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Pericytes, microvasular dysfunction and chronic rejection

Malgorzata Kloc[1], **Jacek Z. Kubiak**[2], **Xian C. Li**[1], and **Rafik M. Ghobrial**[1]

[1]The Houston Methodist Research Institute, Houston TX, USA

[2]Centre National de la Recherche Scientifique Unités Mixtes de Recherche 6290, Institute of Genetics and Development of Rennes, Cell Cycle Group, University of Rennes, Faculty of Medicine, and University of Rennes 1, Faculty of Medicine, Rennes, France

Abstract

Chronic rejection of transplanted organs remains the main obstacle in the long-term success of organ transplantation. Thus, there is a persistent quest for development of anti-chronic rejection therapies and identification of novel molecular and cellular targets. One of the potential targets is the pericytes, the mural cells of microvessels, which regulate microvascular permeability, development and maturation by controlling endothelial cell functions and regulating tissue fibrosis and inflammatory response. In this review we discuss the potential of targeting pericytes in development of microvasular dysfunction and the molecular pathways involved in regulation of pericyte activities for anti-chronic rejection intervention.

Keywords

pericytes; chronic rejection; microvasculature

Introduction

The chronic rejection of transplanted organs represents major hurdle for their long-term function and survival. The symptoms of chronic rejection include accelerated graft atherosclerosis (narrowing and eventual occlusion of the arteries), extensive fibrosis, which obliterates the architecture and function of the organ, and damage to the microvasculature. Clinical and rodent model studies indicate that functional microvasculature is required for the effectiveness of immunosuppressive therapies (1, 2) and that microvascular loss or dysfunction leading to local ischemia may be an important factor in development of tissue fibrosis and an underlying cause of chronic rejection (2–6). Recent studies indicate that adenovirus-mediated hypoxia-inducible factor-1 (HIF-1) gene transfer therapy, which

Address for Correspondence: Malgorzata Kloc or Rafik M. Ghobrial, The Methodist Hospital, Department of Surgery, 6550 Fannin St., Houston, TX 77030, Tel. 713.441.6875, Fax 713.790.3755, mkloc@tmhs.org, RMGhobrial@tmhs.org.

Authors' Contribution:

Malgorzata Kloc - Concept, manuscript writing, figures drawing, mkloc@houstonmethodist.org
Rafik M. Ghobrial - manuscript co-writing, RMGhobrial@houstonmethodist.org
XC Li- manuscript co-writing, XCLi@HoustonMethodist.org
J.Z. Kubiak - manuscript co-writing, jacek.kubiak@univ-rennes1.fr

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through the induction of pro-angiogenic growth factors enhanced microvasculature repair, promotes allograft integrity and alleviates chronic rejection of mouse lung allografts (7). Because, at present, there is no available clinical therapy for chronic rejection, the scientific, medical and pharmaceutical research entities are in relentless pursuit of novel cellular and molecular targets in order to design preventive drugs. In past decades the majority of research in the field has been focused on the most obvious culprits in development of chronic rejection i.e. the immune cells and their regulatory molecules and effectors. However, recently, there are increasing numbers of studies pointing toward greatly underappreciated candidate, the pericytes and the role they play in microvascular dysfunction, fibrosis, inflammation and development of chronic rejection.

1. Pericyte localization, structure and markers

The pericytes (also called the Rouget cells) were discovered in 1871 by German pathologist and bacteriologist C. J. Eberth (8) and subsequently described by Rouget (9) and Zimmerman (10). The pericytes envelop the endothelial wall of the microvessels: capillaries, post-capillary venules, venules and arterioles, and are responsible for microvessel integrity and regulation of blood flow (11,12). The wall of microvessels is built of a single layer of endothelial cells embedded within the basement membrane (which *de facto* is not a membrane but a thin sheet of collagen, laminin, fibronectin and heparan sulfate fibers), (13, 14), which endothelial cells co-produce and share with the pericytes (Fig. 1; 15). Pericytes have a large, round nucleus, small amount of cytoplasm and long cytoplasmic processes, which embrace endothelial wall of the vessel and may extend to the neighboring vessels. Pericytes are connected to the endothelial cells by three major types of intercellular junctions: *1. the peg-and-socket* contacts, which are fingerlike intrusions/protrusions between cells (Fig. 1) in the areas of the low expression regions (LERs) of matrix proteins in the basement membrane; *2. the gap junctions*, which form at the peg-socket contacts (Fig. 1) and allow direct chemical communication between the cytoplasm of neighboring cells through the diffusion of ions and various molecules, and *3*. the spot–like adherence junctions called *the adhesion plaques*, which are connected to actin filament bundles and attach cells to each other and to the extracellular matrix (basement membrane) (Fig.1; 14, 16, 17). The density of pericyte coverage (endothelial cell to pericyte ratio) ranges between 1:1 (in brain) and 10:1 (in muscle) and seems to correlate positively with the microvessel permeability barrier requirement within the particular tissue (the tighter the barrier the higher pericyte density). This ratio also depends on the dynamics of endothelium renewal and vertical topography (and corresponding blood pressure) of the microvessels within the body (12, 18).

The pericytes develop during embryogenesis from mesenchymal cells present within the differentiating tissues induced by contact with the endothelial tubes of locally forming microvessels. Local differences in cellular environments within tissues and organs may explain the functional variance and tissue specificity between pericytes belonging to different microvessels (16). Recent studies indicate that during vascular development in the perinatal mouse heart the vessel endothelium initiates the pericyte ensheathment through brain-derived neurotrophic factor BDNF/ neurotrophic tyrosine kinase TrkB signaling, which is sensitive, in turn, to the small GTPase RhoA/ROCK kinase inhibitor Y-27632 (Fig. 2), (19).

Pericytes are not only involved in *de novo* formation of microvessels (microvasculogenesis) within differentiating tissues but also in microvessel angiogenesis (sprouting from the preexisting vessels) within fully differentiated tissues and organs. Microvessel angiogenesis starts with the formation of primitive capillary tube (angiogenic sprout) derived from proliferating and migrating endothelial cells, which produce immature basement membrane. Subsequently, endothelial cells recruit the pericytes via fibroblast growth factor (FGF-2), platelet-derived growth factor (PDGF), heparin binding epidermal growth factor (HB-EGF) and Interleukin-6 signaling (20–22). Upon contact with the epithelium, the pericytes suppress endothelial cell proliferation and migration, stabilize the vessel and co-participate in maturation of the basement membrane of the microvessel wall. In turn, the contact with endothelial cells triggers synthesis of contractile proteins in the pericytes (14, 23, 24).

One of the major challenges in pericyte research is the lack of an unequivocal pericyte - specific marker. So far, all molecules, which have been found to be expressed by pericytes are also present in various other cell types. The most common markers used to identify pericytes are described below. **Alpha-actin-2 (ACTA2)** also called the smooth muscle or aortic smooth muscle actin (α-SMA, SMactin, alpha-SM-actin, ASMA) is one of six different actin isoforms involved in cell structure, contractility and motility. The expression level of α-SMA in pericytes is regulated *in vitro* and *in vivo* by various growth factors (25, 26). α-SMA is expressed not only in pericytes (27–34) but also in smooth muscle cells, myofibroblasts, monocytes, macrophages and cardiac microvascular endothelial cells (35–37).

Desmin, a type III intermediate filament required for mechanical elasticity of contractile cells (38) expressed in pericytes (33), is also present in skeletal and smooth muscle cells (39). **Chondroitin sulfate proteoglycan (nerve/glia antigen-2/ NG2)** also called chondroitin sulfate proteoglycan 4, melanoma chondroitin sulfate proteoglycan and melanoma-associated chondroitin sulfate proteoglycan has multiple signaling and regulatory functions (40) including recruitment of small GTPases (41). In addition to presence in pericytes (42, 43) NG2 is also expressed in neural progenitor cells, human melanoma cells and stem cells (44–46). Murfee et al. (47) analyzed NG2 and α-SMA co-expression in pericytes along the microvessel walls within rat mesenteric tissue, subcutaneous tissue, spinotrapezius muscle, and gracilis muscle. They found that NG2/ α-SMA co-expression was restricted to perivascular cells along arterioles and capillaries but it was absent in the pericytes of venules. This led to the identification of two distinct pericyte subsets: $NG2^{+}$α-SMA^{+} pericytes found along arterioles and capillaries and $NG2^{-}$α-SMA^{+} pericytes located along postcapillary venules (47). Further studies using confocal intravital microscopy in inflamed tissues showed that these two subsets of pericytes have two distinct functions in interstitial leukocyte migration: $NG2^{-}$α-SMA^{+} pericytes facilitate transmigration of neutrophils from post capillary venules (26, 48, 49) and $NG2^{+}$α-SMA^{+} pericytes control migration of leukocytes that had exited from the venules (26 49). **Aminopeptidase N (ANPEP)** also known as APN, CD13 and leukocyte surface antigen gp150, is a cell-membrane metalloprotease involved in metabolism of regulatory peptides and regulation of angiogenesis (50). ANPEP is present in pericytes, epithelial cells, macrophages, granulocytes, synaptic membranes of neural system and stem cells (51). **Platelet-derived**

growth factor receptor beta (PDGFβ), a tyrosine kinase receptor that regulates cell proliferation, growth and differentiation plays a role in the recruitment of pericytes during angiogenesis (52, 53). In addition to its presence in pericytes, it is also expressed by smooth muscle cells, mesenchymal cells and neuronal progenitors. Additional pericyte markers are listed in reference 12 and 54 (although some of these markers still need verification).

In summary, positive identification of pericytes, their subtypes and diverse functions may rely on the multiple criteria, such as morphology, localization and co-expression of several different pericyte markers.

2. Pericyte Stemness/Pluripotency/Transdifferentiation

Research on pericyte functions in various tissues, organs and model systems suggests that they are highly versatile, functionally pluripotent cells with a great phenotypic plasticity. Pericytes express pluripotency and stem cell markers (55–57). Depending on their tissue/organ-specific location and the specificity of signaling they receive from their surroundings, they exhibit diverse activities, ranging from structural to progenitor-cell like phenotypes and functions. Although pericytes cultured *in vitro* can differentiate into neural cells, smooth and skeletal muscle cells, adipocytes, chondrocytes and osteoblasts (56, 58–60), the lack of unequivocal pericyte-specific markers hinders the ability to follow their pluripotency, differentiation and ultimate fate *in vivo*. One of the more successful approaches utilized a transgenic approach combined with kinetic modeling microscopy in murine system enabling the ability to track the fate of fluorescent-labeled pericytes *in vivo*. These studies (61, 62) demonstrated that pericytes are able to differentiate into collagen-producing myofibroblasts and participate in the development of fibrosis (see below).

3. Role of Pericytes in Fibrosis

Pathogenic fibrosis, which occurs during chronic tissue injury is characterized by profound remodeling and excessive production and deposition of fibrillar extracellular matrix (ECM) containing collagen types I, III and IV, fibronectin, laminins, heparan sulfate proteoglycans and glycosaminoglycans (63, 64). Fibrosis results in disruption of tissue architecture and proper microrperfusion leading to organ failure. It is well established that myofibroblasts are the major ECM-producing cells during inflammation and organ injury. Myofibroblasts develop from myofibroblast progenitors (derived from either resident mesenchymal cells, fibroblasts, fibrocytes, bone marrow-derived cells, epithelial cells or endothelial cells) after they had become activated by pro-fibrotic cytokines and growth factors secreted by lymphocytes upon injury of the endothelium (12, 63, 65, 66). However, recent studies indicate that the ECM-producing myofibroblasts can originate almost exclusively from microvascullar pericytes (61, 67). Lin et al. (61) analyzed the origin of collagen type I, α1 (coll11α1), chondroitin sulfate proteoglycan (NG2 marker) and αSMA-expressing cells in the kidney of transgenic (coll11α1-GFP) mice. Using kinetic modeling microscopy, they showed that kidney injury induces the pericytes to detach from the microvessels and differentiate into the collagen producing-myofibroblasts, the major source of fibrotic ECM. Similarly, detachment of pericytes (labeled with R26R-yellow fluorescent protein) and their contribution to fibrosis was shown in spinal cord injury mouse model (62). Studies of

fibrogenesis in kidney, liver and systemic sclerosis also point to the pericytes as the precursors of myofibroblasts (68 69, 70, 71). It is known that fibrosis depends on activation of c-Jun NH2-terminal kinase (JNK) (72, 73) and the TGFβ/Smad pathway (74, 75). Studies show that there is also crosstalk between the RhoA/ ROCK and JNK and TGFβ/Smad pathways (76, 77) and that inhibition of RhoA kinase (ROCK) by fasudil hydrochloride alleviates myocardial fibrosis and production of type-I and type-III collagen in diabetic rats via inhibition of JNK and TGFβ/Smad (Fig. 2; 77). Although further studies are needed to show how this relates to the collagen-deposition function of pericytes, it is plausible that fibrosis-related pericyte functions may be a potential target for novel anti-chronic rejection therapies (78).

4. Pericyte Contractility and blood flow facilitation

Since their initial characterization as " the contractile cells" or "the microvascular smooth muscle cells" the ability of pericytes to contract and regulate blood flow within capillaries remained controversial for many decades (29, 79, 80). Some studies suggest that pericytes are noncontractile, however through their transformation into smooth muscle cells (34, 81, 82) they progressively, through the intermediate phenotypes, acquire an ultimate contractile phenotype. However, many studies showed that in addition to noncontractile proteins the pericytes also contain several contractile proteins such as smooth muscle- and nonmuscle-specific isoforms of actin, myosin, tropomyosin (31,33, 83–85) and a cyclic guanosine monophosphate (cGMP)-dependent protein kinase that regulates muscle contraction (86). Recent real-time studies of pericytes and dynamics of capillary diameter in mice cortex using intra vital two-photon laser scanning microscopy showed unequivocally that pericytes are contractile and are able to modulate capillary blood flow in the brain (87). The contraction of smooth muscle cells is regulated by the RhoA/Rock signaling pathway, which is a master regulator of actin-related cell functions (88, 89). Recent studies indicate that this is also true for the pericytes and that treatment with the RhoA pathway inhibitor Y-27632 or expression of a dominant-negative or dominant-active Rho severely alters pericyte contractility via interference with their actin filaments (Fig. 2); (23, 90, 91). Interestingly, studies from our laboratory show that the Y-27632 inhibitor abrogates chronic rejection via interference with actin cytoskeleton in rat and mouse cardiac allograft model system (92–95), and our recent (unpublished) data indicate that inhibition of chronic rejection prevents alteration of pericyte shape in the microvessels of transplanted hearts. This may indicate that abrogation of chronic rejection by RhoA pathway inhibitor may depend, at least partially, on the restoration of functions controlled by pericytes such as regulation of blood flow and/or microvasculature permeability.

5. Vessel integrity and pericyte-endothelium cross talk

One of the well-recognized functions of pericytes is their role in securing integrity and regulating permeability of microvessel wall. The loss of pericytes, decrease in pericyte coverage or change of their shape during inflammation distort vessel permeability, lead to vascular hyperdilation and hemorrhage (14, 96). Numerous studies indicate that there is an extensive and multilevel network of crosstalk between the endothelial cells and their pericytes. The major and most comprehensively studied crosstalk circuits consist of

transforming growth factor beta (TGFβ), platelet-derived growth factor subunit B (PDGF-B) and the angiopoietin signaling pathways (11, 12 97).

The **TGFβ signaling pathway** consists of TGFβ superfamily of ligands that include: TGFβ (isoforms β1-3), bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), anti-müllerian hormone (AMH), activin and nodal, and their receptors, which belong to two major types: TGFβ R type I and II. The TGFβ pathway signals either through canonical Smad protein signaling or through non-cannonical non-Smad signaling pathways such as ERK, p38 MAPK, Jun N-terminal kinase (JNK), PI3K-Akt and small GTPases including RhoA/ ROCK and Rac pathways (Fig. 2); (98, 99). Interestingly, RhoA also modulates Smads translocation to the nucleus and acts as a modulator of Smad activation in alpha actin expressing smooth muscle cells, thus regulating TGFβ induced cell differentiation (100). Because pericytes are also contractile and produce alpha actin, this suggests that RhoA may also modulate TGFβ signaling in the pericytes. Although the effects of TGFβ pathway signaling are extremely broad, the principle of canonical Smad signaling is quite simple: the ligands of the TGFβ superfamily interact with a type II receptor, which phosphorylates a type I receptor. These receptors then phosphorylate receptor-regulated R-SMADs, which can now bind the common-mediator Smad (coSMAD). Subsequently, the R-SMAD/coSMAD complexes translocate to the nucleus where they act as transcription factors regulating expression of TGFβ/Smad target genes (101, 102). One of the TGFβ superfamily ligands, the TGFβ protein is highly expressed in various cell types including microvessel endothelial cells and pericytes and it is a secreted protein, which controls a plethora of cell functions including differentiation and proliferation. TGFβ is produced in an inactive pro-form bound to the latent TGF-beta binding protein (LTBP) and latency-associated peptide (LAP), and the release of active TGFβ from this complex is catalyzed by proteinases. Latent TGFβ and TGFβ receptors are expressed both by endothelial cells and pericytes and, at least in bovine cultured cells, a direct contact between these two cell types is necessary for the release of the active form of TGFβ (103). Mouse knockouts of various TGFβ pathway genes including smad4, smad5 and activin receptor-like kinase 1 and 5 genes (alk1 and alk5) result in embryonic lethality and profound defects in vasculature and pericyte development (12, 104–107). Studies in brain endothelium-specific smad4 knockout mice show that the canonical TGFβ pathway is involved in endothelial cell proliferation determining the density of pericyte coverage, via expression of adhesion molecule N-cadherin, which mediates endothelium - pericyte contact (12, 108, 109). In addition, both pericytes and endothelial cells express type I TGFβ activin receptors: receptor-like kinase Alk1 and Alk5, which seem to have antagonistic effects on microvessel maturation and development. Alk-1 signaling promotes cell migration and proliferation and Alk-5 regulates extracellular matrix production and vessel stabilization (12, 110).

The Angiopoietin signaling pathway

Angiopoietins (Ang1, Ang2, Ang3, Ang4) and Tie receptor tyrosine kinases (Tie1 and Tie 2) are involved in all steps of angiogenesis (111,112). Pericytes express Ang1, while endothelial cells and some pericyte subtypes express its main receptor Tie-2 (113, 114). Studies in rodent systems showed that Ang1 and Tie-2 deficient mouse embryos do not form pericytes (115,116). Aditionally, the injection of Ang2, which is an antagonist of Tie-2, into

the eyes of normal rats results in a dose-dependent loss of pericytes, while heterozygous Ang2 deficiency prevents diabetes-induced pericyte loss (117). Pfister et al. (118) showed that in a mouse model of diabetic retinopathy, pericyte loss resulted from the overexpression of Ang2 and was completely blocked in Ang-2 deficient mice. However, studies on ang1 conditional knockout, and mice with defective Tie-2 receptors indicate that Ang1/Tie-2 signaling, while critical for regulation of number and diameter of vessels, is dispensable for the pericyte recruitment to newly forming vessels (119, 120). Pericyte detachment from the microvessel wall is not only regulated by Ang /Tie signaling, but probably also by the interactions between focal adhesion kinase (FAK), p21 protein activated kinase (PAK), integrins and the actin–myosin cytoskeleton (65, 118, 121). Ang1/Tie-2 signaling also regulates proliferation and chemotactic migration of endothelial cells during angiogenesis by modulation of RhoA and Rac1-dependent cytoskeletal rearrangements (Fig. 2). Cascone et al. (122) showed that Ang1 modulates the RhoA/Rac1 pathway via phosphoinositide 3-OH kinase/son of sevenless signaling and that chemotactic motility of endothelial cells is drastically reduced upon treatment with phosphoinositide 3-OH kinase inhibitor or in cells carrying dominant-negative mutants of RhoA and Rac1.

PDGF-B pathway signaling

The PDGF family consists of PDGF-A, -B, -C and -D, which bind to the protein tyrosine kinase receptors, PDGF receptor-α and -β. PDGF-B is produced by the endothelium and during angiogenesis is released from endothelial cells binding PDGFRβ on pericytes to recruit them to the vessel sprout. Depending on the organ, PDGF and PDGFRβ deficient mice either partially or completely lack pericytes, while constitutive activation of PDGFRβ promotes pericyte proliferation and inhibits their differentiation (12, 123). On the other hand, over-proliferation of pericytes accompanied by the loss of endothelial cells from the microvessel wall, observed during kidney injury, results from disrupted VEGF/PDGF signaling between the pericytes and endothelium (124). Endothelial cells produce not only PDGF but also the vascular endothelial growth factor (VEGF) receptor 2, while the pericytes produce VEGF in addition to PDGF receptor-β. Lin et al (124) showed that pericyte proliferation accompanied by endothelial cell loss, reduction of capillaries density and development of fibrosis, which occur during mouse kidney injury can be reversed by blocking of VEGF/PDGF signaling using circulating adenovirus-mediated soluble receptors. In addition they showed that during kidney injury pericytes switch the expression of VEGF isomers (from angiogenic VEGF 164 to dysangiogenic VEGF120 and 188). Interestingly, they also showed that this blockade also mitigates recruitment of inflammatory macrophages into injured kidney, which points to an unexpected functional link between microcapillaries, fibrosis and inflammation (65, 124). Using intra vital microscopy in mouse cremaster muscles and an ear skin inflammation model, Wang et al. (125) showed that inflammatory extravasation of the polymorphonuclear neutrophils (PMNs) through the venular wall depends on a direct contact between the PMNs and pericytes. The PMNs induce relaxation of the pericyte cytoskeleton, which results in the formation of spaces between pericytes and enlargement of the low expression regions (LERs) of matrix proteins in the basement membrane. These changes in turn allow PMNs to transverse the vascular wall. The authors also showed that PMNs induced relaxation of the pericyte cytoskeleton is mediated by inhibition of the RhoA/ROCK signaling pathway in pericytes and suggested that pericytes

may be a potential target for anti-inflammatory therapies. Recent studies indicate that, at least in tumor-induced angiogenesis, the regulation of proliferation and differentiation of pericytes, as well as overall vascular permeability involves Semaphorin 4D (SEMAD4D) and its receptor Plexin-B1. This regulates the Rho A pathway through the guanine nucleotide exchange factors leukemia-associated Rho GEF (126) and angiopoetin-like protein 4 (ANGPTL4) (127). SEMA4D induces expression of PDGF and ANGPTL4 in endothelial cells in a Plexin-B1/RhoA-dependent manner (Fig. 2). These and other studies, mentioned above, point to the importance of the RhoA pathway in regulation of proliferation and differentiation of pericytes and vascular permeability. Other pathways recently found to be involved in signaling between pericytes and endothelium include: Notch, sonic hedgehog (Shh), stromal-derived factor 1-a (SDF-1a)/CXCR4 and heparin-binding epidermal growth factor (HB-EGF)/EGF receptors (ErbBs) signaling pathways (12).

6. Pericytes as master sensors of inflammation and instructors of leukocyte proinflammatory and prosurvival programs

Alteration of microvessel integrity may be one of the most important and underappreciated steps leading to tissue ischemia and the cascade of stimulatory changes driving tissue inflammation and development of chronic rejection. It is known that the leakiness of microvasculature signals T cells, macrophages, monocytes, mast cells and fibroblasts to produce cytokines, chemokines and growth factors, which, in turn, switch on and escalate inflammatory response. One of the most crucial steps in the immune response is the penetration (diapedesis, extravasation) of leukocytes through the capillary walls into the surrounding tissues. Over the years a number of studies have explored mutual endothelium-leukocyte interactions and it is well established that the endothelial wall occupancy and eventual breaching of endothelial wall enhances effector functions of extravasating leukocytes. Recent studies indicate that pericytes play a crucial role in endothelial wall breaching, which is a lengthy (15–40 min) and elaborate process (128). Using intravital confocal microscopy to study neutrophil migration from the venules of the inflamed mouse tissues, Proebst et al. (48) showed that after squeezing between endothelial cells and exiting from the endothelial wall the neutrophils crawl (so called abluminal crawling) along pericyte processes and extravasate the venular wall through the enlarged (by inflammation) gaps between the adjacent pericytes (Fig. 3). Leukocyte crawling depends on the pericyte-expressed intercellular adhesion molecule-1 (ICAM-1) and its leukocyte integrin ligands, Mac-1 and LFA-1. The formation and selection of inter-pericyte exit gaps is coordinated by signaling between the proinflammatory cytokines TNF and IL-1β and their receptors expressed by the pericytes (48). The most recent studies indicate an unexpected and quite refine role of capillary wall pericytes in sensing inflammatory cues and directing leukocyte trafficking into the inflammatory foci and also behaving as *per se* immune cells (25, 26, 48, 49). *In vitro* studies of brain-derived pericytes response to artificial inflammation/infection induced by TNF-α, IL-1β, IFN-γ cytokines or LPS stimulation showed that IL-1β induces production of iNOS, RONS (reactive oxygen or nitrogen species) and prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2 or Cox-2). This mechanism is, traditionally, applied by specialized immune cells (such as macrophages) of the innate immune system to kill pathogens at early time points after the infection. On the other hand, the IFN-γ treatment

of pericytes induces phagocytosis and expression of MHCII and macrosialin / CD68 / Gp110 protein, which allows macrophage homing and plays a role in cell-cell and cell-pathogen interactions (i.e. induces mechanisms involved in adaptive immune responses involving antigen presentation) by binding to lectins and selectins. The TNF-α treatment of pericytes induces both types of immune responses (synthesis of iNOS and RONS and the phagocytosis rate increase) in the pericytes (25). These findings suggest that pericytes' ability to behave like the immune cells of the innate and adaptive immune system may contribute to various circuits of immune response. The active role of pericytes in the innate immune response was also shown *in vivo* in a mouse model of sterile inflammation. Using intravital two-photon microscopy Stark et al. (26) demonstrated that in response to inflammation the arteriolar and capillary NG2+ pericytes produce the chemoattractant MIF (macrophage migration inhibitory factor) and upregulate synthesis of the intercellular adhesion molecule, ICAM-1. These molecules allowed pericytes to attract interstitial neutrophils and macrophages after they had extravasated from postcapillary venules and enhanced their scanning and effector functions at the inflammation sites (Fig. 3). Contact with pericytes "tells" leukocytes to express matrix metalloproteinases, β1 integrins, and formyl peptide receptors (FPR, which bind products of cell and tissue degradation). This allows them to acquire prosurvival signals and directionally migrate toward the inflammation source rich in damage-associated molecular pattern molecules (DAMPs) exposed on cell surfaces following tissue injury (26,49). The authors suggest that the role of NG2+ pericytes is to create the migratory roadways for the movement of extravasated immune cells along arterioles and capillaries and to enhance their ability to scan for and respond to tissue damage and inflammation cues (Fig. 3; 26,49). All these studies indicate that pericytes act as sensors of inflammation, effectors of immunosurveillance and serve various functions of immune cells playing an active role in the immune response and a crucial role in development of chronic rejection.

Below we summarize how the pericyte may contribute to chronic rejection and how the available inhibitors of the RhoA pathway may be effective in modulating the chronic rejection-related functions of pericytes (Fig. 4).

Pericyte functions in inflammation and tissue injury leading to development of chronic rejection, which are known (or predicted) to be sensitive to RhoA pathway inhibitors.

1. **Vessel integrity and permeability**
    - Pericyte ensheathment of the vessels is initiated by the vessel endothelium through the BDNF/ TrkB signaling, which in turn is sensitive to small GTPase RhoA/ROCK kinase inhibitor Y-27632 (19)
    - Pericyte coverage and recruitment is regulated by Angiopoietins (Angs) via endothelial cell interaction. The Ang1 modulates RhoA/Rac1 pathway and endothelial cells functions are changed in cells carrying dominant-negative mutant of RhoA (122)
2. **Blood flow**

- The contraction of pericytes is regulated by RhoA/Rock signaling pathway, and a treatment with RhoA pathway inhibitor Y-27632 or expression of a dominant-negative or dominant-active Rho severely alters pericyte contractility via interference with their actin filaments (23, 90, 91).

3. **Leukocyte extravasation and transmigration**

   - Extravasation of the polymorphonucler neutrophils (PMNs) through the venular wall depends on relaxation of pericyte cytoskeleton and is mediated by the RhoA/ROCK signaling pathway (125)

   - Transmigration of neutrophils from post capillary venules and migration of leukocytes that had exited from the venules depends on NG2, which has multiple signaling and regulatory functions one of them being a recruitment of small GTPases (such as RhoA), (41).

4. **Pathogenic fibrosis**

   - Excessive deposition of ECM by myofibroblasts, which originate from microvascular pericytes is regulated by JNK and TGFβ/Smad pathways. There is a crosstalk between RhoA/ ROCK and JNK and TGFβ/Smad pathways and inhibition of RhoA kinase ROCK by Fasudil alleviates myocardial fibrosis (77).

In summary, although the pericytes and their RhoA pathway regulated functions emerge as the major players in development of chronic rejection it remains to be determined whether they can be used as a critical targets of anti-chronic rejection therapies. Most probably the simultaneous inhibition of several molecular and cellular targets will be required to inhibit chronic rejection of transplanted organs.

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Abbreviations

ACTA2	Alfa-actin-2
Alk	Activin receptor-like kinase
AMH	Anti-müllerian hormone
Ang	Angiopoietin
ANPEP	Aminopeptidase N
Akt	Protein kinase B
α-SMA	Alfa smooth muscle actin
BDNF	Brain-derived neurotrophic factor

CD68	Cluster of differentiation 68
cGMP	cyclic guanosine monophosphate
DAMPS	damage-associated molecular pattern molecules
ECM	Extracellular matrix
FGF-2	Fibroblast growth factor
FPR	formyl peptides receptor
GDFs	Growth and differentiation factors
Gp110	Envelope glycoprotein
GSK429286A	inhibitor of ROCK1 and ROCK2
HB-EGF	Heparin binding epidermal growth factor
HIF-1	Hypoxia-inducible factor-1
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon gamma
iNOS	Inducible Nitric oxide synthase
IL-1β	Interlukin 1 beta
JNK	c-Jun NH2-terminal kinase
LAP	Latency-associated peptide
LERs	Low expression regions
LPS	Lipopolysaccharide
LTBP	Latent TGF-beta binding protein
MHCII	Major histocompatibility complex) class II
MIF	Macrophage migration inhibitory factor
NG2	Nerve/glia antigen-2
PDGF	Platelet-derived growth factor
PDGFβ	Platelet-derived growth factor receptor beta
PI3K	Phosphatidylinositde-3 Kinase
PMNs	Polymoprhonuclear neutrophils
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RKI-1447	inhibitor of ROCK1 and ROCK2
ROCK	Rho-associated protein kinase (ROCK1 and ROCK2)
RONS	Receptor tyrosine kinases

SEMAD4D	Semaphorin 4D
SMAD	homolog of both the Drosophila protein, mothers against decapentaplegic (MAD and the Caenorhabditis elegans protein SMA (small body size)
coSMAD	common-mediator Smad
R-SMAD	receptor-regulated Smad
TGFβ	Transforming growth factor beta
Trk	Thyrosine kinase
VEGF	Vascular endothelial growth factor
Y-27632	Selective ROCK1 (p160ROCK) inhibitor

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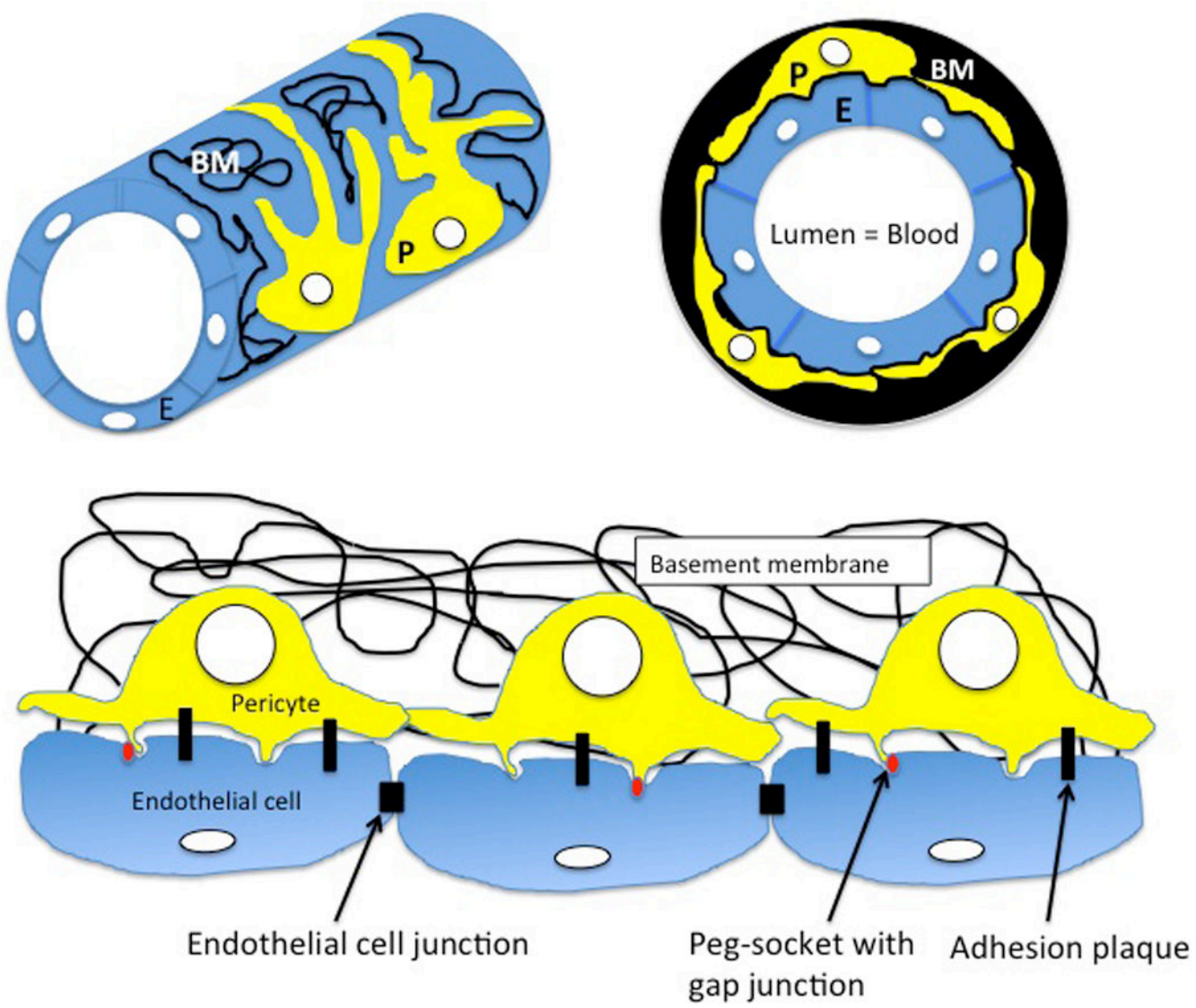

Figure 1. Microvessel structure and pericytes

The microvessels: capillaries, post-capillary venules, venules and arterioles are built of a single layer of endothelial cells (E, blue) and covering pericytes (P, yellow), which are both embedded within fibrous, commonly produced, basement membrane (BM, black). Endothelial cells are inter-connected by cell junctions and are connected to the pericytes by adhesion plaques and peg-sockets with the gap junctions (red).

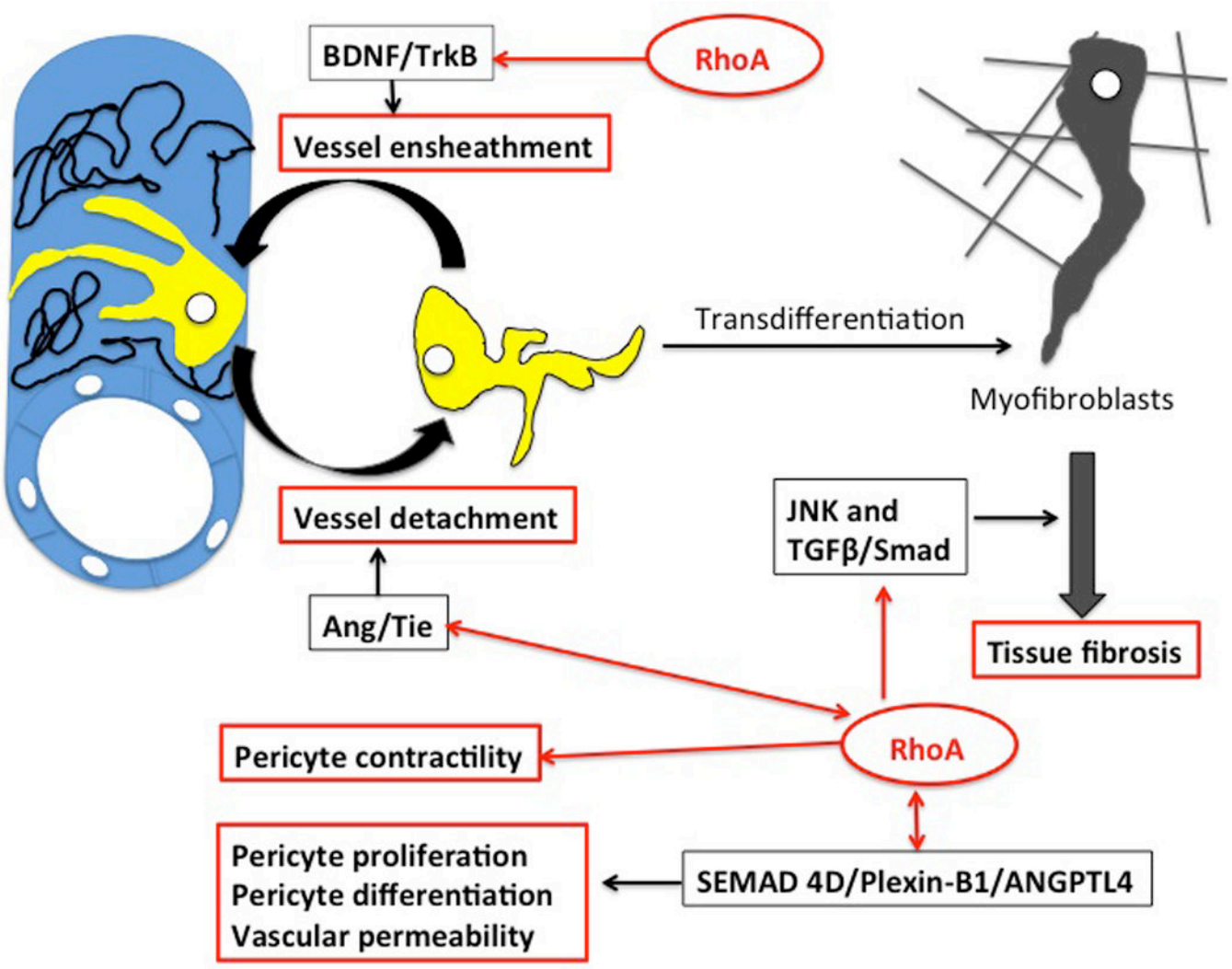

Figure 2. Pericyte functions regulated by small GTPase RhoA pathway

Microvessel ensheathment is initiated by BDNF/TrkB signaling, which is regulated by RhoA pathway and is sensitive to RhoA kinase (ROCK) inhibition. During inflammation and tissue injury pericytes are able to detach from the microvessel wall and transdifferentiate into fibrillar extracellular matrix (ECM)-producing myofibroblasts. Deposition of ECM leads to fibrosis and organ failure. Fibrosis is regulated by JNK and TGFβ/Smad pathways, which cross-talk with RhoA pathway. Inhibition of JNK and TGFβ/Smad through the inhibition of RhoA kinase ROCK abrogates collagen production and fibrosis. Detachment of pericytes from the microvessel wall is regulated by the Ang/Tie pathway, which cross-talks with RhoA signaling. Because RhoA is a master regulator of actin-related cell functions it also regulates pericyte contractility. By crosstalking to Ang/ Tie-2 and SEMAD4D/Plexin-B1/ANGPTL4 pathways, RhoA signaling influences pericyte differentiation and pericyte coverage, which in turn regulate blood flow and overall vessel permeability.

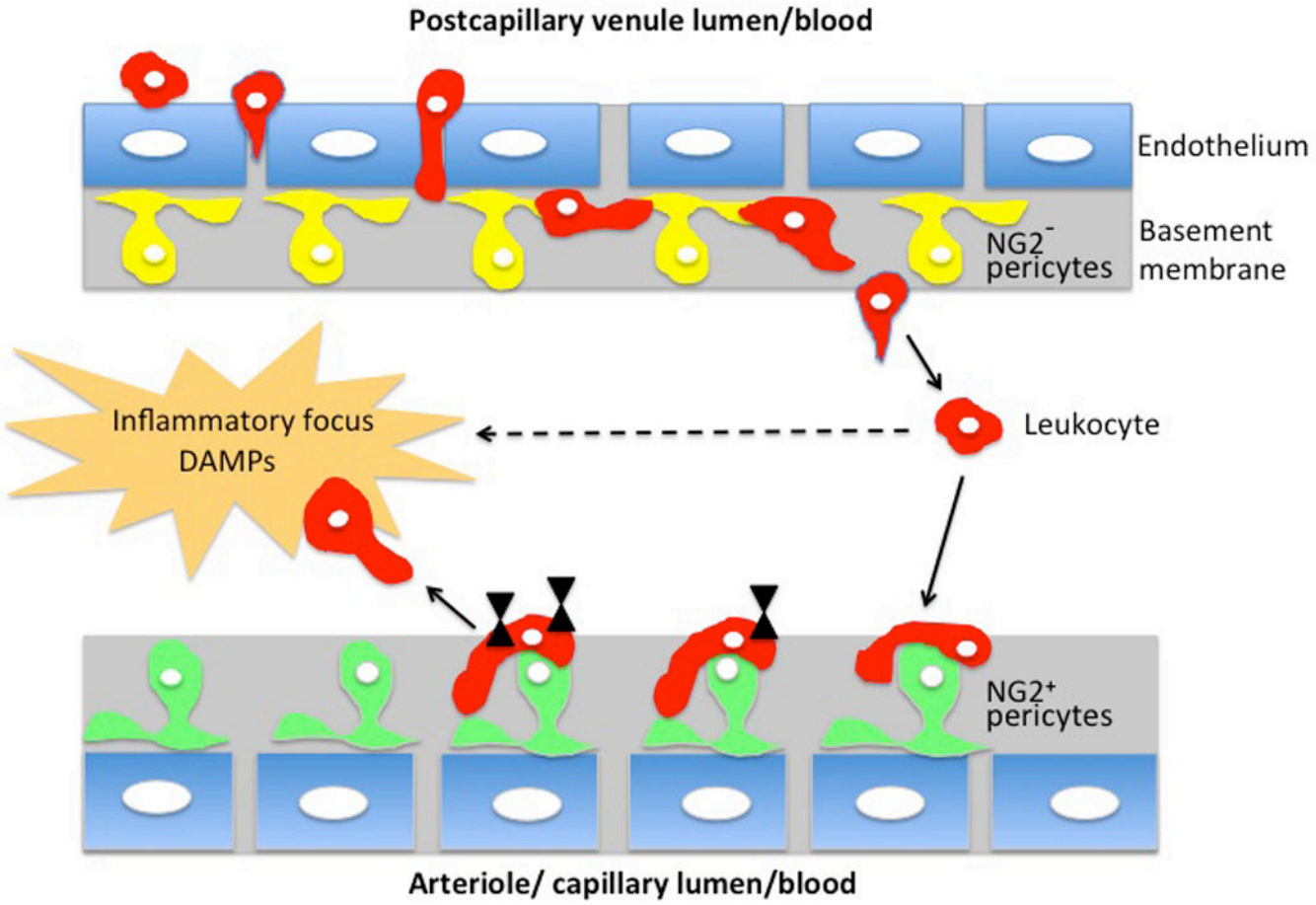

Figure 3. Pericytes participate in endothelial wall breaching by leukocytes, sensitize them and direct them toward the inflammation foci

Depending on the type of microvessel there are two subtypes of pericytes: venules are covered by pericytes lacking NG2 marker (yellow), and arterioles and capillaries are covered by NG2 positive pericytes (green). During tissue inflammation the leukocytes (red) enter the venular wall and after squeezing between endothelial cells (blue) migrate (abluminal migration) along the processes of $NG2^{-}$ pericytes until they find large inter-pericyte gaps where they are able to exit (extravasate) the vessel wall. Leukocyte migration is ICAM-1, Mac-1 and LFA-1 dependent and a selection of the large-size exit gaps is TNF/IL-1β dependent. Subsequently, after exiting from the venules some of the leukocytes are attracted toward ICAM-1; MIF positive $NG2^{+}$ pericytes present on neighboring arterioles and capillaries. Contact with $NG2^{+}$ pericytes and treking along pericyte processes induce leukocytes to produce matrix metalloproteinses, integrins and FPRs (black triangles), progressively acquiring prosurvival signals, upregulating expression of a promigratory receptors and migrating toward the inflammation sites, which are rich in damage-associated molecular pattern (DAMPs) compounds.

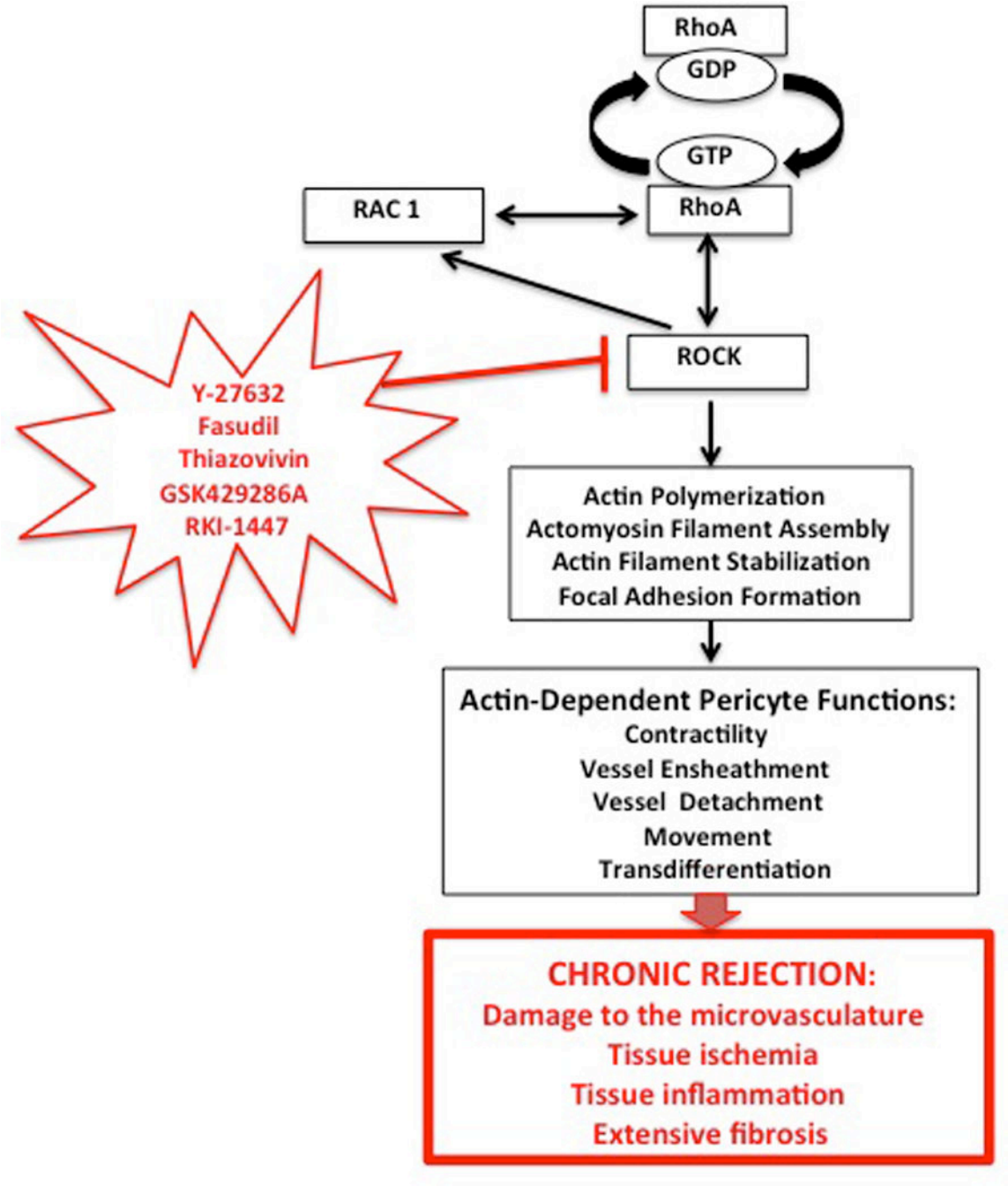

Figure 4. RhoA pathway regulation of actin-related pericyte functions involved in development of chronic rejection

RhoA regulates, through its downstream effector ROCK (containing ROCK 1 and ROCK 2 isoforms) actin filament polymerization and organization, which in turn regulate various functions of pericytes. In addition, RhoA (either directly or through ROCK) cross talks with Rac1 pathway. There are several inhibitors of ROCK (ROCK 1, ROCK 2 or both), which are known, from rodent or human clinical studies, to influence actin-related cellular functions in immune response and transplantation (129, 130). Because many of pericyte functions such as movement, contractility, interaction with vessel endothelium and

transdifferentiation into collagen producing myofibroblasts also depend on actin cytoskeleton, these inhibitors, via targeting pericytes, have a potential to be used as the anti-inflammation and anti-chronic rejection therapeutics.