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Review

Rho-Associated Coiled-Coil Kinase (ROCK) in Molecular Regulation of Angiogenesis

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Abstract

Identified as a major downstream effector of the small GTPase RhoA, Rho-associated coiled-coil kinase (ROCK) is a versatile regulator of multiple cellular processes. Angiogenesis, the process of generating new capillaries from the pre-existing ones, is required for the development of various diseases such as cancer, diabetes and rheumatoid arthritis. Recently, ROCK has attracted attention for its crucial role in angiogenesis, making it a promising target for new therapeutic approaches. In this review, we summarize recent advances in understanding the role of ROCK signaling in regulating the permeability, migration, proliferation and tubulogenesis of endothelial cells (ECs), as well as its functions in non-ECs which constitute the pro-angiogenic microenvironment. The therapeutic potential of ROCK inhibitors in angiogenesis-related diseases is also discussed.

Key words: Rho-associated coiled-coil kinase (ROCK), angiogenesis, endothelial cell, microenvironment, therapeutic target

Introduction

Angiogenesis is the formation of new capillaries from pre-existing blood vessels. It occurs more frequently in ischemic/hypoxic tissues, with the increased demand of oxygen and nutrients. Physiological angiogenesis is well regulated and plays an indispensable role in embryonic development, wound healing and female menstrual cycle [1-3]. Uncontrolled angiogenesis is characterized by the development of immature capillaries in diseases tumors, diabetes, age-related macular degeneration and atherosclerosis [4-7]. Thus, inhibiting angiogenesis has become a promising strategy for the treatment of these diseases. In contrast, therapeutic angiogenesis promoting vessels formation helps improve the function of ischemic hearts or limbs caused by occlusion of coronary or peripheral arteries [8, 9]. Insights into the underlying mechanisms of angiogenesis may reveal novel targets for angiogenesis-related diseases.

As a well-known effector of small GTPase RhoA, Rho-associated coiled-coil kinase (ROCK) regulates actin reorganization during cell adhesion, migration, contraction and proliferation [10]. Angiogenesis is initiated by activation of ECs, followed by EC migration and proliferation, thus it involves the function of ROCK. ROCK is also necessary for constitution of pro-angiogenic microenvironment by regulating gene expression in non-ECs. In this review, we summarized our current understanding of the diverse mechanisms of ROCK in angiogenesis, highlighting the therapeutic potential of ROCK inhibitors.

Molecular structure of ROCK1 and ROCK2

ROCK belongs to the AGC (protein kinase A, G

and C, PKA/PKG/PKC) family of serine-threonine kinases [11]. There are 2 subtypes of ROCK, ROCK1 (ROCKβ) and ROCK2 (ROCKα). They have a high degree of sequence homology, with 65% amino acid sequences in common and 92% homology within their kinase domains [12]. Each ROCK isoform consists of 5 domains, a catalytic kinase domain at the N-terminus, followed by a central coiled-coil domain containing a Rho-binding domain (RBD) and a C-terminal pleckstrin-homology (PH) domain including an internal cysteine-rich domain (Figure 1). Inside cells, ROCK locates primarily in the cytoplasm, but also distributes to the nucleus and membrane [13, 14]. ROCK1 and ROCK2 are widely expressed in tissues of embryos and adults [15]. While ROCK1 is preferentially expressed in kidney, liver, spleen and testis, ROCK2 is enriched in brain, heart, lung and skeletal muscle [15, 16]. Both isoforms are expressed in ECs, in which ROCK1 is mainly distributed in the plasma membrane and ROCK2 is localized in cytoplasm [17].

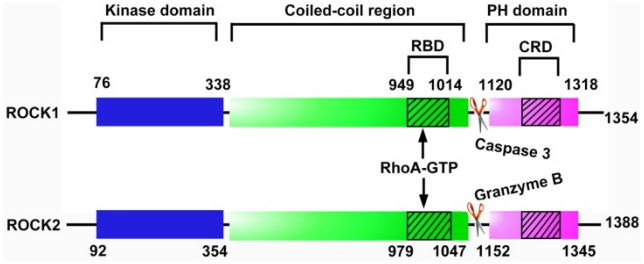


Figure 1. Structural domains of ROCK protein. Each ROCK isoform contains 5 domains: a kinase domain, a coiled-coil domain containing a Rho-binding domain (RBD), a pleckstrin-homology (PH) domain containing an internal cysteine-rich domain (CRD). The binding of RhoA-GTP to RBD alters the inhibitory fold structure and frees the kinase domain, leading to the activation of ROCK. In addition, caspase-3-mediated C-terminus cleavage of ROCK1 and granzyme-mediated cleavage of ROCK2 contribute to the activation of ROCK by disrupting the auto-inhibitory intramolecular fold. CRD: cysteine-rich domain; PH: pleckstrin-homology; RBD: Rho-binding domain.

Knockout and transgenic mouse models of *ROCK1/ROCK2* genes

The *ROCK2* $^{-/-}$ mice were generated in 2003, and 90% of *ROCK2* null embryos died due to severe thrombus formation, placental dysfunction and consequent intrauterine growth retardation [18]. The few survivors were born runts and subsequently developed without gross abnormality, and were fertile [18]. In contrast to *ROCK2*$^{-/-}$ mice, fetal death did not occur in *ROCK1*$^{-/-}$ mice. However, most of *ROCK1*$^{-/-}$ newborns died immediately due to omphalocele, and exhibited eyelids open at birth (EOB) [19]. Further studies indicated that both *ROCK2*$^{-/-}$ and *ROCK1*$^{+/-}$*ROCK2*$^{+/-}$ mice displayed phenotypes similar to that of *ROCK1*$^{-/-}$ mice due to disrupted assembly of actin bundles in epithelial cells of the eyelids and umbilical rings [20]. All the *ROCK1*$^{-/-}$*ROCK2*$^{-/-}$ mouse embryos died in utero between E3.5 and E9.5 [21]. In addition, *ROCK1*$^{+/-}$/*ROCK2*$^{-/-}$ mice, or *ROCK1*$^{-/-}$/*ROCK2*$^{+/-}$ mice, exhibited embryonic lethality around E9.5 due to impaired vasculature development in the yolk sac [21]. Treatment with ROCK inhibitor Y-27632 also destroyed vascular remodeling in wild-type embryos *ex vivo* [21]. These findings provide direct evidence that ROCK is indispensible for developmental angiogenesis. In adults, *ROCK2*$^{+/-}$ mice exhibited a modest, yet significantly decreased vessel density in the lung [22], while *ROCK1*$^{+/-}$ mice showed decreased cardiac fibrosis and neointima [23, 24].

In addition to *ROCK* deletion mouse models, Samuel *et al*. generated *cytokeratin14 (K14)-ROCKII: mER*™ mice for inducible expression of ROCK2 [25]. The expression of the fusion protein ROCK2:mER (ROCK2 with the Rho-binding domain substituted by the modified hormone-binding domain of estrogen receptor) was driven by the promoter of *cytokeratin14*, a gene specifically expressed in skin keratinocytes. Estrogen analogues, such as 4-hydroxytamoxifen (4HT) and tamoxifen, were then administrated to elicit kinase activity of ROCK [25]. Compared with the wild-type mice, transgenic mice with constitutive ROCK activation in skin keratinocytes show an increase of epidermal hyperplasia and a conversion of cutaneous papillomas to invasive carcinomas [26]. The healing of full thickness skin wounds is also accelerated in the *K14-ROCKII:ER* mice [27]. To extend the application of the ROCK2:ER system to more tissues, a two-stage system was developed to allow for the conditional activation of ROCK2 in a tissue-selective manner [28]. *LSL-ROCK2:ER* transgenic mice were generated by placing *loxP* flanked transcription termination cassette (STOP) sequence (LSL) between a cytomegalovirus early enhancer-chicken β-actin (CAG) promoter and the coding sequence for ROCK2:ER. By crossing with tissue-specific CRE recombinase-expressing mouse lines, CRE-mediated recombination between *loxP* sites removes the STOP sequence to allow the expression of ROCK2:ER fusion protein. Upon stimulation with 4HT, the kinase activity of ROCK2 is triggered, and the activation of ROCK2 was verified in various tissues [28]. The 4HT-induced ROCK2 activation in the whole tissues resulted in cerebral hemorrhage and death within 7 days of induction [28]. By crossing *LSL-ROCK2:ER* transgenic mice with genetically modified mice with pancreatic ductal adenocarcinoma, ROCK2 level is specifically elevated in the pancreas, which promotes the growth and invasion of adenocarcinoma [29]. To date, conditional ROCK2 activation in vascular ECs has not been reported.

Kinase activity and substrates of ROCK

The activation of ROCK depends on RhoA-GTP, which is transformed from RhoA-GDP by Rho guanine nucleotide exchange factor (RhoGEF) (Figure 2). In the absence of RhoA-GTP, the C-terminal RBD and PH domains exert an auto-inhibitory effect on the kinase domain by formation of an intramolecular fold [30]. The binding of RhoA-GTP to RBD alters the inhibitory fold structure and frees the kinase domain; hence ROCK is activated [30]. ROCK is also activated in Rho-independent ways. For instance, caspase-3-mediated C-terminus cleavage of ROCK1 and granzyme-mediated C-terminus cleavage of ROCK2 contribute to the activation of ROCK by disruption of the auto-inhibitory intramolecular fold [31, 32]. Furthermore, phospholipids such as arachidonic acid directly activate ROCK in the absence of RhoA-GTP [33, 34].

The RhoA/ROCK signaling is a major regulator of actin reorganization since various cytoskeletal regulatory proteins are substrates of ROCK (Figure 2).

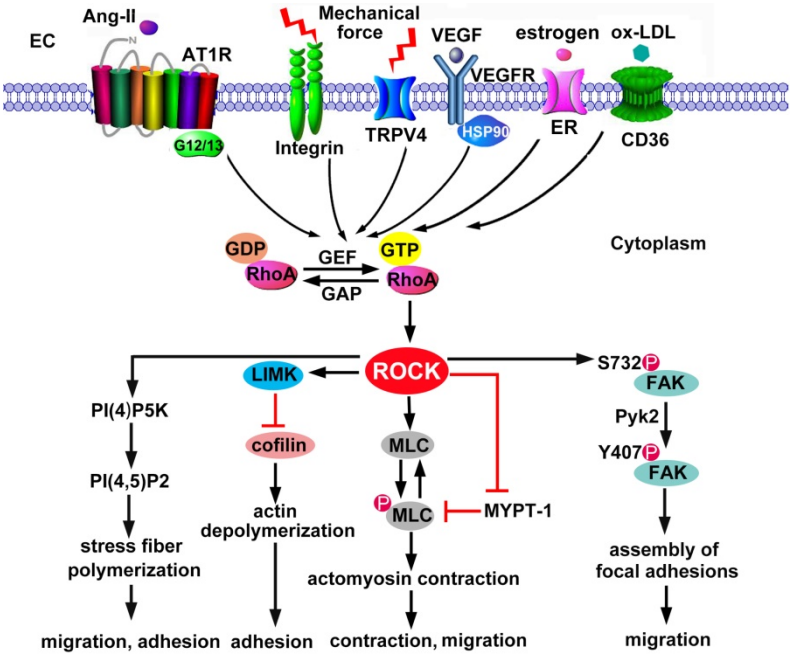


Figure 2. ROCK activation in endothelial cytoskeleton. ECs are activated by a wide range of stimuli, including chemical molecules and physical mechanical forces. The activated receptors recruit and activate GEFs via adaptor proteins. GEFs stimulate the exchange of GDP for GTP, resulting in RhoA activation. In contrast, GAPs abrogate the GTPase activity of RhoA by accelerating the hydrolysis of bound GTP to GDP. ROCK is an effector of RhoA-GTP. Substrates of ROCK include MLC, MLCP and LIMK. Phosphorylation of MLC and LIMK is involved in actin depolymeriztion and actomysion contraction, thus regulating EC adhesion, contraction and migration. In addition, ROCK phosphorylates PI4P5K. As a main product of PI(4)P5K, PI(4,5)P(2) interacts with actin-associated proteins to stimulate reorganization of the actin cytoskeleton and trigger stress fiber polymerization. ROCK also facilitates the phosphorylation of FAK2 by Pyk2, which mediates the assembly of focal adhesions. Ang II: angiotensin II; AT1R: Ang II type I receptors; ER: estrogen receptor; FAK: focal adhesion kinase; GEF: guanine nucleotide exchange factor; GAP: GTPase-activating protein; LIMK: LIM motif-containing protein kinase; MLC: myosin light chain; MLCP: MLC phosphatase; TRPV4: transient receptor potential vanilloid 4; Pyk2, proline-rich tyrosine kinase-2; VEGF: vascular endothelial growth factor.

These regulatory proteins include LIM motif-containing protein kinase (LIMK), myosin light chain (MLC) and MLC phosphatase. ROCK-activated LIMK phosphorylates cofilin and inactivates its actin-depolymerization activity, leading to stabilization of actin filaments [35, 36]. On the other hand, ROCK promotes the phosphorylation of MLC through phosphorylation and inactivation of MLC phosphatase or direct phosphorylation of MLC, leading to the activation of myosin II and the actomyosin-driven contractility [37]. Besides, ezrin/ radixin/moesin [38], adducin [39, 40], and eukaryotic elongation factor 1-α1 (eEF1a1) [41] are downstream targets of ROCK and involved in actin cytoskeleton assembly.

Activation of ROCK in ECs by pro-angiogenic stimuli

The onset of angiogenesis or the 'angiogenic switch' is triggered by the imbalance between pro-angiogenic and anti-angiogenic molecules [42]. Under ischemic or hypoxic microenvironment, pro-angiogenic molecules are mainly produced and secreted from non-ECs. These molecules include vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs) and epidermal growth factors (EGFs) [43-46]. In addition, inflammatory factors, hormone and lipid act as the pro-angiogenic stimulators [47-50]. Mechanical stress caused by cell-ECM or cell-cell interaction changes also promotes angiogenesis [51, 52]. In ECs, ROCK mediates both chemical and mechanical pro-angiogenic stimuli-induced cellular functions (Figure 2).

VEGF

VEGF is the most robust angiogenic growth factor [53]. In VECs, VEGF binds to two types of receptor tyrosine kinase, VEGF receptor (VEGFR) 1 (also known as fms-like tyrosine kinase-1, Flt-1) and VEGFR2 (also known as fetal liver kinase-1, Flk-1 or kinase insert domain-containing receptor, KDR) [54]. VEGFR2 activation contributes to VEGF-stimulated EC migration and proliferation, whereas VEGFR1 negatively regulates these angiogenic effects of VEGF [54]. RhoA is quickly activated by VEGF and recruited to the endothelial cytoplasm membrane [55]. Studies have shown that the Tyr951 residue of VEGFR2, phospholipase C and the G protein Gq/11 are required for

VEGF-stimulated RhoA activation and EC migration [56]. The function of RhoA in EC migration is mediated by ROCK, as inhibition of either RhoA or ROCK abolishes VEGF-induced F-actin stress fiber formation and EC migration *in vivo* and *in vitro* [22, 54, 55, 57]. In addition, the protective effect of VEGF against apoptosis also requires the activation of ROCK [57]. In-depth studies explored the specific contributions of each ROCK isoform. In response to VEGF, ECs with knockdown of *ROCK2* exhibit a drastic reduction in migration and tube formation, which has not been observed in ECs with *ROCK1* knockdown, suggesting that VEGF-induced EC activation is largely mediated through ROCK2 signaling [22].

Oxidized low-density lipoprotein (ox-LDL)

In response to hypoxia and inflammation, angiogenesis is induced within the atherosclerosis plaque [58]. Oxidized low-density lipoprotein (ox-LDL) is a key factor for angiogenesis in atherosclerotic vessels. Both pro-angiogenic (low concentration) and anti-angiogenic (high concentration) effects of ox-LDL have been reported [49, 59]. The angiogenic effect of low concentrations of ox-LDL is partially mediated through ROCK signaling. Oh *et al*. discovered that low concentrations (10-100 μg/ml) of ox-LDL promote angiogenesis of human aortic ECs via receptor CD36-mediated RhoA/ROCK activation [50]. Moreover, cholesterol reverses oxLDL-induced endothelial network formation through preventing RhoA/ROCK activation [50]. This observation furtherly confirms the significance of ROCK in ox-LDL induced angiogenesis.

Angiotensin II

Angiotensin II (Ang II) type 1 and type 2 receptors (AT1R and AT2R) modulate angiogenesis by opposing effects in the microvascular endothelium [60]. VEGF-induced angiogenesis is augmented by Ang II both *in vitro* and *in vivo*, exclusively through AT1R via the activation of RhoA/ROCK signaling. In contrast, selective activation of AT2R by its specific agonist or Ang II with AT1R antagonist inhibits VEGF-driven EC migration by repressing RhoA activity [60]. As G-protein-coupled receptors, AT1R and AT2R activate two types of G-proteins to regulate RhoA/ROCK signaling. $G\alpha_{12/13}$ proteins are activated by AT1R to mediate RhoA activation, whereas $G\alpha_i$ protein activation is required for AT2R-induced RhoA inhibition [60].

Table 1. Summary of phenotypes of *ROCK* knockout mice

Genotype	Genetic background	Phenotype	Mechanism
ROCK1$^{-/-}$	C57BL/6	Eyelid open at birth (EOB), omphalocele, neonatal death (>90%); survivors subsequently develop normally, fertile and apparently healthy [19].	Disorganized actin bundles in the epithelial cells of eyelid and umbilical ring [19].
ROCK2$^{-/-}$	C57BL/6	EOB, omphalocele, fetal death (~90%) [20]; thrombus formation in the labyrinth layer, placental dysfunction, intrauterine growth retardation; survivors are born runts and subsequently develop without gross abnormality, and are fertile [18].	Disorganized actin bundles [20]; dysfunction of embryo-placenta interaction [18].
ROCK1$^{-/-}$*ROCK2*$^{-/-}$	C57BL/6	All embryos die in utero between 3.5 and 9.5 days postcoitum (dpc) [21].	Seriously impaired mouse development [21].
ROCK1$^{+/-}$	C57BL/6	Viable and fertile without obvious phenotypic abnormalities [18]; decreased cardiac fibrosis [23]; reduced neointima formation after vascular injury [24].	Decreased expression of CTGF [23]; decreased ICAM and VCAM expression and leukocyte recruitment [24].
ROCK2$^{+/-}$	C57BL/6	Viable and fertile without obvious phenotypic abnormalities [18]; decrease of blood vessel density in the lung compared with *ROCK1*$^{+/-}$ mice [22].	Blood vessel formation in the lung is largely dependent on ROCK2 signaling [22].
ROCK1$^{+/-}$*ROCK2*$^{+/-}$	C57BL/6	EOB and omphalocele [20].	Disorganized actin bundles [20].
ROCK1$^{+/-}$*ROCK2*$^{-/-}$ or *ROCK1*$^{-/-}$*ROCK2*$^{+/-}$	C57BL/6	Embryos die during a period from 9.5 to 12.5 dpc with defective vasculogenesis and impaired body turning in the yolk sac [21].	Impaired actomyosin contractility appears to contribute to endothelial dysfunctions during vasculogenesis [21].

Table 2. Summary of the phenotypes of *ROCK* transgenic mouse.

Genotype	Genetic background	Activator of mouse ROCK activity	Tissues with conditional ROCK activation	Phenotype
K14-ROCKII-mER™ [25]	FVB/N	4HT	Keratinocytes in the skin	Induces epidermal hyperplasia and the conversion of cutaneous papillomas to invasive carcinomas [26]; accelerates the healing of full thickness skin wounds [27].
K14-CRE; LSL-ROCK2-ER	C57BL/6	tamoxifen	Keratinocytes in the skin	Not mentioned [28].
MMTV-CRE; LSL-ROCK2-ER	C57BL/6	tamoxifen	Mammary epithelium	Not mentioned [28].
Ah-CRE; LSL-ROCK2-ER	C57BL/6	tamoxifen	Intestinal epithelium	Not mentioned [28].
CAG-CRE-ER; LSL-ROCK2-ER	C57BL/6	tamoxifen	Most tissues in whole mice	Induces cerebral hemorrhagic lesions and death [28].
LSL-Kras^{G12D}*;LSL-p53*^{R172H}*; Pdx1-Cre; LSL ROCK2-ER*	Not mentioned	tamoxifen	Pancreatic ductal adenocarcinoma	Promotes tumor growth and invasion, reduces survival [29].

Ah: cytochrome P450 1A1; CRE: Cre recombinase; ER: estrogen receptor; 4HT: 4-hydroxytamoxifen; K14: cytokeratin 14; LSL: *loxP-STOP-loxP*; MMTV: murine mammary tumor virus; Pdxl: pancreatic and duodenal homeobox gene 1; CAG: a synthetic promoter consisting of a cytomegalovirus early enhancer element, a chicken β-actin gene promoter with the first exon and intron, and a splice acceptor from the rabbit β-globin gene.

Estrogen

Estrogens have been proven to initiate angiogenesis via ROCK activation. Simoncini *et al*. first reported that estrogen receptor alpha (ERα) interacts with G protein $G\alpha_{13}$ to drive actin remodeling and EC migration via the RhoA/Rho kinase/moesin pathway [61]. In another study, estradiol enhances the transcription, protein expression, and activity of RhoA in an estrogen receptor-dependent manner [62]. Inhibition of ROCK abrogates the expression of cell cycle-related proteins raised by estradiol and hence interrupted EC migration and proliferation [62]. 17β-estradiol increases horizontal migration of human umbilical vein endothelial cells (HUVECs), which is mediated by ROCK2 activation-dependent activation of c-Jun and c-Fos [63]. c-Jun and c-Fos translocate into the nucleus and stimulate the transcription of endothelial plasminogen activator inhibitor-1 (PAI-1), which is implicated in HUVEC horizontal migration [63]. Formononetin, an isoflavone that displays estrogenic properties, binds to ERα and activates ROCK2/matrix metalloproteinase (MMP) 2/MMP9 signaling, contributing to EC migration, dramatic actin cytoskeleton spatial modification and angiogenesis [48]. Considering the importance of estrogen in the regulation of the menstrual cycle, estrogen-dependent RhoA/ROCK activation may be essential in vascular reconstruction of the endometrium through regulating angiogenesis.

Mechanical forces

In addition to chemical factors, ECs are mainly subject to two types of mechanical force, stretch produced by deformation of blood vessels and shear stress arising from changes in blood flow [64]. Under physiological conditions, these mechanical forces maintain the morphology, signal transduction and function of VECs. During pathological process such as vascular obstruction or injury, the changed mechanical forces remodel the conformation of ECM and ECs [65]. In response, ECs express mechanosensors such as integrin receptors at cell-cell junctions and cell-matrix adhesions to sense and translate the mechanical forces into electrical or biochemical signals and influence angiogenesis [66, 67]. During this process, RhoA/ROCK pathway acts as a transducer of mechanical signals from integrins to cytoskeleton [68]. In confluent cultures of bovine aortic endothelial cells (BAECs), ROCK mediates shear stress-induced cell alignment and stress fiber formation [69]. Shear-induced increase in RhoA/ROCK activity also facilitates EC migration by enhancing the traction force between ECs and the underlying substrate [70]. By employing a stretchable three-dimensional (3D) cell culture model, Fischer *et al*. reported that RhoA/ROCK is required in 3D EC branching guided by local cortical tension [51]. By using a similar culture model, Wilkins *et al*. demonstrated that cyclic uniaxial stretch alone significantly increases endothelial sprouts that aligned perpendicular to the direction of the stretch in a ROCK-dependent way [52]. The sprouting number and alignment induced by stretch are suppressed by the ROCK inhibitor Y-27632. Despite of the potent pro-angiogenic activity of VEGF, combination of the stretch and VEGF does not show a synergistic effect on angiogenesis. Moreover, unlike cyclic stretch or VEGF alone, the combination of stretch and VEGF induces angiogenesis through a ROCK-independent mechanism [52]. It is likely that receptor tyrosine kinase (RTK) rather than ROCK mediates the new sprouts formation induced by the combination of cyclic stretch and VEGF [52].

Studies in tumor capillary ECs further support the importance of RhoA/ROCK in mechanical stress-associated angiogenesis. Unlike normal ECs, tumor capillary ECs exhibit aberrantly high levels of RhoA and ROCK, leading to abnormal mechanosensing and excessive angiogenesis in response to cyclic strain [71]. Mechanosensitive ion channel transient receptor potential vanilloid 4 (TRPV4) is functionally expressed in ECs, and acts as a mechanosensor of cyclic stretch and flow [72, 73]. In normal ECs, TRPV4 senses mechanical force and induces optimal RhoA/ROCK activation necessary for endothelial migration and contraction which is required for partial cell rounding. However, reduction of TRPV4 level in ECs activates Rho/ROCK signaling, resulting in enhanced proliferation, migration, and aberrant tube formation, which could be decreased by the ROCK inhibitor Y-27632 [74]. Given that TRPV4 is a calcium ion (Ca^{2+})-permeable nonselective cation channel and TRPV4 activation increases intracellular Ca^{2+} influx [75], Ca^{2+} might involve in TRPV4 regulated ROCK activation.

To summarize, ROCK mediates both soluble chemical factors- and physical mechanical force-induced angiogenesis. As these two types of stimulators exist simultaneously *in vivo*, ROCK may function as an integrator through overlapping or additive effects in ECs. In addition to RhoA, multiple Rho GTPases affect ROCK activities [76]. In response to VEGF, the expression of RhoB increases in HUVECs [77]. Interference of RhoB mitigates VEGF-induced vessel sprouting and cord formation, partially through activating RhoA-ROCK signaling [77]. RhoJ also inhibits RhoA-ROCK signaling in HUVECs [78]. Knockdown of RhoJ promotes the activation of RhoA-ROCK signaling and the phosphorylation of MLC, leading to disrupted EC

migration and tube formation [78]. It is not clear how RhoB and RhoJ suppress RhoA-ROCK signaling, whereas RhoE binds directly to ROCK1 and inhibits RhoA from binding to ROCK1 [79]. Although RhoE competes with RhoA for binding with ROCK1, the RhoE binding site on ROCK1 protein is different from that of RhoA [79]. It appears that angiogenesis is regulated by a dynamic balance of various Rho GTPases, and ROCK might be the convergence point.

ROCK in fundamental stages of angiogenesis

In response to pro-angiogenic stimuli, ECs are shifted from a quiescent state to an active angiogenic state [53]. First, EC junctions in the existing vessels dissociate and plasma proteins leak into the extracellular matrix (ECM). The accumulated plasma proteins provide a new ECM for migrating ECs [80]. In addition to growth factors, proteases such as MMPs, are secreted by both parenchymal cells and ECs [81]. The proteases break down the basement membrane and release the proangiogenic factors that are anchored in ECM [81]. These changes facilitate the migration of tip cells under the guidance of a gradient of VEGF or other growth factors. Tip cells are specialized ECs with polarized filopodia, which sense and guide the sprout towards the angiogenic stimulus [82]. Following tip cells, the specialized endothelial stalk cells proliferate during sprout extension to form the nascent vascular lumen [83]. These sprouts ultimately form capillary-like structures. After vessel fusion and pruning, pericytes and smooth muscle cells are recruited to wrap around and stabilize it. In this section, we dissect the roles of ROCK in the basic stages of angiogenesis, including hyperpermeability, migration, proliferation and tubulogenesis.

Vascular permeability

VEGF, originally known as vascular permeability factor (VPF) [84], is the main contributor of vascular hyperpermeability through activating ROCK. ROCK inhibitor Y-27632 blocks VEGF-induced microvascular hyperpermeability in a dose-dependent manner [85]. Pretreatment with exoenzyme C3, an inhibitor of RhoA, significantly represses the albumin trans-endothelial flux [86]. In addition, ROCK increases endothelial permeability mainly through promoting the cellular contraction via MLC phosphorylation and contractile fibers formation. The ROCK regulates cell junctions. ROCK promotes the dissociation of cell-cell adherens junctions (AJs) by suppressing VE-cadherin expression and its membrane localization [87, 88]. In addition to VE-cadherin, ROCK inhibits expression of occludin and claudin-1, the major components of tight junctions, leading to lipopolysaccharide-induced endothelial barrier dysfunction [89].

Several mechanisms are found to constrain ROCK activity and its ability of increasing vascular permeability under physiological conditions. Generally, Krev interaction trapped protein 1 (KRIT1)/cerebral cavernous malformations 2 protein (CCM2) complex maintains the endothelial barrier function by suppressing RhoA/ROCK signaling [90]. However, the interaction between KRIT1 and CCM2 is disrupted in endothelium of human cerebral cavernous malformations (CCMs), resulting in hyper-activation of ROCK and serious vascular leakage, which could be ameliorated by the ROCK inhibitor fasudil (also known as HA1077) [90]. In addition to KRIT1, MEKK3 (MAPK/ERK kinase kinase 3) forms a protein complex with CCM2 to maintain vascular integrity in embryonic organs and neonatal brains [91]. Both CCM2 and MEKK3 exert an inhibitory effect on ROCK. Y-27632 partially rescues the disrupted neurovascular integrity caused by MEKK3 deficiency, indicating that MEKK3/ CCM2-mediated repression of the Rho/ROCK pathway is required for maintenance of vascular integrity [91].

Migration

EC migration requires the contraction of actomyosin, the rearrangement of stress fibers and the formation of focal adhesions [92], all of which are regulated by ROCK. First, ROCK activation enhances actomyosin contraction in ECs through promoting the phosphorylated of MLC. Besides, ROCK phosphorylates LIMK, which phosphorylates and inactivates its downstream substrate, cofilin, leading to inhibition of actin depolymerization and stress fiber rearrangement in VEGF-stimulated ECs [35]. Cell migration requires dynamic assembly and disassembly of focal adhesions [93]. FAK phosphorylated at Tyr407 forms a complex with paxillin and vinculin to promote the formation of focal adhesion [94]. Le Boeuf *et al*. found that VEGF-induced VEGFR2-HSP90 interaction activates RhoA/ROCK signaling, which in turn promotes the phosphorylation of FAK on Tyr407 [94]. Although ROCK does not directly phosphorylate Tyr407, it phosphorylates Ser732 of FAK and modifies the conformation of FAK, leading to the phosphorylation of FAK on Tyr407 by proline-rich tyrosine kinase 2 (Pyk2) [95].

In addition, Rho/ROCK-dependent generation of PI(4,5)P2 by phosphatidylinositol 4-phosphate 5-kinase (PI(4)P5K) induces activation of phospholipase C and elevation of intracellular calcium ion, facilitating Semaphorin 4D-induced cytoskeletal polymerization in HUVECs [96]. As a scaffold for RhoA/ROCK, α-catulin localizes on vimentin

intermediate filaments and increases migration of pulmonary vascular ECs in a ROCK-dependent manner [97]. Recent studies showed that ECs migrate as a group joined via cadherin-containing AJs in sprouting angiogenesis [98]. The association of ROCK2 with Raf is required for the recruitment of ROCK2 to AJs, which promotes the maturation of AJs by activation of junctional myosin. Disruption of the Raf/ROCK complex impairs the maturation of AJs, leading to angiogenesis defects [98].

Proliferation

The effects of ROCK on EC proliferation is attributed to its role on cytoskeleton. For instance, reduction of FAK promotes RhoA/ROCK activities, which in turn, augments cytoskeletal tension, creating a pro-proliferative condition [99]. Tumor necrosis factor-α (TNF-α) has been reported to promote angiogenesis [47]. However, studies also showed that TNF-α suppresses EC proliferation in a ROCK-dependent way. TNF-α exposure leads to ROCK-mediated phosphorylation of cytoskeletal protein ezrin, which translocates to the nucleus and represses the transcription of cyclin A, therefore, inhibiting proliferation [100].

In addition, ROCK regulates EC proliferation by its targeted gene expression associated with cell cycle. In the hypoxic condition, ROCK2 promotes proliferation of pulmonary arterial ECs though upregulating the expression of cyclin A and cyclin D1 [101]. Rho/ROCK signaling mediates oxLDL-induced EC proliferation through downregulation of cell cycle inhibitor p27 [102]. Integrin cytoplasmic domain–associated protein-1 (ICAP1) inhibits ROCK, and increases p21/p27, resulting in suppression of EC proliferation [103].

Tubulogenesis

Lumen formation or tubulogenesis is an essential process in angiogenesis [104]. Initially, clearance of cell-cell junctions between ECs to the lateral borders is required for the proper formation of a lumen. The apical junctions is controlled by F-actin and non-muscle myosin II (NMII) contractility mediated by the signaling of Ras interacting protein 1 (Rasip1) [105]. Rasip1 promotes the activity of Cdc42, which in turn stimulates Pak4 to activate NMII. The latter controls remodeling of pre-apical junctions, allowing the lumen to open. In addition to apical junction clearance, Rasip1 binds to its GTPase activating protein, Arhgap29, which subsequently inhibits activity of RhoA [105, 106]. Signaling of RhoA/ROCK contributes to tubulogenesis through regulation of vessel diameter by suppressing lumen expansion via activity of NMII. A loss of RhoA/ROCK signaling results in an overexpansion of the lumen, thus compromising the structural integrity of the vessel. Therefore, these two signaling pathways (Rasip1/ Cdc49/ Pak4) and (Rasip1/ Arhgap29/ RhoA/ ROCK) converge on NMII to regulate apical junction remodeling and lumen expansion respectively that are essential for tubulogenesis.

Together, ROCK promotes endothelial proliferation, permeability, migration and tubulegenesis. In addition, ROCK was reported to drive proliferation by spatiotemporally governing cytokinesis [107-109] and by increasing centrosome amplification in epithelial cells [110, 111]. ROCK also promotes the progression of G1 to S phase of vascular smooth muscle cells (VSMCs) through manipulating cyclin expression [112, 113]. RhoA/ROCK activation shortens the half-life of endothelial nitric oxide synthase (eNOS) mRNA and hence inhibits the expression of eNOS in ECs [114]. In contrast, overexpression of eNOS suppresses Ang II-induced migration of VSMCs by inhibition of $G_{12/13}$-RhoA-ROCK signaling, suggesting ROCK as an eNOS downstream target [115]. These mechanisms need to be furtherly studied in ECs.

Crosstalk between RhoA/ROCK signaling and other signaling pathways in ECs

NF-κB

In ECs, nuclear factor-κB (NF-κB) signaling regulates transcription of genes to favor angiogenesis. Inhibition of NF-κB blocks capillary tube formation by suppression of pro-angiogenic factors such as IL-8, MMPs, intercellular cell adhesion molecule (ICAM) and VEGFR2 [116-119]. Both ROCK and NF-κB are activated by lysophosphatidic acid (LPA), a well-known inflammation mediator. NF-κB functions downstream of ROCK to mediate LPA-induced expression of monocyte chemotactic protein-1 (MCP-1) [120]. Although LPA-induced IL-8 expression is ROCK dependent, it is independent of NF-κB signaling [120].

NF-κB is a key regulator of thrombin- and TNF-α-induced ICAM-1 gene transcription in ECs [121]. In thrombin-induced ICAM-1 expression, RhoA/ROCK pathway increases phosphorylation of IκB kinase (IKK), leading to IκBα degradation, RelA/p65 subunit phosphorylation and translocation of NF-κB into the nucleus [121]. Distinct from thrombin, TNF-α-induced NF-κB activation and ICAM-1 gene transcription in ECs are RhoA/ROCK independent, despite of the fact that TNF-α activates RhoA/ROCK in ECs [121]. Furthermore, a regulatory pattern occurs in mesangial cells, in which TNF-α

promotes nuclear uptake of RelA/p65 by the aid of ROCK-mediated cytoskeletal organization, which is independent of IκBα degradation or p65/RelA phosphorylation [122]. Whether such mechanism exists in ECs needs to be elucidated.

MRTF-A

Myocardin-related transcription factor (MRTF) serves as an actin-regulated transcription coactivator for the serum response factor (SRF) [123]. MRTF-SRF signaling elevates transcription of VE-cadherin (*CDH5*) [124] and cysteinerich 61 (*CYR61*, also called *CCN1*) [125], thereby regulates vascular integrity, vessel growth and maturation. During sprouting, SRF/MRTF specifically enhances development of filopodia protrusion in tip cells by activating *Myl9* gene expression, which encodes myosin regulatory light polypeptide 9 [126].

The activation of MRTF-SRF is mediated by ROCK [127]. Briefly, the interaction between globular actin (G-actin) and MRTF retains MRTF within the cytoplasm of resting cells [123]. Upon activation, Rho GTPase and ROCK induce G-actin to polymerize into filamentous actin (F-actin) via ROCK-LIMK-cofilin pathway, dissociating MRTF from G-actin to enter the nucleus [127]. In the nucleus, MRTF interacts with the transcription factor SRF to induce target gene transcription [127]. In addition, ROCK promotes phosphorylation of MRTF-A, which facilitates the expression of SRF-responsive genes [128].

Upon activation by VEGF, Rho/ROCK signaling promotes nuclear translocation of MRTF-A in mesenchymal stem cells (MSCs), which differentiate into ECs [129]. RhoA/ROCK/MRTF-A signaling also regulates differentiated multipotent MSCs by increasing expression of integrins. Treatment with either Rho inhibitor C3 transferase or ROCK inhibitor Y-27632 markedly reduces expression of integrin α1, α5 and β1, and completely abrogates sprouting of MSC-derived ECs [130].

Hypoxia inducible factor-1 (HIF-1)

Advanced solid tumors and ischemic tissues are featured by hypoxic microenvironment [131]. Hypoxia activates transcriptional factor HIF which promotes transcription of pro-angiogenic genes [131]. In normoxic condition, HIF-1α is ubiquitinated and degraded rapidly [132]. However, HIF-1α is stabilized in hypoxic condition [133]. Takata *et al*. reported that low oxygen conditions stimulate RhoA activity in HUVECs. Interfering with RhoA or ROCK2 by small interfering RNAs, as well as fasudil pretreatment, promote degradation of HIF-1α by proteasome. Therefore, HIF-1α-dependent expression of VEGF is disrupted [133]. In this study, although protein levels of HIF-1α and VEGFR2 are inhibited by fasudil in ECs, their mRNA levels remain unchanged, suggesting that RhoA/ROCK pathway involves in posttranscriptional regulation of HIF-1α/VEGFR2 expression. These results emphasize the role of Rho/ROCK in regulating protein degradation or translation. Further studies are needed to determine the mechanism by which Rho/ROCK pathway modulates HIF-1α ubiquitination.

ERK

The negative regulation between ERK and ROCK signaling has been reported in ECs. ERK signaling antagonizes ROCK during angiogenesis [134]. Src family kinases (SFK) activates ERK, which in turn restrains the RhoA/ROCK pathway, leading to the mitigation of endothelial tube regression [134]. Inhibition of ERK signaling reduces tube formation of ECs and suppresses angiogenesis through activating ROCK- MLC pathway, which is abrogated by ROCK inhibition [135]. On the other hand, ROCK acts as a negative regulator of ERK signaling pathway since ROCK inhibitor H-1152P enhances VEGF-induced ERK1/2 activation in ECs [136]. Crosstalk of RhoA/ ROCK signaling in ECs has been summarized in Figure 3.

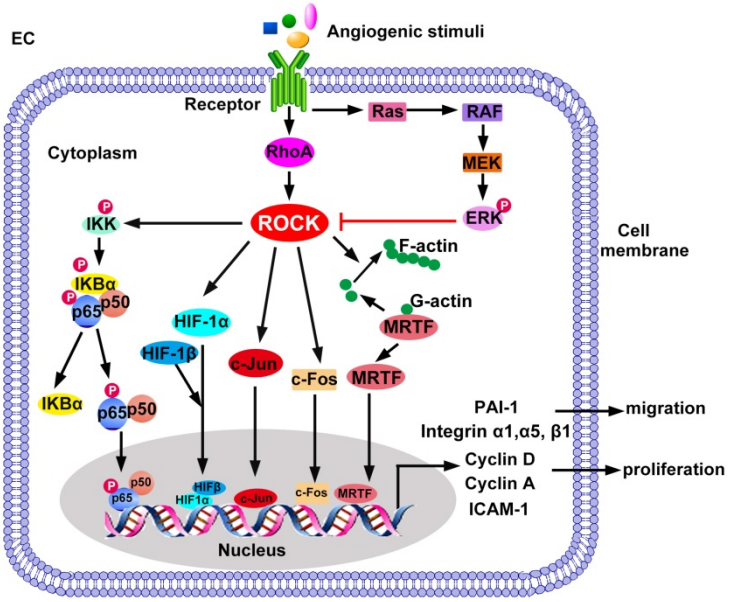


Figure 3. ROCK-regulated gene expression in EC migration and proliferation. ROCK promotes translocation of p65/p50, c-Jun, c-Fos, and HIF-1α/HIF-1β into the cell nucleus. ROCK also releases MRTF from inhibitory G-actin in the cytoplasm and promotes nuclear translocation of MRTF. ERK negatively regulates ROCK signaling. The transcription factors bind to specific DNA elements to activate transcription of target genes essential for EC migration and proliferation. ERK: extracellular regulated protein kinase; HIF: hypoxia-inducible factor; ICAM-1: intercellular adhesion molecule-1; IκB: inhibitor of NF-κB; IKK: IκB kinase; MEK: mitogen-activated protein kinase kinase; MRTF: myocardin-related transcription factor; PAI-1: plasminogen activator inhibitor-1.

ROCK in regulation of the angiogenic microenvironment

The communications between ECs and the surrounding microenvironment are necessary for ECs activation and angiogenesis [4]. The angiogenic microenvironment is constituted by extracellular matrix, various secretary molecules, and adjacent non-ECs including parenchymal cells, perivascular mural cells, and inflammatory cells [4]. In this section, we discuss the roles of ROCK in regulating expression of VEGF and MMPs, release of microvesicles, and behavior of macrophages and pericytes (Figure 4 and Table 3).

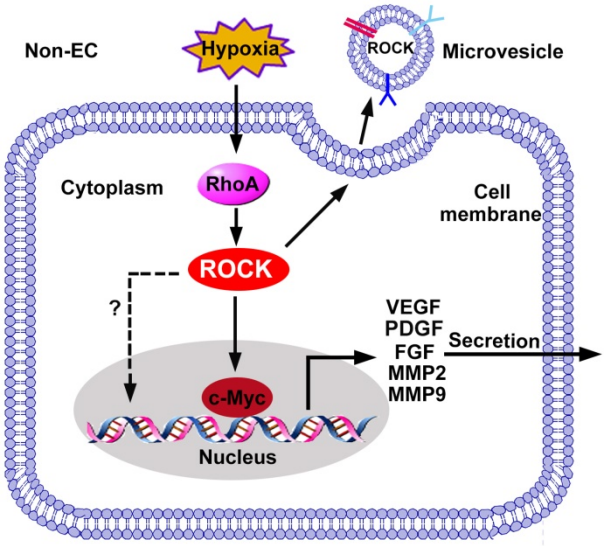


Figure 4. ROCK regulated gene expression in non-ECs. The expression of pro-angiogenic factors in non-ECs is regulated by the RhoA/ROCK pathway. ROCK promotes c-Myc binding to *VEGF* promoter and increases the transcription of *VEGF*. In addition, ROCK increases expression of FGF, MMP2/9 and TGF-β through unknown mechanisms. ROCK is also required for the release of microvesicles, and ROCK can be encapsulated into microvesicles to be received by ECs. FGF: fibroblast growth factor; PDGF: platelet-derived growth factor; MMP: matrix metalloproteinase; VEGF: vascular endothelial growth factor.

VEGF

ROCK is an upstream kinase regulating the expression of VEGF from non-ECs. In colon cancer cells, hypoxia activates RhoA-ROCK signaling, which activates *VEGF* gene transcription by increasing the binding affinity of c-Myc and the *VEGF* promoter [137]. The Rho-ROCK-c-Myc cascade also contributes to VEGF induction in ovarian cancer cells [138]. Moreover, administration of ROCK inhibitor AMA0 428 decreases VEGF levels in the diabetic retina, suggesting the function of ROCK in promoting VEGF expression [139].

MMPs

During angiogenic sprouting, MMPs are required for ECM degradation [140]. Besides, MMPs facilitate release of ECM-bound angiogenic growth factors, cleave the adhesions junctions, proteolyze ECM components to expose the binding site with integrin, all of which promote angiogenesis [141]. Studies demonstrate that ROCK increases the expression and secretion of MMPs from non-ECs. For instance, RhoA knockdown reduces the activities of RhoA and ROCK, and suppresses expression of MMP2 and MMP9 in breast cancer cells [142]. ROCK inhibitor fasudil suppresses gene and protein expressions of MMP2 and MMP9 in glioma cells [143]. Inhibition of Rho or ROCK blocks PDGF-BB-induced MMP2 expression in rat VSMCs [144].

Table 3. Summary of genes and proteins regulated by ROCK in angiogenesis

Gene or protein	Expression	Mechanism	Function of ROCK in angiogenesis
VEGF	↑	Rho/ROCK/c-Myc promotes transcription of *VEGF* gene.	Promotes EC migration, proliferation, increases cell permeability [137, 138]
MMP	↑	Unknown.	Cleavage of ECM [142-144]
Integrins α1, α5 and β1	↑	RhoA/ROCK/MRTF-A increases transcription of genes encoding integrins α1, α5 and β1.	Promotes migration and tube formation [130]
HIF	↑	ROCK inhibits proteasome-mediated degradation of HIF-1α.	Regulates transcription of genes involved in angiogenesis [133]
VEGFR2	↑	Rho/ROCK regulates VEGFR-2 protein level posttranscriptionally.	Promotes ECs migration, proliferation, increases cell permeability [133]
IL-8	↑	ROCK increases transcription of *IL-8* gene through p38 and JNK signaling.	Increases inflammatory responses [120]
MCP-1	↑	RhoA/ROCK increases transcription of *MCP-1* gene through p38/JNK/NF-κB signaling.	Increases inflammatory responses [120]

Extracellular vesicles

Extracellular vesicles are classified as exosomes, microvesicles and apoptotic vesicles [145]. Their cargo varies with respect to proteins, lipids and nucleic acids, depending on the cells which they originate from [146]. After entering recipient cells, they facilitate cell-to-cell communication in diseases including pathological angiogenesis [146]. Yi *et al.* found that exosomes derived from metastatic ovarian cancer enhance viability, migration and tube formation of HUVECs. Mass spectrometry analysis revealed higher levels of 10 pro-angiogenic proteins, including ROCK1 and ROCK2, in exosomes secreted from high-grade ovarian cancers compared to

low-grade ones [147]. In another study, microvesicles generated from rat plasma during cerebral ischemia encapsulate active ROCK and increase permeability of the endothelial barrier *in vitro* [148]. Pre-treatment of either endothelial barriers or microvesicles themselves with Y-27632 before the co-culture attenuates the reduction of transendothelial electrical resistance [148]. It is noteworthy that ROCK is also involved in the generation of microvesicles, which requires cytoskeleton reorganization and actin-myosin contraction [149].

Macrophages

Macrophages synthesize adhesion molecules, and release chemokines, growth factors, and proteases, amplifying angiogenesis in inflammatory diseases [150]. Two types of active macrophages exist *in vivo*. Monocyte-derived non-polarized macrophages (M0) are polarized to M1 and M2 phenotypes under the stimulation of LPS/IFN-γ and IL-4/IL-13, respectively [151]. ROCK regulates polarization and inflammatory cytokine secretion of macrophages (Figure 5). First, ROCK determines whether macrophages polarize into M1 or M2 subtypes. ROCK2 activation preferentially promotes the polarization and function of M2 macrophages [152]. Injection of M2 macrophages enhance choroidal neovascularization, while M1 macrophages reduce it [152], possibly by secretion of IL-12 and TNF-α. Selective ROCK2 inhibition decreases M2-like macrophages and choroidal neovascularization in aged mice with macular degeneration [152]. Similarly, M1 macrophages have anti-angiogenic effects, whereas M2 macrophages promote angiogenesis through secretion of VEGF, FGF, PDGF, and MMPs [153, 154]. RhoA/ROCK signaling has been demonstrated to regulate macrophage phenotype, polarity and function, probably through regulating the actin cytoskeleton [155]. Disruption of RhoA/ROCK pathway induces extreme elongation of M0 and M2 but not M1 macrophages and inhibits expression of M2-specific but not M1-specific molecular markers [155]. Therefore, targeting M2 macrophages by specific ROCK2 inhibitors is promising for anti-tumor therapy.

Pericytes

Pericytes are required for vascular stability and maturation [156]. They are incorporated into the newly formed vessels to support angiogenesis as a constituent of the vessel wall [156]. Durham *et al.* reported that deletion of ROCK-associated myosin phosphatase-RhoA-interacting protein (MRIP) in pericytes enhances contractility and force production, which in turn increases angiogenic sprouting of the co-cultured ECs [157]. In addition, ROCK facilitates the communications between ECs and pericytes. In high glucose-treated ECs, activation of ROCK promotes the shedding of endothelial microvesicles carrying miR-503 [158]. Taken up by recipient pericytes, these microvesicles reduce expression of Ephrin-B2 (EFNB2) and VEGF, resulting in impaired migration and proliferation of pericytes [158].

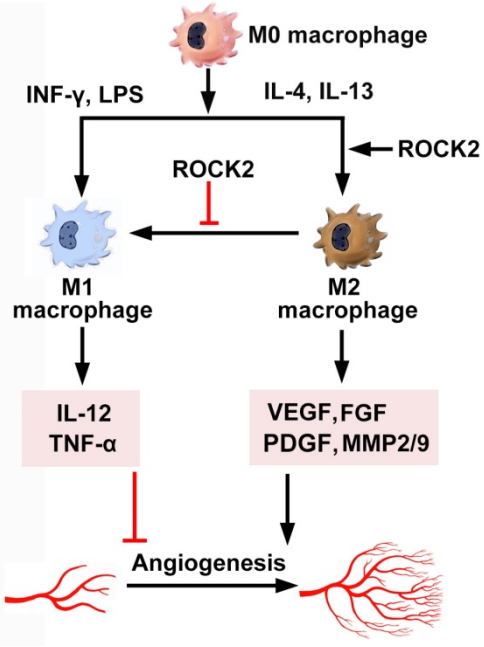


Figure 5. ROCK activation in macrophages. Monocyte-derived non-polarized macrophages (M0) are polarized to M1 and M2 phenotypes under the stimulation of LPS/IFN-γ and IL-4/IL-13, respectively. ROCK2 promotes the polarization of M2 macrophages. Inhibition of ROCK2 modifies the morphological properties of M2 macrophages, thereby inhibits the expression of VEGF, PDGF, FGF and MMPs. The cytokines, proteases and growth factors released by M2 mediate its pro-angiogenic role. Moreover, inhibition of ROCK2 repolarizes M2 to M1, which may repress angiogenesis by secretion of IL-12 and TNF-α. FGF: fibroblast growth factor; IL: interleukin; INF-γ: interferon-γ; LPS: lipopolysaccharide; PDGF: platelet-derived growth factor; MMP: matrix metalloproteinase; TNF: tumor necrosis factor; VEGF: vascular endothelial growth factor.

Therapeutic potential of ROCK inhibitors

Considering the vital role of ROCK in angiogenesis, targeting ROCK may have therapeutic potential for angiogenesis-related diseases. To date, various ROCK inhibitors have been produced. Among these inhibitors, fasudil and ripasudil have been approved clinically for cerebral vasospasm and glaucoma, respectively [159]. The crystal structures of ROCK1 complexes with 4 different ATP-competitive inhibitors, including fasudil (Protein Data Bank code: 2ESM; Figure 6, generated by PyMOL, Schrödinger, Inc.), hydroxyfasudil, Y-27632 and H-1152P, have been revealed [160, 161]. Proposed by Takami *et al.*, the ATP-binding pocket can be divided into 3 regions according to the crystal structures [162]. As shown in

Figure 6C, the isoquinoline ring of fasudil is structurally similar to purine, therefore it binds to the ATP adenine-binding region (A region) at the bottom of the pocket in the kinase domain of ROCK. The sulfonyl group of fasudil serves as a linker and occupies the ATP pentose ring-binding region of the pocket (F region). The linker connects the isoquinoline ring and the piperazine ring of fasudil which locates in the opening area at the top of the pocket (D region).

The development of high-selective inhibitors for ROCK is restricted due to the high homology of ATP-binding region between ROCK and several other protein kinases. Therefore, most of the ROCK inhibitors are not completely specific, and may target other kinases [163-167]. As shown in Table 4, the IC50 values of fasudil (1.65 μM), Y-27632 (3.27 μM) and H-1152P (0.36 μM) against cGMP dependent protein kinase (PKG, also known as protein kinase G) are just 10 to 30 times higher than those against ROCK2 (0.158, 0.162 and 0.012 μM, respectively) [168]. Within the concentration ranges, these inhibitors repress ROCK and PKG. Both angiogenic and anti-angiogenic functions of PKG have been reported [169, 170]. Modulation of PKG might contribute to the effects of fasudil, Y-27632 and H-1152P on angiogenesis. Besides PKG, ROCK inhibitors target kinases such as PKA, PKC and CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) with relatively low IC50 values. This may partially explain the distinct effects of ROCK inhibitors on angiogenesis. Studies using Y-27632 or fasudil as ROCK inhibitors demonstrated conflicting results [135, 136, 171]. The anti-angiogenic function of Y-27632 or fasudil is likely mediated by other kinases together with ROCK under these experimental settings. Therefore, the off-target effects of ROCK inhibitors should be considered when evaluating the role of ROCK on angiogenesis.

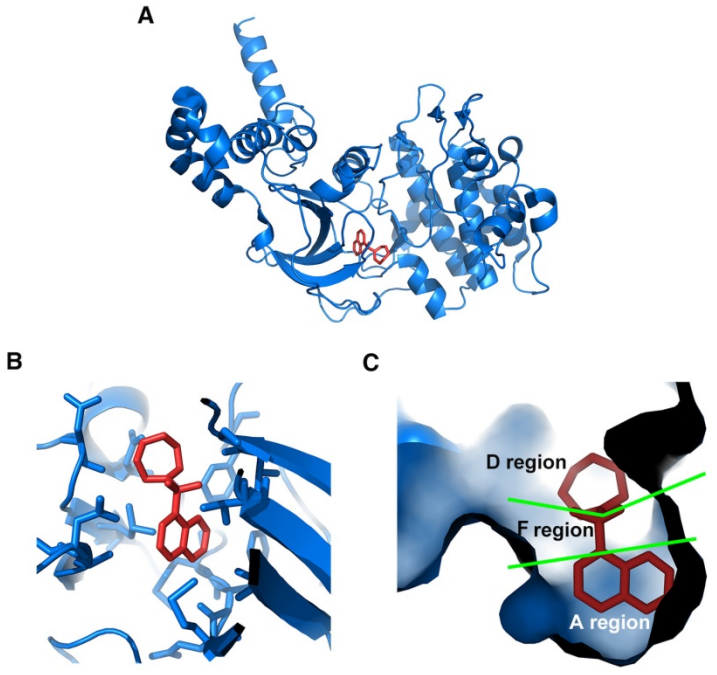


Figure 6. Crystal structure of ROCK bound with fasudil. (A) Ribbon representation of overall crystal structure of ROCK (blue) in complex with fasudil (red); **(B)** Amino acid residues in the ROCK kinase domain interact with fasudil; **(C)** Cavity surface representation of ATP-binding pocket in the ROCK kinase domain. It is composed of A, F, and D regions. The isoquinoline ring of fasudil occupies A region. The piperazine ring occupies D region. The sulfonyl group that links the isoquinoline ring and the piperazine ring binds to F region. Protein Data Bank code: 2ESM. Images were generated using PyMOL (Schrödinger, Inc.).

The ATP-binding regions of ROCK1 and ROCK2 are almost identical, making it difficult to develop ROCK isoform-selective inhibitors [172]. Recently, several ROCK2 specific inhibitors have been discovered by means of various screening methods [172]. These inhibitors have a high selectivity for ROCK2, thus will help investigate the role of each ROCK isoform in diseases.

Emerging drug delivery carriers, including extracellular vesicles, might be used to deliver ROCK inhibitors to enhance the specificity and therapeutic efficacy. Studies have shown that ROCK inhibitor fasudil can be efficiently loaded into liposomes [173, 174]. Recently, Gupta *et al*. developed erythrocytes derived nanoerythrosomes as stable and efficient delivery carriers for slow and continous release of fasudil [175]. The half-life of fasudil *in vivo* is extended in nanoerythrosomes [175], and targeting specificity is improved through conjugating a peptide specially located in pulmonary arterial hypertension lesions with the nanoerythrosomes [176]. This carrier system exerts a significant pulmonary vasodilatory effect by ROCK inhibition, and has minimal effect on systemic arterial pressure [176].

To date, ROCK inhibitors have been examined in animal models of ocular angiogenesis and tumor angiogenesis (Table 4).

Ocular angiogenesis

Angiogenesis within the eyes contributes to vision loss in several ocular diseases, including age-related macular degeneration and diabetic retinopathy [177]. ROCK inhibitor ripasudil (also known as K-115) significantly suppresses migration and proliferation of retinal microvascular endothelial cells (HRMECs) by reduction of VEGF-induced ROCK activation [178]. In mice with oxygen-induced retinopathy (OIR), eye drops with 0.8% ripasudil effectively reduce the area of retinal hypoxia induced by OIR. Moreover, ripasudil normalizes vascularity and increases retinal vascular perfusion [178]. In the corneal micropocket assay, another model of angiogenesis in the mouse eye, ROCK inhibitor

AMA0526 efficiently inhibits corneal neovascularization and decreased inflammatory cell infiltration [179]. In addition, ROCK inhibitor AMA0428 decreases vessel growth and leakage in a mouse model of laser-induced choroidal neovascularization [180].

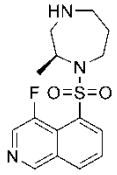
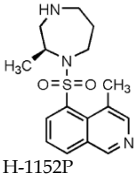
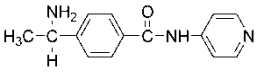
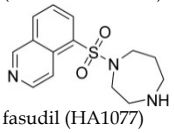
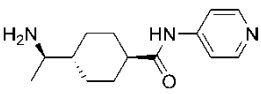
Tumor angiogenesis

Constitutive activation of ROCK is often associated with invasive and metastatic phenotypes of human cancers [181-183]. Tumor-derived ECs display an enhanced ability to form capillary networks, correlating with a constitutively high level of RhoA/ROCK signaling [71]. In a lung cancer cell-transplanted mouse model, Wf-536 (also known as Y-32885), a derivative of Y-27632, inhibits cancer cell invasion and EC migration [184]. Wf-536 and the MMP inhibitor marimastat display a synergistic anti-angiogenic effect in a prostate cancer xenotransplant model [185]. Moreover, ROCK inhibitors normalize function and structure of tumor vasculature [71], which is highly permeable due to lack of uniform pericyte and basement membrane coverage [186, 187].

Conclusions and Perspectives

ROCK conditions tissue microenvironment by promoting the secretion of growth factors and cytokines from non-ECs. These pro-angiogenic factors along with mechanical forces resulting from EC-microenvironment interaction, in turn, modulate ROCK signaling to regulate EC function in angiogenesis, including permeability, migration, proliferation and tubulogenesis. ROCK contributes to these cellular functions largely through regulation of cytoskeleton-associated proteins. In addition, several transcription factors are activated by ROCK to regulate expression of pro-angiogenic genes.

Table 4. Effects of ROCK inhibitors on angiogenesis

Therapeutic Implications	Compound	IC50 values for ROCK (μM)	IC50 values for off-target protein kinases (μM)	Models	Pharmacological effects
Retinal angiogenesis	 Ripasudil (K-115)	ROCK1 (0.051), ROCK2 (0.019) [166]	PKA (2.1), PKC (27), CaMKII (0.37) [166]	oxygen-induced retinopathy	Reduces retinal hypoxia area, normalizes retinal neovasculature [178]
	AMA0526 (structure undisclosed)	Undisclosed.	Undisclosed.	corneal micropocket assay	Inhibits corneal neovascularization, decreases inflammatory cell infiltration [179]
	AMA0428 (structure undisclosed)	Undisclosed.	Undisclosed.	neovascular age-related macular degeneration	Reduces choroidal neovascularization, blocks inflammation and fibrosis [180]
	 H-1152P	ROCK2 (0.012) [168]	PKA (3.03), PKC (5.68), PKG (0.36), MLCK (28.3), CaMKII (0.18), GSK3α (60.7), Src (3.06), MKK4 (16.9), EGFR (50) [168]	oxygen-induced retinopathy	Enhances sprouting angiogenesis driven by VEGF [136]
Tumor angiogenesis	 Wf-536	ROCK kinase domain (0.4) [167]	PKN kinase domain (0.4) [167]	a lung cancer cell-transplanted mouse model	Inhibits invasion and migration of cancer cells, inhibits formation of capillary-like tubes [184]
	Wf-536 and Marimastat (an MMP inhibitor)	Not available.	Not available.	human prostate cancer xenotransplants	Suppresses angiogenesis and growth of tumor, improves survival [185]
	 fasudil (HA1077)	ROCK2 (0.158) [168]	PKA (4.58), PKC (12.3), PKG (1.65), MLCK (21.6), CaMKII (6.7), GSK3α (>100) [168]	an intracerebral human glioma cell xeno-graft mouse model	Suppresses angiogenesis and tumor growth [143]
	 Y-27632	ROCK2 (0.162) [168]	PKA (>100), PKC (25.8), PKG (3.27), MLCK (>100), CaMKII (13), GSK3α (50) [168]	tumor capillary ECs isolated from transgenic TRAMP mice bearing prostate adenocarcinoma	Normalizes the ability of tumor ECs to form tubular networks, restores mechanosensitivity [71]

CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II; GSK3α: glycogen synthase kinase 3 alpha; MKK4: mitogen-activated protein kinase kinase 4; MLCK: myosin light-chain kinase; PKA: protein kinase A; PKC: protein kinase C; PKG: cGMP dependent protein kinase; PKN: protein kinase N; TRAMP: transgenic adenocarcinoma of mouse prostate.

With ongoing efforts to decipher its role in angiogenesis, it may become possible to target ROCK for anti-angiogenic treatment of cancer, ocular neovascularization and other angiogenesis-related diseases. ROCK inhibitors showed significant prospects for chemotherapy based on their anti-angiogenic effects and their ability to normalize tumor vessels. It may be more effective to combine ROCK inhibitors with other angiogenesis antagonists, including MMP inhibitors or VEGF antibodies, to enhance the anti-angiogenic effects.

Although extensive studies on ROCK have been performed, many issues remain unresolved, such as the off-target effect of ROCK inhibitors and the isoform-specific regulation of angiogenesis. There is also an unmet need to develop more specific and clinically suitable ROCK inhibitors to improve anti-angiogenic therapies.

Abbreviations

AJ: adherens junction; AMPK: adenosine monophosphate-activated protein kinase; Ang II: angiotensin II; AT1R: Ang II type 1 receptor; AT2R: Ang II type 2 receptor; CCM2: cerebral cavernous malformations 2; EC: endothelial cell; ECM: extracellular matrix; EGF: epidermal growth factor; eNOS: endothelial nitric oxide synthase; ER: estrogen receptor; ERK: extracellular signal-regulated kinase; FAK: focal adhesion kinase; FGF: fibroblast growth factor; HIF: hypoxia-inducible factor; HMEC-1: human microvascular endothelial cell-1; HNF4A: hepatocyte nuclear factor 4 alpha; HRMEC: human retinal microvascular endothelial cell; HSP90: heat shock protein 90; ICAM-1: intercellular adhesion molecule-1; IL-8: interleukin-8; KRIT1: Krev interaction trapped protein 1; LIMK: LIM motif-containing protein kinase; LPA: lysophosphatidic acid; MAPK: mitogen-activated protein kinase; MCP-1: monocyte chemotactic protein-1; MEKK3: MAPK/ERK kinase kinase 3; MLC: myosin light chain; MMP: matrix metalloproteinase; MS1: mile sven 1; NF-κB: nuclear factor-κB; OIR: oxygen-induced retinopathy; ox-LDL: oxidized low-density lipoprotein; PAI-1: plasminogen activator inhibitor-1; PCNA: proliferating cell nuclear antigen; PI3K: phosphatidylinositol 3-kinase; PP2A: protein phosphatase-2A; PDGF: platelet-derived growth factor; PH: pleckstrin homology; RBD: Rho-binding domain; RhoA: ras homolog gene family member A; RhoGEF: Rho guanine nucleotide exchange factor; ROCK: Rho-associated coiled-coil kinase; SFK: Src family kinase; Shh: sonic hedgehog; TNF: tumor necrosis factor; TRPV4: transient receptor potential vanilloid 4; VCAM-1: vascular cell adhesion molecule-1; VEGF: vascular endothelial growth factor; VEGFR-2: vascular endothelial growth factor receptor-2; VPF: vascular permeability factor.

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Competing Interests

The authors have declared that no competing interest exists.

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