proliferative endothelial cell nuclear BrdU incorporation (αSMA + BrdU: red), the Myc

epitope tag as a marker of transfection (Myc: green), and nuclei by Hoechst (Hoechst: blue). Parallel phase image are provided. Images are 200x. *Arrows:* BrdU-positive, pericyte-contacting proliferating endothelial cell. *Arrowheads:* BrdU-negative, pericyte-contacting quiescent endothelial cell. *B:* Adenoviral Rho GTPase-altered pericytes as well as dominant-active Ras, Rac1, Cdc42, and GTP control-transfected pericytes, were co-cultured for 24 hours with bovine retinal endothelial cells as above. Co-cultures were then scored for nuclear BrdU-positive endothelial proliferation as a function of pericyte contact and GTPase status. Results are expressed as mean percentages ± standard error ($n > 400$ cells/condition, experiments in triplicate). *: $p < 0.05$ for differences between lone and pericyte-contacting endothelial cells with same GTPase status; †, ‡: $p < 0.05$ for differences between lone endothelial cell populations with different Rho GTPase status.

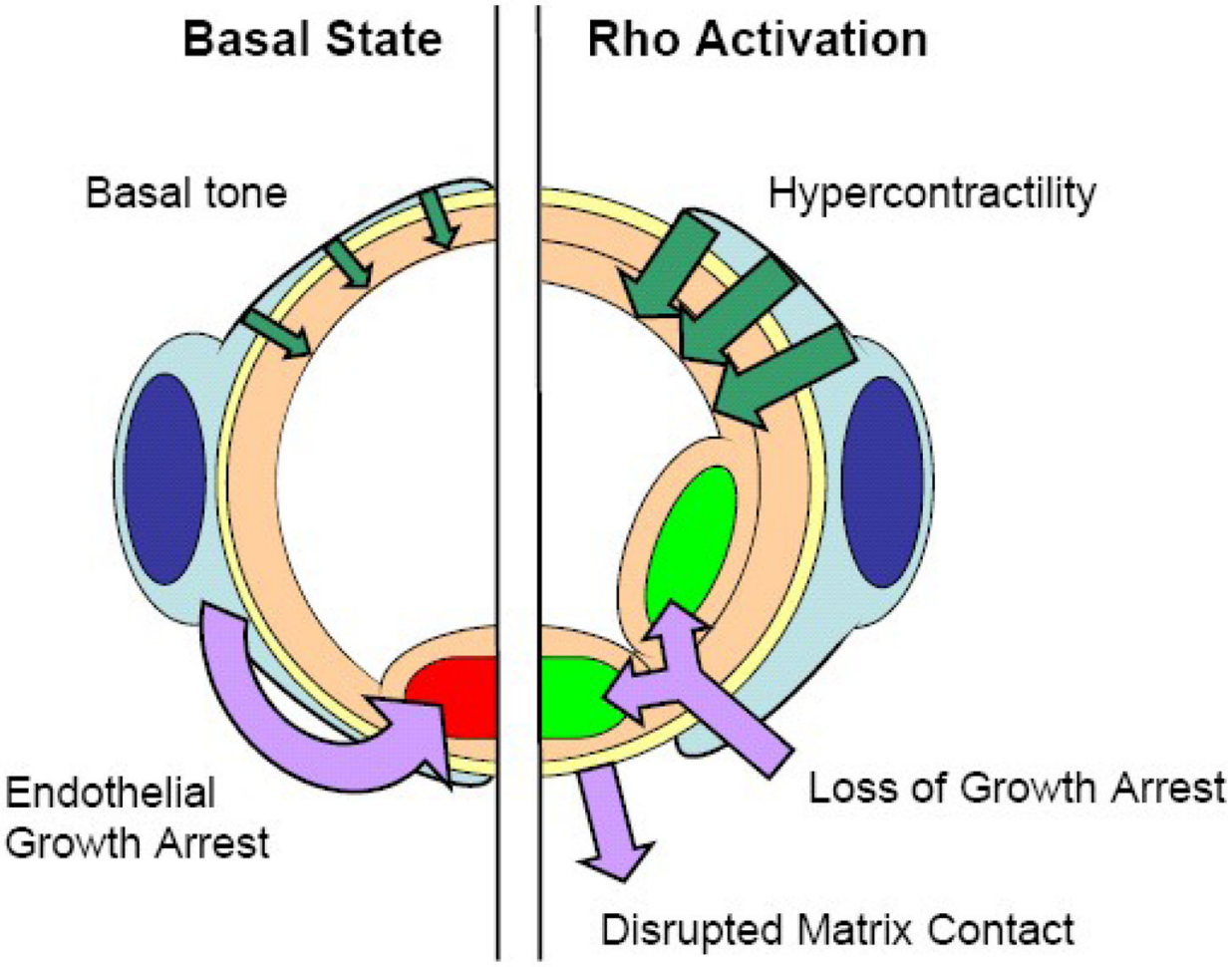

Figure 4. Diagrammatic representation of downstream changes of Rho activation in microvascular pericytes

Rho activation causes increased pericyte contractility, loss of endothelial contact-mediated growth arrest, and disruption of cell-matrix interactions. On the level of the microvascular bed, pathological activation of Rho may lead to microvascular hypertension, pathological angiogenesis, and increased capillary permeability.