

ASSOCIATE EDITOR: RHIAN M. TOUYZ

# Rho Kinases in Health and Disease: From Basic Science to Translational Research

Gervaise Loirand

Institut National de la Santé et de la Recherche Médicale UMR1087, Université de Nantes, CHU Nantes, l'institut du thorax, Nantes, France

Abstract	1075
I. Introduction	1075
II. Rho-Associated Kinase Activity, Targets, and Roles	1075
III. Lessons from Animal Models of Rho-Associated Kinase Deletion	1076
A. Cardiovascular Physiology and Pathophysiology	1077
1. Cardiac Function and Hypertrophy	1077
2. Blood Pressure and Hypertension	1080
3. Pulmonary Arterial Hypertension	1080
4. Atherosclerosis	1080
5. Neointimal Thickening/Restenosis	1081
6. Endothelial Function and Dysfunction	1081
B. Asthma	1081
C. Glucose Metabolism	1082
D. Kidney Function and Renal Disease	1082
IV. Association between <i>Rho-Associated Kinase</i> Variants and Disease Susceptibility	1082
A. Cardiovascular Diseases	1082
B. Cancer	1084
C. Autoimmune Diseases	1084
D. Glaucoma	1084
V. Rho-Associated Kinase Activity or Expression: Candidate Biomarker of Human Diseases	1084
A. Cardiovascular Diseases	1084
B. Autoimmune Diseases	1086
C. Cancer	1086
VI. Pharmacological Rho-Associated Kinase Inhibitors	1087
A. Rho-Associated Kinase Inhibitors	1087
B. Clinical Evaluation and Potential Applications of Rho-Associated Kinase Inhibitors	1087
1. Central Nervous System Disorders	1087
a. Subarachnoid hemorrhage-induced vasospasm	1087
b. Cerebral stroke	1088
c. Spinal cord injury	1088
d. Neurodegenerative Diseases	1089
2. Cardiovascular diseases	1089
a. Systemic hypertension	1089
b. Pulmonary arterial hypertension	1089
c. Spastic and stable effort angina	1089
d. Raynaud phenomenon	1090
3. Glaucoma	1090
4. Autoimmune Diseases	1090
5. Perspectives for Other Diseases	1090

This research was supported by the Institut National de la Santé et de la Recherche Médicale, the French Agence Nationale de la Recherche [Grant ANR-11-BSV1-013-01], and the Fondation de France [Grant 2013 00038590].

**Address correspondence to:** Gervaise Loirand, Institut National de la Santé et de la Recherche Médicale UMR1087, Université de Nantes, CHU Nantes, l'institut du thorax, 8 Quai Moncoussu, 44007 Nantes, France. E-mail: gervaise.loirand@univ-nantes.fr  
dx.doi.org/10.1124/pr.115.010595.

VII. Conclusions .....	1091
References .....	1091

**Abstract**—Rho-associated kinases ROCK1 and ROCK2 are key regulators of actin cytoskeleton dynamics downstream of Rho GTPases that participate in the control of important physiologic functions, S including cell contraction, migration, proliferation, adhesion, and inflammation. Several excellent review articles dealing with ROCK function and regulation have been published over the past few years. Although a brief overview of general molecular, biochemical, and functional properties of ROCKs is included, an effort has been made to produce an original work by collecting and synthesizing recent studies aimed at translating basic

discoveries from cell and experimental models into knowledge of human physiology, pathophysiological mechanisms, and medical therapeutics. This review points out the specificity and distinct roles of ROCK1 and ROCK2 isoforms highlighted in the last few years. Results obtained from genetically modified mice and genetic analysis in humans are discussed. This review also addresses the involvement of ROCKs in human diseases and the potential use of ROCK activity as a biomarker or a pharmacological target for specific inhibitors.

## I. Introduction

Rho-associated kinases (ROCKs) belong to the AGC family of serine/threonine kinases. Originally called ROK $\alpha$ , ROCK2 is the first ROCK isoform that was identified as a ubiquitously expressed RhoA binding protein containing an N-terminal serine/threonine kinase domain, highly related to the human myotonic dystrophy kinase. The ROCK kinase domain is followed by a coiled-coil region containing the Rho-binding domain (RBD) and a pleckstrin homology domain with a cysteine-rich domain toward the C terminus (Manser et al., 1994; Leung et al., 1995). ROCK1 (ROK $\beta$  or p160ROCK) was then discovered as another highly homologous active RhoA binding protein (Leung et al., 1996). Human *ROCK1* and *ROCK2* genes are located on chromosome 18 (18q11.1) and chromosome 2 (2p24), respectively. ROCKs have been extensively studied and are identified as major downstream effectors of RhoA, playing a central role in the regulation of actin cytoskeleton dynamics and generation of actin-myosin contractility. Today, 20 years after their discovery, a PubMed search for “Rho kinase” returns approximately 10,000 references, including approximately 1000 reviews (Loirand et al., 2006; Thumkeo et al., 2013; Knipe et al., 2015). Although initial ROCK studies focused on cell biology and function, subsequent evidence has recognized the role of ROCKs in the regulation of physiologic processes and their involvement in cardiovascular,

metabolic, and neurologic disorders and cancer. Studies of ROCKs, both in humans and in genetically modified mouse models, have enhanced general knowledge of their pathophysiological mechanisms and have defined ROCK1 and ROCK2 as attractive and promising targets of inhibitory molecules for potential applications in a broad range of human diseases.

## II. Rho-Associated Kinase Activity, Targets, and Roles

The overall identity of the human ROCK1 and ROCK2 protein sequences is 64% (Rath and Olson, 2012). ROCK1 and ROCK2 have a similar structure, with 90% identity in the kinase domain, indicating that both isoforms share the same protein targets. More than 30 common ROCK substrates have been identified, most of which are involved in the regulation of cytoskeletal dynamics, cell morphology, and contraction (Schofield and Bernard, 2013). One of the most described targets is the myosin binding subunit (MYPT1) of the myosin light chain phosphatase (MLCP). ROCK-mediated phosphorylation of MYPT1 on Thr697 and Thr855 inhibits the catalytic activity of MLCP, leading to an increase in myosin light chain (MLC) phosphorylation. This mechanism is responsible for nonmuscle cell contraction and for sustained smooth muscle contraction through the process of calcium sensitization.

**ABBREVIATIONS:** AMA0076, 3-[2-(Aminomethyl)-5-[(pyridin-4-yl)carbamoyl]phenyl] benzoate; Ang II, angiotensin II; AR-12286, 2-(dimethylamino)-N-(1-oxo-2H-isoquinolin-6-yl)-2-thiophen-3-ylacetamide; AR-13324, [4-[(2S)-3-amino-1-(isoquinolin-6-ylamino)-1-oxopropan-2-yl]phenyl]methyl 2,4-dimethylbenzoate; methanesulfonic acid; BA-210, 4-N-(3-chloro-7-methoxyacridin-1-yl)-1-N,1-N-diethylpentane-1,4-diamine; dihydrochloride; eNOS, endothelial nitric oxide synthase; GSK269962A, N-(3-[[2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-1H-imidazo[4,5-c]pyridin-6-yl]oxy]phenyl)-4-[[2-(4-morpholinyl)ethyl]oxy]benzamide; H-1152, (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinolyl)sulfonyl]-hexahydro-1H-1,4-diazepine; IL, interleukin; K-115, 4-fluoro-5-[[2-(2S)-2-methyl-1,4-diazepan-1-yl]sulfonyl]isoquinoline; KD-025, 2-(3-(4-((1H-indazol-5-yl)amino)quinazolin-2-yl)phenoxy)-N-isopropylacetamide (formerly SLx-2119); L-NAME, N-nitro-L-arginine methylester; MLC, myosin light chain; MLCP, myosin light chain phosphatase; MYPT1, myosin binding subunit of the myosin light chain phosphatase; NO, nitric oxide; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; OVA, ovalbumin; PAH, pulmonary arterial hypertension; RBD, Rho-binding domain; ROCK, Rho-associated kinase; SAR407899, 6-(piperidin-4-yloxy)isoquinolin-1(2H)-one; SB-772077-B, 4-(7-[[3(S)-3-amino-1-pyrrolidinyl]carbonyl]-1-ethyl-1H-imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-amine; SM-ROCK2, smooth muscle-specific Rho-associated kinase 2—overexpressing; SR3677, N-[2-[2-(Dimethylamino)ethoxy]-4-(1H-pyrazol-4-yl)phenyl]-2,3-dihydro-1,4-benzodioxin-2-carboxamide dihydrochloride; Y-27632, (+)-(R)-*trans*-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexane carboxamide; Y-39983, 4-[(1R)-1-aminoethyl]-N-(1H-pyrrolo[2,3-b]pyridin-4-yl)benzamide hydrochloride (also known as RKI-983 and SNJ-1656).

Although both ROCK isoforms regulate MLCP activity and MLC phosphorylation, the mechanisms involved are likely different because only ROCK2, but not ROCK1, binds directly to MYPT1 (Wang et al., 2009). Furthermore, depending on the cell type, downstream ROCK effects would depend on both the ROCK isoforms and ROCK targets expressed. Both ROCK isoforms are ubiquitously expressed; however, ROCK1 is abundantly expressed in the liver, lung, kidney, spleen, testis, and circulating inflammatory cells, whereas ROCK2 is mainly found in the heart, muscle (including smooth muscle), and brain. ROCK1 and ROCK2 also differ by their subcellular location (Schofield and Bernard, 2013). ROCK2 is found in the cytoplasm, is associated with vimentin and actin stress fibers, and can be translocated to the plasma membrane (Leung et al., 1995; Sin et al., 1998; Chen et al., 2002). Subcellular localization of ROCK1 is less clear. ROCK1 is thought to be associated with the catenin/E-cadherin complex in the apical junctions of endothelial cells and with the microtubule organizing center (Chevrier et al., 2002; Nishimura and Takeichi, 2008).

Both ROCK1 and ROCK2 contain a single RBD in the central coiled-coil region of the protein that binds RhoA, RhoB, and RhoC, which promotes activation (Fig. 1). However, identity of the RBD of ROCK1 and ROCK2 is only 59%, suggesting that these kinases can be regulated differently (Rath and Olson, 2012) (Table 1). The C-terminal region maintains ROCKs in an autoinhibited state in basal conditions, likely through direct binding to the kinase domain, either by intramolecular or homodimeric interactions (Amano et al., 1999; Doran et al., 2004; Shimizu et al., 2005). Activation of ROCK must release the protein from this autoinhibitory conformation. This is achieved by several mechanisms, including Rho protein binding to RBD (Amano et al., 2000), lipid (arachidonic acid, phosphatidylinositol-phosphates) interaction with the pleckstrin homology domain (Feng

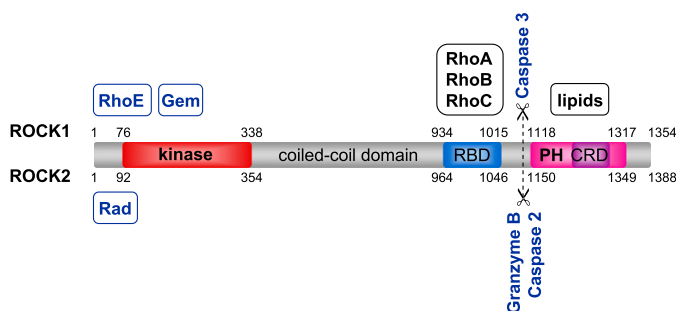
et al., 1999; Yoneda et al., 2005; Lowery et al., 2007; Wen et al., 2008), and enzymatic removal of the C terminus by caspases or granzyme B (Sebbagh et al., 2001, 2005; Sapet et al., 2006), which can be different for ROCK1 and ROCK2 (Table 1). In addition, ROCK1 is subjected to negative control by the small G protein RhoE, which binds to its N terminus and prevents RhoA binding to RBD (Riento and Ridley, 2003). This negative regulation is counteracted by phosphoinositide-dependent kinase-1, which prevents RhoE binding (Pinner and Sahai, 2008). The small G proteins Gem and Rad also bind and inhibit ROCK1 and ROCK2, respectively, by unknown mechanisms (Ward et al., 2002). Finally, phosphorylation, including autophosphorylation mechanisms, can either attest activation or modulate ROCK activity (Lowery et al., 2007; Lee et al., 2010; Chuang et al., 2012).

In summary, despite the strong identity of ROCK1 and ROCK2, different upstream activating/regulating mechanisms, different subcellular distributions of ROCK1 and ROCK2, and coexpression of different targets with ROCK1 and/or ROCK2 in different cell types could all contribute to specific ROCK1 and ROCK2 functions. The development of isoform-specific ROCK knockout mice has helped to discriminate common and specific nonredundant functions of ROCK isoforms.

### III. Lessons from Animal Models of Rho-Associated Kinase Deletion

Numerous studies aimed at elucidating the physiologic/pathophysiological roles of ROCKs, relying on the use of pharmacological ROCK inhibitors in animal models, have produced a large body of useful and convincing data. However, interpreting these data is sometimes difficult and limited because of the lack of specificity of commonly used ROCK inhibitors such as fasudil [formerly HA-1077]; and Y-27632 [(+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexane carboxamide]. Indeed, these inhibitors similarly act on both ROCK isoforms and also block other serine-threonine kinases such as protein kinase C-related kinase 1 and protein kinase C-related kinase 2, cAMP-activated protein kinase, and AMP-activated protein kinase (Bain et al., 2007). Mouse models of ROCK deletion have thus been developed to provide powerful tools to discriminate specific and nonredundant functions of ROCK1 and ROCK2 in vivo. Unfortunately, the critical role of ROCK1 and ROCK2 in cell function leads to a high rate of embryonic and perinatal lethality of total homozygous deletion of *Rock1* or *Rock2* (Thumkeo et al., 2003; Shimizu et al., 2005), and the phenotype of both *Rock1*<sup>-/-</sup> and *Rock2*<sup>-/-</sup> mice is influenced by the genetic background.

*Rock1*<sup>-/-</sup> mice in a C57Bl/6 background are born with open eyelids and with an omphalocele, which is



**Fig. 1.** Schematic molecular structure and main regulators of ROCKs. ROCK sequences comprise a kinase domain located at the amino terminus of the protein, followed by a coiled-coil region containing the RBD and a pleckstrin homology domain with a cysteine-rich domain. ROCK-1 and ROCK-2 are highly homologous, with an overall amino acid sequence identity of 65%. Common regulators are indicated in black, whereas specific ROCK1 and ROCK2 regulators are indicated in blue on the corresponding side. CRD, cysteine-rich domain; PH, pleckstrin homology.

TABLE 1  
Regulation of ROCK activity

Mechanism	Effect	ROCK1	ROCK2	Reference
Binding to RBD	Stimulation	RhoA RhoB RhoC	RhoA RhoB RhoC	Amano et al., 1999
Binding to PH domain	Stimulation	PIP <sub>2</sub> , PIP <sub>3</sub>	PIP <sub>2</sub> , PIP <sub>3</sub>	Yoneda et al., 2005; Wen et al., 2008
Binding to N terminus	Inhibition	AA	AA	Feng et al., 1999
Binding	Stimulation (prevents RhoE binding)	RhoE PDK1	—	Riento and Ridley, 2003 Pinner and Sahai, 2008
Binding	Inhibition	Gem	Rad	Ward et al., 2002
Autophosphorylation	Activation marker	Ser1333	Ser1366	Chuang et al., 2012
Phosphorylation	Inhibition	—	Tyr722 (Src)	Lee et al., 2010
	Stimulation (potentiates RhoA effect)		Multiple site (polo-like kinase 1)	Lowery et al., 2007
Cleavage of C terminus	Stimulation	Caspase3	Granzyme B Caspase2	Sebbagh et al., 2001, 2005 Sapet et al., 2006

AA, arachidonic acid; PDK1, phosphoinositide-dependent kinase-1; PH, pleckstrin homology; PIP<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; PIP<sub>3</sub>, phosphatidylinositol-(3,4,5)-trisphosphate.

responsible for the postnatal death of the majority of *Rock1*<sup>-/-</sup> mice (90%) (Shimizu et al., 2005). Surviving *Rock1*<sup>-/-</sup> mice develop normally, with no obvious phenotypic abnormalities other than the eye defects. This phenotype is not observed in *Rock1*<sup>-/-</sup> FVB mice, but 60% of these mice die in utero before embryonic day E9.5 (Zhang et al., 2006). *Rock1*<sup>-/-</sup> FVB mice that are alive at birth have no apparent congenital malformation; this suggests that, in this genetic background, *Rock1* is critical at very early stages of development, before or early after embryo implantation.

Homozygous deletion of *Rock2* in a mixed 129/SvJ and C57BL/6 genetic background leads to fetal death in more than 90% of *Rock2*<sup>-/-</sup> mice, which is attributed to thrombus formation, placental dysfunction, and intra-uterine growth retardation (Thumkeo et al., 2003). In a pure C57BL/6 background, open eyelids at birth and the presence of an omphalocele add to this phenotype, leading to a 99% mortality rate (Thumkeo et al., 2005).

This suggests redundant roles of ROCK isoforms during embryonic development. Nevertheless, in the C57BL/6 mice background, open eyelids at birth and presence of the omphalocele phenotype observed in *Rock1*<sup>-/-</sup> and *Rock2*<sup>-/-</sup> mice indicate that both *Rock1* and *Rock2* are necessary for eyelids and ventricular body wall closure, and each isoform does not compensate for the absence of the other. Interestingly, mice expressing mutated MYPT1 that cannot be phosphorylated by ROCK exhibit a similar omphalocele phenotype, thus supporting a role of ROCK in signaling cascades that regulate closure of the ventral body wall at the umbilical ring (Chen et al., 2015). This particular example suggests that the correlation between the phenotype of mice carrying genetic manipulation of known ROCK targets, as well as upstream ROCK regulators, and ROCK knockout phenotypes can provide useful data to identify ROCK signaling pathway components and to understand their biologic functions. Unfortunately, available data required to establish such phenotypic correlations are still limited. Because

of the high number and diversity of ROCK substrates and upstream ROCK regulators (membrane receptors/ligands; more than 30 RhoA exchange factors), each having numerous different targets in addition to ROCK and different expression profiles according to cell/tissue types, such a correlation is difficult to make and can potentially lead to misinterpretation from what we currently know.

The apparent normal development of *Rock1*<sup>-/-</sup> and *Rock2*<sup>-/-</sup> mice that are born and survive suggests that each ROCK isoform in these mice has been able to compensate for the function of the deleted isoform, which points to the need for careful interpretation of experimental data obtained with these mice. To circumvent these issues, relevant approaches in heterozygous *Rock1*<sup>+/-</sup> and *Rock2*<sup>+/-</sup> mice, as well as conditional and tissue-specific knockout mice, have been developed and prove valuable in elucidating isoform-specific physiologic and pathophysiological ROCK functions.

#### A. Cardiovascular Physiology and Pathophysiology

1. *Cardiac Function and Hypertrophy.* Chronic treatment with ROCK inhibitors limits agonist- or myocardial infarction-induced pathologic cardiac remodeling and hypertrophy in mice, suggesting a role of ROCKs in these processes (Kobayashi et al., 2002; Higashi et al., 2003; Satoh et al., 2003; Hattori et al., 2004; Wang et al., 2005; Loirand et al., 2013). The cardioprotective effects of ROCK inhibitors have been attributed to a reduction of cardiomyocyte hypertrophy induced by increased mechanical strains or soluble hypertrophic signals (Hoshijima et al., 1998; Yanazume et al., 2002; Brown et al., 2006), limitation of fibrosis (Li et al., 2012a; Yang et al., 2012), and inhibition of cardiomyocyte apoptosis (Chang et al., 2006; Shi et al., 2010). Although the mechanisms underlying the involvement of ROCK1 and/or ROCK2 and their respective roles in cardiac hypertrophy have not been completely elucidated, an increase in cardiac ROCK1 activity has been detected in response to pressure

TABLE 2  
Cardiac phenotype in genetically modified mice

Target	Modification	Transgene/Mouse	Phenotype	Reference
<i>Rock1</i>	Haploinsufficiency ( <i>Rock1</i> <sup>+/-</sup> )		Normal heart structure and function No change in cardiac hypertrophy Decreased cardiac fibrosis	Rikitake et al., 2005
	Deficiency ( <i>Rock1</i> <sup>-/-</sup> )		Decreased cardiac fibrosis Decreased cardiomyocyte apoptosis	Zhang et al., 2006
	Deficiency ( <i>Rock1</i> <sup>-/-</sup> )	In <i>Gαq</i> mice <sup>a</sup>	No change in cardiac hypertrophy Preserved left ventricular structure function Decreased cardiomyocyte apoptosis	Shi et al., 2010
<i>Rock2</i>	Cardiac-specific overexpression	ROCK1 in <i>Gαq</i> mice <sup>a</sup>	Accelerated hypertrophic decompensation	Shi et al., 2008
	Cardiac-specific overexpression	Active C-terminal-truncated ROCK1	Extensive cardiac fibrosis	Yang et al., 2012
	Cardiac-specific deletion ( <i>cRock2</i> <sup>-/-</sup> )		Normal heart structure and function	Okamoto et al., 2013
	Haploinsufficiency ( <i>Rock2</i> <sup>+/-</sup> )		Decreased cardiac hypertrophy Normal heart structure	Okamoto et al., 2013
	Haploinsufficiency ( <i>Rock2</i> <sup>+/-</sup> )	In <i>Fhl2</i> <sup>+/-</sup> mice <sup>b</sup>	Decreased cardiac hypertrophy Normal heart structure Cardiac hypertrophy similar to control mice	Okamoto et al., 2013

<sup>a</sup>Mice overexpressing *Gαq* in cardiomyocytes.

<sup>b</sup>Four and a half LIM domains protein 2.

overload (Zhang et al., 2006). The subsequent effect of ROCK activation may be related to the stimulation of hypertrophic gene transcription (Kuwahara et al., 2010), downregulation of endothelial nitric oxide synthase (eNOS), and an increase in oxidative stress (Kobayashi et al., 2002; Mita et al., 2005). *Rock1*<sup>+/-</sup> mice showed normal cardiac structure and function under basal conditions (Rikitake et al., 2005) (Table 2). Surprisingly, cardiac hypertrophy induced by angiotensin II (Ang II) or *N*-nitro-L-arginine methyl ester (L-NAME) assessed by wall thickness, left ventricular mass, cardiomyocyte size, and atrial natriuretic factor expression was also similar in control and *Rock1*<sup>+/-</sup> mice (Rikitake et al., 2005). However, compared with control mice, cardiac fibrosis and expression of fibrosis markers (transforming growth factor- $\beta$ , connective tissue growth factor, and type III collagen) was reduced in *Rock1*<sup>+/-</sup> mice chronically treated with Ang II or L-NAME or after transaortic constriction (Rikitake et al., 2005). A similar observation, associated with a reduction in cardiomyocyte apoptosis, was made in *Rock1*<sup>-/-</sup> mice in response to pressure overload by aortic banding (Zhang et al., 2006) (Table 2). These results are consistent with a major role of ROCK1 in pathologic cardiac fibrosis but not hypertrophy. This profibrotic role of ROCK1 has been confirmed by the extensive cardiac fibrosis due to upregulation of transforming growth factor- $\beta$  and myofibroblast differentiation in a mouse model expressing a truncated activated form of ROCK1 in cardiomyocytes (Yang et al., 2012) (Table 2). The absence of a role of ROCK1 in pathologic hypertrophy was further supported in a mouse model with cardiac-specific overexpression of *Gαq* that develops compensated cardiac hypertrophy. Cardiac overexpression of ROCK1 in these mice results in increased cardiomyocyte apoptosis and accelerated hypertrophic decompensation (Shi et al., 2010). By contrast, deletion

of ROCK1 improves survival, inhibits cardiomyocyte apoptosis, and preserves left ventricular structure and function without ameliorating cardiac hypertrophy in old *Gαq* mice (Shi et al., 2008, 2010) (Table 2). Although ROCK1 appears to play a key role in the transition from hypertrophy to heart failure, its deletion does not mimic the antihypertrophic effect of ROCK inhibitors, suggesting either a role of ROCK2 in the development of cardiac hypertrophy or a ROCK-independent effect of ROCK inhibitors. This latter hypothesis is further supported by the defects in cardiac organogenesis observed after treatment with ROCK inhibitors but not after deletion of *Rock* genes (Wei et al., 2001).

The respective roles of ROCK1 and ROCK2 were further addressed by generating cardiomyocyte-specific *Rock2*<sup>-/-</sup> (*c-Rock2*<sup>-/-</sup>) mice (Table 2). These mice display normal hemodynamic parameters, cardiac anatomy, and function under basal conditions (Okamoto et al., 2013). However, Ang II infusion-induced cardiac hypertrophy is significantly reduced in *c-Rock2* mice compared with control mice (Okamoto et al., 2013). This protective effect of cardiomyocyte *Rock2* deletion is associated with a decrease in hypertrophy-related fetal gene expression, intraventricular fibrosis, cardiac apoptosis, and oxidative stress. The antihypertrophic effect of *Rock2* deletion was abolished by deleting or knocking down *Fhl2* (four and a half LIM domains protein 2), a negative regulator of cardiac hypertrophy that binds and inhibits extracellular signal-regulated kinases and also antagonizes RhoA-mediated activation of serum response factor-dependent transcription (Okamoto et al., 2013).

All of these data thus suggest that ROCK2, but not ROCK1, is an important mediator of cardiac hypertrophy through a mechanism that involves, at least in part, regulation of *Fhl2*, extracellular signal-regulated kinase, and serum response factor signaling.

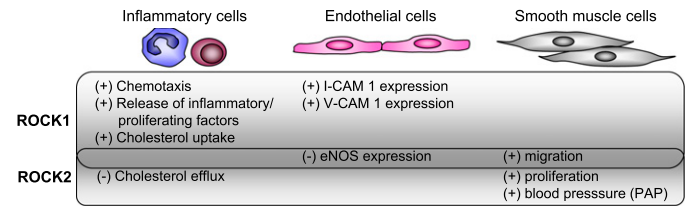
TABLE 3  
Vascular phenotype in genetically modified mice

Target	Modification	Transgene/Mouse	Phenotype	Reference
<i>Rock1</i> BP/endothelial function/hypertension	<i>Rock1</i> <sup>+/-</sup>		Normal basal BP Experimental hypertension similar to control mice Reduced basal systolic BP Increased NO production and endothelial-dependent relaxation Abolition of diabetes-induced rise in BP and endothelial dysfunction	Rikitake et al., 2005 Yao et al., 2013
Atherosclerosis	Bone marrow <i>Rock1</i> <sup>-/-</sup>	Transplanted in <i>Ldlr</i> <sup>-/-</sup> mice	Reduced atherosclerosis Reduced lipid accumulation Reduced macrophage lipid uptake Reduced neointimal formation after vascular injury	Wang et al., 2008
Neointimal thickening	<i>Rock1</i> <sup>+/-</sup>			Noma et al., 2008
<i>Rock2</i> BP/endothelial function/hypertension	Bone marrow <i>Rock1</i> <sup>-/-</sup> <i>Rock2</i> <sup>+/-</sup>	Transplanted in control mice	Reduced neointimal formation after vascular injury	Noma et al., 2008 Noma et al., 2008
			Normal basal systolic BP	Noma et al., 2008
			Reduced basal systolic BP Increased NO production and endothelial-dependent relaxation Reduction of diabetes-induced rise in BP and endothelial dysfunction	Yao et al., 2013
Atherosclerosis	Bone marrow <i>Rock2</i> <sup>-/-</sup>	Transplanted in <i>Ldlr</i> <sup>-/-</sup> mice	Reduced atherosclerosis Reduced lipid accumulation Reduced macrophage cholesterol efflux No change in intimal formation after vascular injury	Zhou et al., 2012
Neointimal thickening PAH	<i>Rock2</i> <sup>+/-</sup> SM- <i>Rock2</i> <sup>+/-</sup> TgSM- <i>Rock2</i>		Reduced PAH Reduced vascular smooth muscle cell proliferation Increased PAH Increased vascular smooth muscle cell proliferation	Noma et al., 2008 Shimizu et al., 2013 Shimizu et al., 2013

BP, blood pressure.

**2. Blood Pressure and Hypertension.** High blood pressure was decreased by treatment with ROCK inhibitors in several experimental models of hypertension in mice and rats (Loirand and Pacaud, 2010) (Table 3). Combined effects of ROCK inhibition, affecting central nervous system blood pressure regulation, arterial smooth muscle contractility, and inflammatory processes, likely account for the beneficial effect of ROCK inhibitors on hypertension (Loirand and Pacaud, 2010; Loirand et al., 2013). Under basal conditions, *Rock1*<sup>-/-</sup>, *Rock1*<sup>+/-</sup>, and *Rock2*<sup>+/-</sup> mice displayed normal hemodynamic parameters, with blood pressure and heart rate values similar to those of control mice (Rikitake et al., 2005; Zhang et al., 2006; Noma et al., 2008). Treatment with Ang II or L-NAME similarly increases blood pressure in *Rock1*<sup>+/-</sup> mice and control mice. However, in these hypertension models, perivascular fibrosis is reduced in the heart of *Rock1*<sup>+/-</sup> mice compared with controls (Rikitake et al., 2005). This observation thus suggests that ROCK1 does not participate in the regulation of blood pressure and high blood pressure, which is likely exclusively dependent on ROCK2. Studies with an experimental model of hypertension in *Rock2*<sup>+/-</sup> mice or conditional models of *Rock2* deletion would be useful to directly address and answer this question. Nevertheless, in contrast with previous publications, basal systolic blood pressure in *Rock1*<sup>+/-</sup> and *Rock2*<sup>+/-</sup> mice was recently described to be approximately 15 mm Hg lower than that of control mice, thus raising the possibility that both ROCK1 and ROCK2 can control blood pressure (Yao et al., 2013). Further studies, particularly with inducible and cell-specific ROCK1 and ROCK2 deletion (smooth muscle, endothelial, and immunoinflammatory cells) or ROCK isoform-specific inhibitors, are necessary to define the respective role of ROCK isoforms in the regulation of blood pressure and hypertension.

**3. Pulmonary Arterial Hypertension.** The efficiency of ROCK inhibitors (Y-27632 or fasudil) in reducing pulmonary arterial hypertension (PAH) in several experimental models in rats and mice suggests that ROCK activation is a critical shared mechanism in the pathogenesis of PAH (Abe et al., 2004; Fagan et al., 2004; Nagaoka et al., 2004, 2005). PAH is characterized by endothelial dysfunction, endothelial and smooth muscle cell proliferation, and increased vasoconstriction of pulmonary arteries. The role of vascular smooth muscle ROCK2 in the pathogenesis of PAH was recently investigated by the generation of smooth muscle-specific *Rock2*<sup>+/-</sup> (SM-*Rock2*<sup>+/-</sup>) mice and smooth muscle-specific Rho-associated kinase 2-overexpressing (SM-ROCK2) mice (Shimizu et al., 2013) (Table 3). Compared with control mice, hypoxia-induced PAH is attenuated in SM-*Rock2*<sup>+/-</sup> mice and potentiated in SM-ROCK2 mice. Lung immunostaining of Ki67 and phosphorylated extracellular signal-regulated kinase indicates that these effects are associated with a decrease



**Fig. 2.** Synthesis of identified ROCK1 and ROCK2 roles in inflammatory, endothelial, and smooth muscle cells potentially involved in vascular disorders (hypertension, remodeling, and atherosclerosis). I-CAM 1, intercellular adhesion molecule 1; PAP, pulmonary arterial pressure; V-CAM 1, vascular cell adhesion molecule 1.

and an increase in pulmonary artery cell proliferation in SM-*Rock2*<sup>+/-</sup> mice and SM-ROCK2 mice, respectively. The change in vascular smooth muscle cell migration and proliferation induced by genetic ROCK2 manipulation has been confirmed in vitro, indicating a primary intrinsic defect of vascular smooth muscle cells (Fig. 2). This study thus highlights the causal role of vascular smooth muscle cell ROCK2 in the pathogenesis of PAH, partly through the regulation of vascular smooth muscle cell migration and proliferation, secondary to oxidative stress induction and cytokine production (Shimizu et al., 2013).

**4. Atherosclerosis.** Y-27632 decreases atherosclerosis in *Ldlr*<sup>-/-</sup> mice and reduces T lymphocyte infiltration and inflammation within plaques, suggesting a role of ROCK1 and/or ROCK2 in atherogenesis (Mallat et al., 2003). The respective role of ROCK1 and ROCK2 in the development of atherosclerosis has been further characterized by *Rock1*<sup>-/-</sup> and *Rock2*<sup>-/-</sup> bone marrow transplantation into irradiated *Ldlr*<sup>-/-</sup> mice (Table 3). Lack of ROCK1 in bone marrow-derived cells limits atherosclerosis and decreases inflammatory cells (macrophages, T cells) and lipid accumulation in the atherosclerotic lesions of *Ldlr*<sup>-/-</sup> mice (Wang et al., 2008). Both chemotaxis and the ability to take up modified low-density lipoprotein and undergo foam cell formation are impaired in bone marrow-derived *Rock1*<sup>-/-</sup> macrophages. *Rock2* deletion or haploinsufficiency in bone marrow-derived cells also protects *Ldlr*<sup>-/-</sup> mice from atherosclerosis and reduces lipid deposition in atherosclerotic plaques (Zhou et al., 2012). Foam cell formation is reduced in macrophages from bone marrow of *Rock2*<sup>+/-</sup> mice, although cholesterol uptake is not decreased. By contrast, the loss of ROCK2 in macrophages stimulates cholesterol efflux by upregulating the peroxisome proliferator-activated receptor- $\gamma$ /liver X receptor/ATP-binding cassette transporter A1 pathway (Zhou et al., 2012). Although these studies show that both ROCK isoforms are involved in the development of atherosclerosis in macrophages, these data reveal distinct functions of ROCK1 and ROCK2. Although they both contribute to lipid accumulation in atherosclerotic lesions and foam cell formation, macrophage ROCK1 favors macrophage chemotaxis and cholesterol uptake, whereas macrophage ROCK2 inhibits peroxisome

proliferator-activated receptor- $\gamma$ -dependent cholesterol efflux (Fig. 2).

5. *Neointimal Thickening/Restenosis*. Long-term inhibition of ROCKs reduces neointimal thickening induced by coronary stent implantation in pigs (Matsumoto et al., 2004) or balloon injury in rats (Sawada et al., 2000). Moreover, maintained activation of ROCK signaling is observed in human arteries after stent implantation (Guérin et al., 2005). Neointima formation and vascular inflammation induced by carotid artery ligation were reduced in *Rock1*<sup>+/-</sup> mice but remained similar to controls in *Rock2*<sup>+/-</sup> mice (Noma et al., 2008) (Table 3). Bone marrow transplantation experiments then demonstrated a predominant role of ROCK1 in mediating neointima formation in leukocytes compared with that of ROCK1 in vascular wall cells. ROCK1 in circulating leukocytes appears to be the primary determinant of leukocyte recruitment to the endothelial surface and vascular cell proliferation via the subsequent release of inflammatory and proliferating factors. ROCK1 in endothelial cells participates in the induction of endothelial adhesion molecule expression (intercellular adhesion molecule 1, vascular cell adhesion molecule 1), whereas ROCK1 in vascular smooth muscle cells is involved in vascular smooth muscle cell migration (Noma et al., 2008) (Fig. 2). In agreement with data obtained in atherosclerosis models, these findings point to the important contribution of leukocyte ROCK1 in the pathogenesis of vascular diseases characterized by an inflammatory component (Fig. 2).

6. *Endothelial Function and Dysfunction*. A tight spatiotemporal regulation of ROCK activity in endothelial cells is essential for normal cell migration, lumen formation, and normal angiogenesis (Whitehead et al., 2009; Stockton et al., 2010; Davis et al., 2011). Defective vascular remodeling in the yolk sac and in utero death before embryonic day E9.5 of homozygous mouse embryos deficient in both isoforms of ROCK (*Rock1*<sup>-/-</sup>; *Rock2*<sup>-/-</sup>) confirm the role of ROCKs in developmental angiogenesis and suggest a redundant role of ROCK isoform functions in vascular development during embryogenesis (Kamijo et al., 2011).

Numerous studies have addressed the role of ROCKs in endothelial function, in part through the use of ROCK inhibitors. Endothelial ROCK activity has thus been shown to downregulate eNOS expression by decreasing eNOS mRNA stability (Laufs and Liao, 1998; Zhou and Liao, 2009). Moreover, ROCKs decrease eNOS catalytic activity by inhibiting the phosphoinositide 3-kinase/Akt pathway and stimulating arginase activity (Ming et al., 2002, 2004; Wolfrum et al., 2004). Indeed, some of the beneficial effects of ROCK blockers on the cardiovascular system, as well as the pleiotropic effects of statins, have been attributed to their positive effects on nitric oxide (NO) production and availability as well as endothelial function (Rikitake and Liao, 2005). ROCKs also control endothelial barrier function. Basal endothelial ROCK activity is required for proper expression

of VE-cadherin and endothelial barrier integrity (van Nieuw Amerongen et al., 2007). By contrast, by inducing actomyosin contraction in endothelial cells, stimulation of ROCK activity disrupts endothelial cell junctions, increases endothelial permeability (van Nieuw Amerongen et al., 2007), and facilitates leukocyte trans-endothelial migration (Heemskerk et al., 2014).

In agreement with these functions of ROCK in endothelial cells, in vivo pharmacologic ROCK inhibition ameliorates endothelial function by restoring endothelium-dependent relaxation and eNOS expression and activity in a variety of animal models of pathophysiological conditions associated with endothelial dysfunction, such as hypertension or diabetes (Higashi et al., 2003; Bivalacqua et al., 2004; Shah and Singh, 2006a,b; Horowitz et al., 2007; Arita et al., 2009; Nuno and Lamping, 2010; Tsounapi et al., 2012). Nevertheless, surprisingly, the use of genetically modified mice to dissect the role of endothelial cell ROCKs in endothelium function and dysfunction in vivo is limited to diabetic conditions.

Vascular diseases are the principal causes of death and disability in people with diabetes (Creager et al., 2003). Hyperglycemia-induced endothelial dysfunction, resulting from decreased NO production and the subsequent induction of oxidative stress and inflammation, is the initial trigger of the predisposition to atherosclerosis in patients with diabetes. In addition to the beneficial effect of ROCK inhibitors, the 2.5-fold increase in ROCK2 expression observed in the endothelium of diabetic rats further supports a role of ROCK in diabetes-induced endothelial dysfunction (Cicek et al., 2013). In the streptozotocin model of diabetes in mice, although the increase in glucose levels was not modified, *Rock1* or *Rock2* haploinsufficiency abolished the diabetes-induced rise in blood pressure (Yao et al., 2013). Maximal contractile response to phenylephrine is not modified in *Rock1*<sup>+/-</sup> and *Rock2*<sup>+/-</sup> aortic rings but the sensitivity was significantly decreased. Endothelial-dependent relaxation and NO production are strongly enhanced in aortas from *Rock1*<sup>+/-</sup> and *Rock2*<sup>+/-</sup> mice compared with controls, and diabetes-induced endothelial dysfunction and decreased NO production are completely and partially abolished by *Rock1* and *Rock2* haploinsufficiency, respectively. These results suggest a dominant role of ROCK1 in diabetes-induced endothelial dysfunction. This effect of ROCK1 has been attributed to its stimulating action of arginase activity and/or expression (Yao et al., 2013) (Table 3).

### B. Asthma

Asthma is characterized by bronchoconstriction, airway hyperresponsiveness, and inflammation. In animal models of allergic asthma, exposition to the allergen ovalbumin (OVA) induces ROCK activation in the lungs (Zhu et al., 2011). The ROCK inhibitors Y-27632 and fasudil attenuate airway hyperresponsiveness and reduce inflammation, suggesting a causal role of ROCK1 and/or ROCK2 in the pathogenesis of asthma (Schaafsma et al.,



2006, 2008). OVA-induced airway hyperresponsiveness is virtually abolished in both *Rock1*<sup>+/-</sup> mice and *Rock2*<sup>+/-</sup> mice compared with control mice. By contrast, OVA-induced lung inflammation is not modified in either *Rock1*<sup>+/-</sup> or *Rock2*<sup>+/-</sup> mice, although *Rock1* or *Rock2* haploinsufficiency attenuates mast cell degranulation and reduces the number of eosinophils in bronchoalveolar lavages compared with controls (Zhu et al., 2011). Moreover, ROCK2, but not ROCK1, is involved in mucous cell hyperplasia in the OVA model of allergic asthma (Kasahara et al., 2015). These results suggest that both ROCK1 and ROCK2 are independently required for allergen-induced airway hyperresponsiveness, likely through their effects in airway smooth muscle cell contraction and mast cell degranulation.

### C. Glucose Metabolism

Studies of the effects of ROCK inhibitors on glucose homeostasis in vivo have produced conflicting results. In animal models of diabetes, chronic treatment with the ROCK inhibitor fasudil has been shown to either improve glucose tolerance or to have no effect on blood glucose levels (Kanda et al., 2006; Kolavennu et al., 2008). By contrast, in normal mice, acute ROCK inhibition promotes insulin resistance by reducing insulin-mediated glucose uptake in skeletal muscle (Furukawa et al., 2005). In agreement with this observation, *Rock1*<sup>-/-</sup> mice exhibit systemic insulin resistance (Lee et al., 2009). However, they show normal glucose tolerance, but with a 65% increase in glucose-induced insulin secretion. Insulin resistance induced by *Rock1* deficiency was attributable to an impairment of the insulin receptor substrate 1/phosphoinositide 3-kinase/Akt signaling pathway and glucose transport into skeletal muscle (Lee et al., 2009).

Despite their insulin resistance, *Rock1*<sup>-/-</sup> mice display an increase in proximal insulin signaling in adipose tissue, and adipose tissue-selective deletion of *Rock1* slightly ameliorates insulin sensitivity in the high-fat diet model of insulin resistance (Lee et al., 2014b). Thus, in adipose tissue, ROCK1 negatively controls insulin action in vivo. However, loss of adipose ROCK1 leads to increased insulin receptor signaling, with a minor effect on whole-body metabolism and glucose homeostasis (Lee et al., 2014b). These data also collectively confirm the complex and tissue- or cell-specific role of ROCKs in glucose metabolism and insulin signaling. In addition, a potential in vivo role of ROCK2 in glucose metabolism remains to be explored, since it has been described to regulate insulin receptor signaling in vitro (Farah et al., 1998).

### D. Kidney Function and Renal Disease

In addition to its role in the regulation of renal arteriolar contraction and glomerular blood flow and filtration, ROCK activity participates in the control of tubular cells, podocytes, and mesangial cell structure

and function (Hayashi et al., 2006). Furthermore, involvement of the ROCK signaling pathway in the development of kidney disease is supported by the protective action of ROCK inhibitors in a variety of animal models of nephropathy (Nagatoya et al., 2002; Satoh et al., 2002; Kanda et al., 2003; Nishikimi et al., 2004). Surprisingly, *Rock1*<sup>-/-</sup> mice are not protected against renal fibrosis in the unilateral ureteral obstruction model (Fu et al., 2006). Indeed, *Rock1* deletion enhances transforming growth factor- $\beta$ /Smad signaling in the obstructed kidney, which can explain, at least in part, why *Rock1* deletion fails to limit renal fibrosis. A role of ROCK2 and/or a nonspecific action of ROCK inhibitors could account for the discrepancy between the effect of pharmacological and genetic inhibition of ROCK signaling.

By contrast, deletion of *Rock1* protected against the development of albuminuria in the model of streptozotocin-induced diabetic kidney disease (Zhou et al., 2011). This effect is associated with a decrease in the activation of transforming growth factor- $\beta$  signaling and fibrosis in the diabetic kidney. Therefore, in this model, deletion of *Rock1* mimics the protective effect of ROCK inhibition on diabetic nephropathy (Kikuchi et al., 2007). Analysis on *Rock2*<sup>-/-</sup> or *Rock2*<sup>+/-</sup> mice or even mice with combined genetic manipulation of both *Rock* isoforms would be useful to decipher their respective role in the kidney, which remains controversial.

## IV. Association between *Rho-Associated Kinase* Variants and Disease Susceptibility

Although the number of studies aimed at addressing the genetic contribution of *ROCK1* and *ROCK2* to disease susceptibility or protection is increasing, the overall conclusion that can be drawn is still not definitive, mainly because of the absence of replication studies. Most studies have used a candidate gene approach, and neither *ROCK1* nor *ROCK2* was identified to be associated with human disease in Western or Asian populations by genome-wide association studies (Table 4).

### A. Cardiovascular Diseases

A genetic analysis showed that the Thr431Asn *ROCK2* variant (rs2230774, C/A) influences blood pressure. The AA genotype (Asn/Asn *ROCK2*) is associated with high basal blood pressure and increased systemic vascular resistance (Seasholtz et al., 2006). The association of *ROCK2* with hypertension was also evaluated in a large prospective cohort of hypertensive patients and healthy, normotensive controls (Rankinen et al., 2008). A lower risk of high blood pressure is associated, in a recessive manner, with a major haplotype block, defined by four single nucleotide polymorphisms (rs965665, rs10178332, rs6755196, and rs10929732) at the *ROCK2* locus. Homozygotes for the minor alleles

TABLE 4  
ROCK polymorphisms

Gene	Single Nucleotide Polymorphism	Allele	Association	Reference	
ROCK1 18q11	rs73963110	C/T	Associated with colorectal cancer	Sari et al., 2013	
	rs35996865	T/G			
	rs288980	A	Associated with the risk of ischemic stroke	Zee et al., 2014	
	rs7239317	A			
	rs2127958	G			
	rs1481280	A			
	rs1006881	A			
	rs11874761	A			
	rs10083915	G			
	rs11873284	G			
	rs288979 (Tyr269 = )	C/T			
	ROCK2 2p24	rs1515210	G/C	T allele in a protective haplotype against vasospastic angina	Yoo et al., 2012
		rs978906	A/G	G allele in a protective haplotype against vasospastic angina Not associated with coronary disease and blood pressure A allele associated with stiffer arteries; Alteration of miR-1183 binding, modulation of Rock2 expression	Yoo et al., 2012 Liu et al., 2013 Liao et al., 2015
		rs2290156	G/C	Associated with colorectal cancer Not associated with blood pressure	Sari et al., 2013 Seasholtz et al., 2006
rs2271621		T/G	T allele in a protective haplotype against vasospastic angina	Yoo et al., 2012	
rs34945852 (Lys1083Met)		T/A	Associated with colorectal cancer	Sari et al., 2013	
rs35768389 (Asp601Val)		T/A	Associated with Behcet disease Associated with colorectal cancer	Oguz et al., 2012 Sari et al., 2013	
rs726843		C/T	Not associated with blood pressure	Seasholtz et al., 2006	
rs2230774 (Thr431Asn)		C/A	A allele associated with high basal blood pressure	Seasholtz et al., 2006	
(Thr431Ser)		C/G	C allele in a protective haplotype against vasospastic angina C allele associated with arterial stiffness and with high Rock2 activity Not associated with coronary disease and blood pressure Associated with breast cancer metastasis	Yoo et al., 2012 Liao et al., 2015 Liu et al., 2013 Kalender et al., 2010	
rs1130157 Arg83Lys		G/A	Not associated with primary open-angle glaucoma Not associated with breast cancer metastasis Not associated with primary open-angle glaucoma	Demiryürek et al., 2015 Kalender et al., 2010 Demiryürek et al., 2015	
rs1515219		C/T	Associated with Behcet disease Not associated with blood pressure	Oguz et al., 2012 Seasholtz et al., 2006	
rs2230773 (Ala145Ala)		G/A	Not associated with coronary disease and blood pressure	Liu et al., 2013	
rs3771106		G/A	G allele in a protective haplotype against vasospastic angina	Yoo et al., 2012	
rs965665		C/G	Associated with high blood pressure	Rankinen et al., 2008	
rs10178332		A/C	Associated with colorectal cancer Associated with high blood pressure	Sari et al., 2013 Rankinen et al., 2008	
rs6755196		A/G	Associated with high blood pressure	Rankinen et al., 2008	
rs10929732		A/G	Associated with colorectal cancer Associated with high blood pressure	Sari et al., 2013 Rankinen et al., 2008	

have an 85% lower risk of hypertension than carriers of the common allele (Rankinen et al., 2008). By contrast, no statistically significant associations have been detected between multiple variations within the *ROCK2* locus (rs978906, rs2230774, and rs56304104) and coronary disease and blood pressure in a Chinese population (Liu et al., 2013). Several reasons can account for these inconsistent results for *ROCK2* association with blood pressure, including patient selection/stratification, ethnic differences, and genotyping errors. Indeed, the complex pathogenesis of hypertension and the possible low effect of individual gene variations may also explain the lack of association between *ROCK2* variants and this condition. Furthermore, considering the complex genetic network between *ROCK2* and other genes within the same pathway, the potential roles of *ROCK2* variations might be diluted or masked by gene–gene and/or gene–environment interactions.

Recently, two functional polymorphisms of *ROCK2* (rs978906 and rs2230774) were found to be associated with stiffer arteries through modulation of *ROCK2*

activity and expression (Liao et al., 2015). Leukocyte *ROCK* activity is higher in subjects with the CC genotype at rs2230774 (Thr<sup>431</sup>/Thr<sup>431</sup> *ROCK2*) than AC and AA, without a change in the expression level of *ROCK2*. By contrast, the 3' untranslated region rs978906 polymorphism affected the expression of *ROCK2* by interfering with miR-1183 binding.

In Korean individuals with vasospastic angina, an analysis of *ROCK2* polymorphisms (rs978906, rs2271621, rs2230774, rs1515210, and rs3771103) showed that the G-T-C-T-G haplotype is protective against vasospastic angina (Yoo et al., 2012).

A recent prospective study addressed the potential association of *ROCK1* and *ROCK2* with the risk of ischemic stroke in participants from the Women's Genome Health Study (Zee et al., 2014). In this large cohort of 23,294 initially healthy Caucasian women, 7 single polymorphisms in *ROCK1* show significant association with the risk of ischemic stroke, but none of the 15 *ROCK2* single nucleotide polymorphisms tested were associated with ischemic stroke.

The role of ROCK polymorphisms has also been investigated in cardiac malformations. The potential genetic factors in the development of nonsyndromic tetralogy of Fallot and the key role of ROCK1 in planar cell polarity provided a rational basis to analyze the role of genetic variation of ROCK1 in the risk of tetralogy of Fallot. Two rare frequency variants of *ROCK1* (rs56085230 and rs288979) were thus found to significantly increase the risk of tetralogy de Fallot in a test cohort including 458 cases and 1331 controls (Palomino Doza et al., 2013).

**B. Cancer**

Somatic mutations in *ROCK1* and *ROCK2* genes leading to constitutive activation have been identified in human cancer cell lines and primary tumors (Greenman et al., 2007; Lochhead et al., 2010). In addition, genetic polymorphisms of ROCK genes are found to be associated with cancer.

Both *ROCK1* and *ROCK2* polymorphisms have been addressed in colorectal cancer development. A significant association was found between *ROCK2* gene polymorphisms rs2290156, rs10178332, rs35768389 (Asp601Val), rs10929732, and rs34945852 (Lys1083-Met) and colorectal cancer in Turkish patients (Sari et al., 2013). The disease is also associated with *ROCK1* polymorphisms (rs73963110 and rs35996865). In addition, immunohistochemical staining indicated increased expression of both ROCK1 and ROCK2 in tumor tissues (Sari et al., 2013).

By analyzing genotype distributions and allele frequencies for the *ROCK2* polymorphisms rs2230774 (Thr431Asn) and rs1130157 (Arg83Lys) in patients with breast cancer, it has been shown that although the Arg83Lys polymorphism has no effect, Thr431 polymorphism of *ROCK2* is a risk factor for metastases of breast cancer (Kalender et al., 2010).

**C. Autoimmune Diseases**

The association between *ROCK2* gene polymorphisms and Behcet disease, a multisystemic vasculitis, was screened in a Turkish population (Oguz et al., 2012). The Asp601Val *ROCK2* variant (rs35768389) was found to be associated with the disease. A significant association was also identified for rs1515219, whereas eight other variations tested (rs726843, rs2290156, rs965665, rs10178332, rs2230774, rs6755196, rs1029732, and rs34945852) did not show an association. In addition, an increase in mRNA *ROCK2* expression was found in peripheral blood from patients with Behcet disease. These data thus suggest that *ROCK2* polymorphisms may affect susceptibility to Behcet disease.

**D. Glaucoma**

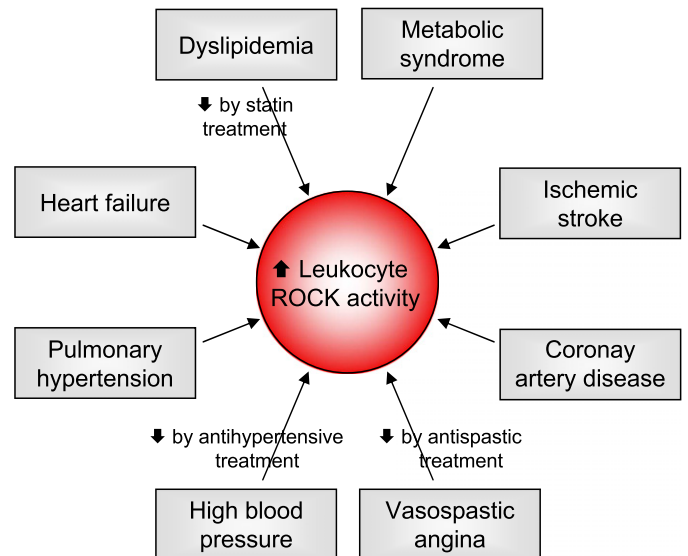
Accumulating evidence suggests that ROCK participates in the regulation of aqueous outflow, including

pharmacological data from clinical trials showing that ROCK inhibitors decrease intraocular pressure and demonstrate beneficial effects in glaucoma patients (Inoue and Tanihara, 2013). *ROCK* gene polymorphisms were thus recently investigated in primary open-angle glaucoma, the most common form of glaucoma (Demiryürek et al., 2015). However, neither *ROCK1* nor *ROCK2* polymorphisms were associated with the increased risk of primary open-angle glaucoma development (Table 4).

**V. Rho-Associated Kinase Activity or Expression: Candidate Biomarker of Human Diseases**

**A. Cardiovascular Diseases**

In agreement with in vitro and animal studies, both genetic and pharmacological studies support a role of ROCK activation in the development of cardiovascular pathologies such as hypertension, pulmonary hypertension, atherosclerosis, and restenosis in humans (Fig. 3; Table 5). Therefore, it would be clinically relevant to assess the level of ROCK activity in patients with cardiovascular diseases or risk factors. In 2007, a seminal article reported that the metabolic syndrome is associated with increased leukocyte ROCK activity, and that ROCK activity in leukocytes could be used as an independent predictor and a surrogate marker for diagnosis of the metabolic syndrome (Liu et al., 2007). Increased ROCK activity was then observed in dyslipidemic subjects without cardiovascular disease and was reduced by statin treatment (Liu et al., 2009a). Leukocyte ROCK activity is also higher in patients with cardiovascular diseases than in healthy individuals (Hata et al., 2011a). Interestingly, in patients with cardiovascular disease, ROCK activity in leukocytes



**Fig. 3.** Leukocyte ROCK activity according to cardiovascular diseases and treatments.

TABLE 5  
Assessment of ROCK activity or expression in pathologic conditions

Condition	Change in ROCK activity of expression	Cell type	Reference
Cardiovascular disease			
Metabolic syndrome	Increased activity	Leukocytes	Liu et al., 2007
Dyslipidemia	Increased activity	Leukocytes	Liu et al., 2009a
Cardiovascular diseases	Increased activity	Leukocytes	Hata et al., 2011a
Vasospastic angina	Increased activity	Neutrophils	Kikuchi et al., 2011
Heart failure	Increased activity	Leukocytes	Hung et al., 2012
Hypertension	Increased activity	Leukocytes	Ocaranza et al., 2011
Pulmonary hypertension	Increased activity	Lung, leukocytes	Dong et al., 2012
Ischemic stroke	Increased activity	Leukocytes	Do e et al., 2013
			Hata et al., 2011b
			Do e et al., 2009
			Feske et al., 2009
			Cheng et al., 2014
Autoimmune disease			
Systemic lupus erythematosus	Increased activity	Leukocytes	Isgro et al., 2013
Cancer			
Bladder cancer	Increased expression	Cancerous tissue	Kamai et al., 2003
Breast cancer	Increased expression of Rock1	Cancerous tissue	Liu et al., 2009b
Hepatocellular carcinoma	Increased expression of Rock2	Cancerous tissue	Wong et al., 2009
Osteosarcoma	Increased expression of Rock1	Cancerous tissue	Liu et al., 2011
Vulvar cancer	Decreased expression of Rock1	Cancerous tissue	Akagi et al., 2014

correlates with the increased forearm blood flow induced by the ROCK inhibitor fasudil, indicating that leukocyte ROCK activity is an index of vascular ROCK activity (Hata et al., 2011a). ROCK activity was also found to be increased in leukocytes or neutrophils from patients with coronary vasospastic angina and was decreased by antispastic treatment (Hung et al., 2012; Kikuchi et al., 2011). Indeed, ROCK activity in leukocytes independently predicted the presence of coronary vasospastic angina and correlated with disease severity (Hung et al., 2012). Increased leukocyte ROCK activity was even greater in patients with concomitant peripheral and coronary arterial disease, suggesting that ROCK activity may be a potential marker of atherosclerotic burden for patients with polyvascular disease (Dong et al., 2013).

Regarding ischemic stroke, it has been reported that peripheral leukocyte ROCK activity is elevated in patients 24–72 hours after stroke onset (Feske et al., 2009). Recently, a prospective study was carried out to assess the association of leukocyte ROCK activity with outcomes after acute ischemic stroke (Cheng et al., 2014). It was thus further shown that leukocyte ROCK activity is higher in patients with acute ischemic stroke than in the cardiovascular risk–matched control group without a stroke history (Cheng et al., 2014). Leukocyte ROCK activity was not found to correlate with inflammatory markers such as white blood cell count, serum level of high sensitive C-reactive protein, interleukin (IL)-6, or tumor necrosis factor- $\alpha$ . However, leukocyte ROCK activity upon admission was significantly associated with lower stroke-free event survival after acute ischemic stroke, and it was thus defined as an independent predictor for recurrent stroke in patients who had an atherosclerotic stroke (Cheng et al., 2014).

Similarly, ROCK activity in peripheral blood mononuclear cells is increased in hypertensive patients and

can be reduced by antihypertensive treatments, particularly calcium channel blockers (Hata et al., 2011b). Leukocyte ROCK activity in treated hypertensive patients is similar to that of controls (Ocaranza et al., 2011).

In patients with pulmonary hypertension, ROCK activity is significantly higher in both lung tissues and circulating neutrophils compared with controls (Do e et al., 2009). A significant correlation was established between neutrophil ROCK activity and the severity and duration of pulmonary hypertension (Do e et al., 2009).

Compared with healthy individuals, leukocyte ROCK activity is increased in patients with stable chronic congestive heart failure, as well as in individuals with acute heart failure (Ocaranza et al., 2011; Dong et al., 2012; Do e et al., 2013). Upregulation of ROCK1, ROCK2, and RhoA expression was also detected in leukocytes from patients with congestive heart failure compared with controls (Dong et al., 2012). Whether increased ROCK activity is a marker of the deterioration of systolic function is still unclear, because reports have suggested both that a correlation with left ventricular ejection fraction exists (Ocaranza et al., 2011; Dong et al., 2012) and that there is no correlation (Do e et al., 2013). Nevertheless, the combination of ROCK activity and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) was found to be more useful for predicting mortality in patients with chronic heart failure than NT-proBNP alone. These results suggest that although NT-proBNP is a more sensitive biomarker than ROCK activity, ROCK activity may be an index of inflammation, endothelial dysfunction, and vasoconstriction associated with chronic heart failure (Dong et al., 2012).

In healthy subjects as well as those with cardiovascular risk factors but no established cardiovascular or

cerebrovascular diseases, leukocyte ROCK activity significantly correlates with body mass index, systolic blood pressure, low-density lipoprotein cholesterol level, flow-mediated vasodilation, and Framingham risk factor score (an index of cumulative cardiovascular risk used for estimating individual 10-year risk of developing cardiovascular disease) (Soga et al., 2011). This later correlation thus suggests that leukocyte ROCK activity possibly predicts cardiovascular mortality and morbidity. This possibility was addressed in a recent prospective study in a large population (633 subjects) over 42 months of follow-up (Kajikawa et al., 2014). Elevated leukocyte ROCK activity correlated with first major cardiovascular events, including myocardial infarction, stroke, and death from cardiovascular causes. Therefore, ROCK activity in leukocytes seems to be a potential biomarker for predicting cardiovascular events.

Taken together, all of these studies highlight the potential for using leukocyte ROCK activity as a biomarker of cardiovascular risk factors, mortality, and morbidity; however, these findings also raise several questions. Why is leukocyte ROCK activated in patients with cardiovascular diseases? Is there a link between the mechanisms responsible for this increased ROCK activity in leukocytes and those responsible for the pathogenic action of ROCK overactivation in vascular or cardiac cells? Is there a causal relationship between the activation of ROCK in leukocytes and the development/progression of cardiovascular diseases? Moreover, another methodologic issue should be addressed before implementing a clinical application of the quantification of leukocyte ROCK activity: the duration and complexity of the Western blot protocols currently used to determine leukocyte ROCK activity is not compatible with a routine biological test. New appropriate quantitative methods should thus be developed before clinical use of leukocyte ROCK activity as a biomarker becomes possible.

### *B. Autoimmune Diseases*

Contribution of the ROCK pathway to autoimmunity and autoimmune disease is emerging (Reedquist and Tak, 2012). This is supported by *in vitro* demonstration of the role of ROCK signaling in T cell development and function, including adhesion, chemotactic responses, and antigen-dependent activation, as well as the beneficial effect of ROCK inhibition in experimental models of rheumatoid arthritis and lupus (Li et al., 2007; He et al., 2008; Stirzaker et al., 2012). In an attempt to assess the ROCK pathway in human autoimmune diseases, measurement of leukocyte ROCK activity shows that 60% of patients with systemic lupus erythematosus exhibit an increase in ROCK activity (Isagro et al., 2013). This observation is consistent with a role of dysregulation of ROCK signaling in the pathogenesis of autoimmune disorders. It also suggests that ROCK

could be a novel therapeutic target for the treatment of systemic lupus erythematosus, and measurement of leukocyte ROCK activity may be useful to assess the efficacy of therapies in patients treated for this disease.

### *C. Cancer*

Several studies described the inhibitory effects of ROCK blockers on *in vitro* adherent tumor cell migration and invasion (Gutjahr et al., 2005; Torika et al., 2006; Patel et al., 2014), although recent data showed that ROCK inhibition increases the metastatic potential of nonadherent breast tumor cells by increasing their reattachment efficacy (Bhandary et al., 2015). ROCKs have been implicated in the pathogenesis of cancer and several aspects of human tumor progression (Rath and Olson, 2012). The prognosis value of ROCKs has thus been assessed by examining ROCK expression in association with histopathologic characteristics of human tumors. It should be kept in mind that because ROCK activity is controlled by numerous positive and negative regulators, the expression of ROCKs does not obligatorily reflect their activity.

In bladder cancer, ROCK1 and ROCK2 expression is significantly greater in primary tumors than in the non-neoplastic bladder. Tumor ROCK expression is associated with poor differentiation, muscle invasion, lymph node metastasis, and short survival, suggesting that ROCKs could be involved in the progression of bladder cancer and may be valuable prognostic markers (Kamai et al., 2003).

A similar observation has been reported for ROCK1 in breast cancer, with a high expression of ROCK1 in metastatic breast tumors compared with nonmetastatic tumors (Liu et al., 2009b) and a correlation with clinicopathologic parameters and overall patient survival (Lane et al., 2008; Bottino et al., 2014).

ROCK2, but not ROCK1, is also overexpressed in human hepatocellular carcinoma compared with the nontumorous liver, and the overexpression of ROCK2 is positively associated with the presence of intrahepatic metastasis and is thus a sign of more aggressive biologic behavior (Wong et al., 2009).

ROCK1 is highly expressed in various tumor cell lines and tumor tissues from patients with osteosarcoma. Moreover, high levels of ROCK1 expression are associated with shorter overall patient survival (Liu et al., 2011).

Surprisingly, opposing results were recently reported in vulvar cancer, in which the level of ROCK1 expression was lower than in adjacent normal tissue and lower expression of ROCK1 correlated with a low survival rate, suggesting that ROCK1 expression is a marker of good prognosis in vulvar cancer (Akagi et al., 2014).

Although these data suggest a role of ROCKs in cancer progression and invasiveness and show that ROCKs could represent potential prognosis markers and therapeutic targets, further studies are needed to

understand the specific roles of ROCK1 and ROCK2 in different human cancers.

## VI. Pharmacological Rho-Associated Kinase Inhibitors

### A. Rho-Associated Kinase Inhibitors

Fasudil (formerly HA-1077), an isoquinoline derivative initially developed as an intracellular calcium antagonist, and the pyridine derivative Y-27632 were the first ATP-competitive ROCK inhibitors described. They have been used extensively as experimental pharmacological tools to explore in vivo ROCK functions and their involvement in a large panel of processes and diseases. Nevertheless, both compounds have a low potency (micromolar) compared with clinically approved kinase inhibitors (less than nanomolar) and they inhibit other kinases involved in the same cellular functions as ROCKs (Bain et al., 2007). These limitations have prompted the development of new more selective and more potent ROCK inhibitors that are more suitable as human therapeutic agents. Given the different roles of ROCK1 and ROCK2 highlighted in the past few years, an additional challenge is to discover isoform-specific ROCK inhibitors. Active research has thus been carried out recently to develop a new series of ROCK inhibitors based on different scaffolds (Guan et al., 2013; Pan et al., 2013; Feng and LoGrasso, 2014). Here, we summarize typical examples of these new compounds.

H-1152 [(S)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-hexahydro-1*H*-1,4-diazepine], a derivative of fasudil showing better potency and selectivity, has been developed but only used in preclinical in vitro and in vivo studies (Sasaki et al., 2002). The Y-27632 derivative Y-39983 [also known as RKI-983 and SNJ-1656; 4-[(1*R*)-1-aminoethyl]-*N*-(1*H*-pyrrolo[2,3-*b*]pyridin-4-yl)benzamide hydrochloride] is 30-fold more potent on ROCK activity than Y-27632 and seems promising for future therapeutic applications (Tokushige et al., 2007). AMA0076 [3-[2-(Aminomethyl)-5-[(pyridin-4-yl)carbamoyl]phenyl]benzoate] is another Y-27632 derivative equipotent to Y-39983 that similarly inhibited ROCK1 and ROCK2, with IC<sub>50</sub> values of 3.7 and 2.3 nM, respectively. AMA0076 displays soft drug properties, characterized by its conversion into its rapid functionally inactive metabolite, and it is expected to have better pharmacokinetic parameters and decreased side effects (Van de Velde et al., 2014).

Other new classes of ATP-competitive ROCK inhibitors such as aminofurazan-based compounds GSK269962A [*N*-(3-[[2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-1*H*-imidazo[4,5-*c*]pyridin-6-yl]oxy]phenyl)-4-[[2-(4-morpholinyl)ethyl]oxy]benzamide] and SB-772077-B [4-(7-[[3*S*]-3-amino-1-pyrrolidinyl]carbonyl)-1-ethyl-1*H*-imidazo[4,5-*c*]pyridin-2-yl)-1,2,5-oxadiazol-3-amine] potently inhibit ROCK activity, with IC<sub>50</sub> values of 1.6 and 4 nM and 5.6 and 6 nM

for recombinant human ROCK1 and ROCK2, respectively; these compounds also block several other kinases (Doe et al., 2007). They both relax arterial contraction in vitro and decrease blood pressure in rats in vivo (Doe et al., 2007). The Sanofi compound SAR407899 [6-(piperidin-4-yloxy)isoquinolin-1(2*H*)-one], with IC<sub>50</sub> values of 276 nM and 102 nM for ROCK1 and ROCK-2, respectively, was 4-fold more active than Y-27632 and 8-fold more active than fasudil (Löhn et al., 2009). Despite its ability to potently relax precontracted arteries, its efficient antihypertensive properties after oral administration in rodents, and testing in clinical trials, the development of this compound has been discontinued.

Among new ROCK1 and ROCK2 inhibitors developed, AR-12286 [2-(dimethylamino)-*N*-(1-oxo-2*H*-isoquinolin-6-yl)-2-thiophen-3-ylacetamide], which originated from a 6-aminoisoquinoline amide series, and the isoquinolinesulfonamide K-115 [4-fluoro-5-[[2*S*]-2-methyl-1,4-diazepan-1-yl]sulfonyl]isoquinoline] appeared as promising compounds. K-115 was characterized as a highly selective ROCK inhibitor, with IC<sub>50</sub> values of 51 nM and 19 nM for ROCK1 and ROCK2, respectively, whereas IC<sub>50</sub> values for other kinases such as protein kinase C are in the micromolar range (Isobe et al., 2014).

Various classic approaches of screening as well as fragment-based drug discovery methods and optimization have led to the identification of compounds with different selectivity for ROCK1 and ROCK2 (Boerma et al., 2008; Ray et al., 2011; Li et al., 2012b). Among them, KD-025 [2-(3-(4-((1*H*-indazol-5-yl)amino)quinazolin-2-yl)phenoxy)-*N*-isopropylacetamide; formerly SLx-2119] was the first and remains the only ATP-competitive isoform-selective ROCK2 inhibitor. Its selectivity is approximately 200-fold better toward ROCK2 than ROCK1, with IC<sub>50</sub> values of 105 nM and 24 μM, respectively (Boerma et al., 2008), and KD-025 has potential therapeutic applications for ischemic stroke (Lee et al., 2014a) and autoimmune disease (Zanin-Zhorov et al., 2014).

### B. Clinical Evaluation and Potential Applications of Rho-Associated Kinase Inhibitors

Fasudil was approved in Japan in 1995, and it is used as a vasodilator to prevent cerebral vasospasm after surgery for subarachnoid hemorrhage and to improve blood flow after acute ischemic stroke (Shibuya et al., 1992). The favorable safety profile of fasudil, the results from experiments in ROCK-deficient mice, and the improvement obtained by pharmacological ROCK inhibition in animal models of human diseases provided solid bases to translate the benefits of ROCK inhibitors to humans in different clinical indications.

#### 1. Central Nervous System Disorders.

a. *Subarachnoid hemorrhage-induced vasospasm.* Since 1995, several large-scale clinical trials have been conducted to assess the safety and efficacy of intrarterial administration of fasudil to prevent or reverse

cerebral vasospasm in patients with subarachnoid hemorrhage after trauma or rupture of a cerebral aneurysm. Fasudil has thus demonstrated tolerability, safety, and efficacy in a large number of patients ( $n = 1462$ ) undergoing surgery for SAH (Suzuki et al., 2007). A meta-analysis including eight clinical series recently reported that fasudil decreased, in a statistically significant fashion, the incidence of angiographic and clinical vasospasm (Liu et al., 2012). The beneficial effect of fasudil has been attributed to ROCK inhibition and the resulting decrease of MLC phosphorylation and vascular smooth muscle cell contraction, amelioration of endothelial function, and reduction of inflammation. However, nonspecific inhibition of other kinases could not be excluded.

*b. Cerebral stroke.* Ischemic stroke is a common complication of atherosclerosis, leading to brain damage associated with a high rate of death or severe disability. In animal models of acute cerebral ischemia, ROCK inhibitors increase cerebral blood flow and reduce the area of infarct, likely through stimulation of eNOS activity (Shin et al., 2007). Inhibition of ROCK also prevents ischemia/reperfusion-induced ROS-dependent blood–brain barrier damage (Kahles et al., 2007). In addition to these vascular effects, inhibition of ROCK also preserves cerebral tissue from damage by decreasing neuronal apoptosis via stimulation of Akt signaling (Wu et al., 2012). This experimental evidence thus points to a promising protecting effect of fasudil against acute ischemic stroke–induced neuronal loss in humans (Satoh et al., 2001; Vesterinen et al., 2013). This hypothesis has been addressed in a phase 3 clinical studies in 160 patients with acute ischemic stroke. Intravenous fasudil administration within 48 hours after ischemic stroke for 14 days significantly improved neurologic function at 2 weeks and clinical outcomes at 1 month after the onset of symptoms (Shibuya et al., 2005). Additional trials in larger populations and longer follow-up would be useful to confirm these results.

Recently, the novel ROCK2-selective kinase inhibitor KD-025 orally administered by gavage 1–3 hours after

ischemia onset showed good efficacy on cerebral ischemic outcomes in mice (Lee et al., 2014a). Improved tissue outcomes persisted for at least 4 weeks. Moreover, contrary to previously tested isoform-nonspecific ROCK inhibitors, KD-025 did not cause severe hypotension. This suggests that the beneficial effect of ROCK inhibition in acute ischemic stroke mainly resulted from vascular, brain, and/or leukocyte ROCK2 inhibition and that specific ROCK2 inhibitors, with limited hypotensive effects, could have a more favorable safety profile than isoform-nonspecific ROCK inhibitors. In a phase 1 study in healthy volunteers, KD-025 showed no major side effects, raising hopes that clinical trials in patients with ischemic stroke could be performed in the near future (Lee et al., 2014a).

*c. Spinal cord injury.* Induction of neural plasticity and regeneration is a challenge for the treatment of traumatic or nontraumatic spinal cord injury. RhoA/ROCK signaling activation downstream of Nogo receptor family members or chondroitin sulfate proteoglycan receptors promotes axon growth inhibition, thus suggesting that inhibition of this pathway may be a promising strategy for axon regeneration (Fujita and Yamashita, 2014). Indeed, treatment with the ROCK inhibitors fasudil or Y-27632 stimulates axonal regrowth and functional recovery in animal spinal cord injury models (Hara et al., 2000; Dergham et al., 2002; Fournier et al., 2003; Sung et al., 2003). A recent meta-analysis of 30 preclinical studies reported that RhoA/ROCK inhibition improves by 15% locomotor recovery after experiment spinal cord injury (Watzlawick et al., 2014). A phase 1/2 clinical trial (Japan Primary Registries Network identifier UMIN00000825) designed to assess the safety and feasibility of the combination of fasudil and olfactory mucosa autograft into patients with injured spinal cords has been completed in Japan but no results have been disclosed (Table 6). Nevertheless, the increase neurologic recovery observed in patients with cervical or thoracic spinal cord injury treated with a single dose of Rho inhibitor Cethrin [BA-210; 4-N-(3-chloro-7-methoxyacridin-1-yl)-1-N,1-N-diethylpentane-1,4-diamine;dihydrochloride] further

TABLE 6  
Clinical trials of ROCK inhibitors

Indication	Compound	Target	Phase	Status	Identifier
Glaucoma	AMA0076	ROCK1/ROCK2	2	Completed	NCT02136940; NCT01693315; NCT02003547
	PG324	ROCK1/ROCK2	2	Completed	NCT025057575
	Y-39983 (SNJ-1656; RKI-983)	ROCK1/ROCK2	2	Completed	NCT00846989; NCT00515424
	AR-13324	ROCK1/ROCK2	3	Recruiting	NCT02207621
Psoriasis	KD025 (SLx2119)	ROCK2	2	Recruiting	NCT02317627
			2	Completed	NCT02106195
Diabetic retinopathy	K-115 (Ripasudil)	ROCK1/ROCK2	2	Completed	JapicCTI-142456
	Fasudil	ROCK1/ROCK2	3	Recruiting	NCT01823081
Erectile dysfunction	SAR407899	ROCK1/ROCK2	2	Completed	NCT00914277
Amyotrophic lateral sclerosis	Fasudil	ROCK1/ROCK2	2	Recruiting	NCT01935518
Spinal cord injury	Fasudil	ROCK1/ROCK2	1/2	Completed	JPRN-UMIN00000825
Atherosclerosis	Fasudil	ROCK1/ROCK2	2	Completed	NCT00120718
	Fasudil	ROCK1/ROCK2	2	Recruiting	NCT00670202
Chronic kidney disease	SAR407899	ROCK1/ROCK2	1	Completed	NCT01485900

supports that ROCK is promising molecular target for the treatment of spinal cord injury (Fehlings et al., 2011).

*d. Neurodegenerative Diseases.* Alzheimer disease is characterized by the extracellular deposition of  $\beta$ -amyloid aggregates. Several studies have demonstrated the involvement of ROCKs in the cleavage of amyloid precursor protein to the toxic  $\beta$ -amyloid peptide (Salminen et al., 2008). In murine models of Alzheimer disease, treatment with ROCK inhibitors reduces the level of toxic  $\beta$ -amyloid peptide (Zhou et al., 2003), promotes elongation of dendrite arbors (Couch et al., 2010), and improves learning dysfunction (Hou et al., 2012). Furthermore, the ROCK inhibitor fasudil reduces cognitive impairment and hippocampal neurodegeneration induced by  $\beta$ -amyloid peptide by suppressing the inflammatory response (Song et al., 2013). The anti-inflammatory, neuroprotective, and neuroregeneration-stimulating activities of ROCK inhibitors can be an added value in this pathologic context (Mueller et al., 2005). However, ROCK1 and ROCK2 knockdown experiments in human neuroblastoma cells resulting in increased and decreased  $\beta$ -amyloid peptide secretion, respectively, have revealed opposing effects of ROCK isoforms (Herskowitz et al., 2013). Accordingly, the net effect of nonselective ROCK inhibitors on  $\beta$ -amyloid peptide secretion is limited, and selective ROCK2 inhibition by SR3677 [N-[2-[2-(Dimethylamino)ethoxy]-4-(1H-pyrazol-4-yl)phenyl]-2,3-dihydro-1,4-benzodioxin-2-carboxamide dihydrochloride] results in a significantly greater effect (Herskowitz et al., 2013). These data support the need to develop isoform-selective ROCK2 inhibitors in this particular pathologic context.

A role of ROCKs has also been proposed in other neurodegenerative diseases, including Parkinson disease, Huntington disease, and amyotrophic lateral sclerosis. Fasudil prevents dopaminergic neuron degeneration and improves motor performance in a mouse model of Parkinson disease through stimulation of the Akt-mediated neuroprotective pathway (Tonges et al., 2012). This neuroprotection also supports the beneficial effect of pharmacologic ROCK inhibition in the R6/2 HD mouse model of Huntington disease (Li et al., 2009). In the mouse model of amyotrophic lateral sclerosis (hSOD1<sup>G93A</sup>), neuron loss has been attributed to ROCK-mediated inhibition of Akt signaling, and inhibition of this pathway by fasudil slows disease progression and prolongs survival time (Takata et al., 2013). In an experimental autoimmune encephalomyelitis animal model of multiple sclerosis, RhoA-ROCK signaling in autoreactive Th1/Th17 helper T cells has been identified as a potential target for treatment of multiple sclerosis and related neurodegenerative disorders (Paintlia et al., 2012).

These preclinical studies in rodents suggest neuroprotective and neuroregeneration-stimulating activities

of ROCK inhibitors that make them promising candidates for the treatment of diverse neurodegenerative disorders. As a logical outcome, the ROCK inhibitor fasudil is currently in a phase 2 clinical trial (ClinicalTrials.gov identifier NCT01935518) for the treatment of amyotrophic lateral sclerosis (Table 6).

*2. Cardiovascular diseases.* As the major downstream RhoA effector and a key regulator of vascular smooth muscle cell contraction and endothelial function, ROCK was identified as an interesting target for cardiovascular pharmacology. After numerous and positive preclinical studies, several clinical trials have demonstrated some benefits of ROCK inhibition in cardiovascular diseases in humans.

*a. Systemic hypertension.* Intra-arterial administration of fasudil induces a stronger decrease in forearm vascular resistance in patients with essential hypertension than in normotensive patients, suggesting that hypertension-associated vascular dysfunction depends, at least in part, on ROCK activation (Masumoto et al., 2001). Recently, it was shown that ROCK activity and ROCK-dependent vasoconstriction of cutaneous vessels are upregulated in patients with essential hypertension compared with normotensive subjects, thus confirming the potential role of increased ROCK-dependent arterial tone in hypertension (Smith et al., 2013). However, unexpectedly, the blood pressure-lowering effect of ROCK inhibitors generally decreases after 7–10 days of chronic treatment for reasons that are unknown (Feng and LoGrasso, 2014).

*b. Pulmonary arterial hypertension.* Acute intravenous or inhaled fasudil treatment significantly also reduces pulmonary vascular resistance and mean pulmonary arterial pressure in patients with severe PAH (Fukumoto et al., 2005; Ishikura et al., 2006; Fujita et al., 2010); More recently, chronic effects have been analyzed by the use of AT-877ER, an extended-release formulation of fasudil (Fukumoto et al., 2013). Three months of treatment with AT-877ER led to significant improvement in patients with PAH, with a correlation with serum levels (Fukumoto et al., 2013).

*c. Spastic and stable effort angina.* Intracoronary administration of fasudil reduces myocardial ischemia by preventing coronary artery spasm in patients with vasospastic angina (Masumoto et al., 2002). The anti-anginal effect of fasudil was further documented in larger clinical trials in patients with stable effort angina. Long-term oral treatment with fasudil reduces myocardial ischemia and ameliorates exercise tolerance (Shimokawa et al., 2002; Vicari et al., 2005). Supported by these effects of ROCK inhibitors in humans and their beneficial actions in animal models of atherosclerosis, two phase 2 clinical trials (ClinicalTrials.gov identifiers NCT00120718 and NCT00670202) are assessing the effect of fasudil in atherosclerosis (Table 6).



*d. Raynaud phenomenon.* In light of the observed antivasospastic effect of fasudil, the exacerbated cold-induced vasospasm in patients with Raynaud phenomenon represents a potential interesting clinical application of ROCK inhibitors. In a small clinical trial in 17 patients with Raynaud phenomenon secondary to systemic sclerosis, a single dose of fasudil demonstrated a significant benefit in terms of digital blood flow after a cold challenge (Fava et al., 2012).

*3. Glaucoma.* Glaucoma is a frequent optic neuropathy identified as the second leading cause of vision loss worldwide. Lowering intraocular pressure is the only therapeutic strategy shown to be effective in the treatment of glaucoma. Accumulating preclinical evidence shows that activation of RhoA/ROCK in the outflow pathway (trabecular meshwork, Schlemm canal) reduces aqueous humor outflow, thereby increasing intraocular pressure (Inoue and Tanihara, 2013). By contrast, ROCK inhibition increases aqueous humor outflow and decreases intraocular pressure, an effect mainly attributed to the relaxing action of ROCK inhibitors on smooth muscle–like trabecular meshwork cells shown to express both ROCK1 and ROCK2 (Wang et al., 2013). Several phase 1 and phase 2 clinical trials have been conducted to assess the safety and efficacy of topical application of various ROCK inhibitors in patients with glaucoma or ocular hypertension. Although results from some of these studies have not yet been published (Table 6), clinical studies of K-115 (ripasudil; Tanihara et al., 2013, 2015) and AR-13324 [XXX] (Bacharach et al., 2015) demonstrated safety and efficacy of once- or twice-daily administration of the inhibitor to decrease intraocular pressure. A phase 3 clinical trials for AR-13324 is in progress (Table 6). After its phase 3 trial, ripasudil (ophthalmic solution 0.4%; Glanatec; DWTI, Nagoyashi, Japan) was approved in Japan at the end of 2014 for the treatment of glaucoma and ocular hypertension when other therapeutic agents are not effective or cannot be administered. A phase 2 study is in progress to assess the effect of ripasudil for the treatment of diabetic retinopathy.

*4. Autoimmune Diseases.* ROCK activity was reported to be upregulated in patients with autoimmune disease (rheumatoid arthritis and systemic lupus erythematosus), and ROCK inhibition reduces autoimmune responses in animal models (He et al., 2008; Biswas et al., 2010). By controlling IL-21 and IL-17 production, ROCK2 plays a major role in the control of autoimmunity in mice (Biswas et al., 2010). ROCK2 seems to also be important for autoimmunity in humans, because the selective ROCK2 inhibitor KD-025 inhibited IL-21 and IL-17 secretion in T cells derived from healthy subjects or patients with rheumatoid arthritis and promoted the suppressive function of regulatory T cells (Zanin-Zhorov et al., 2014). By regulating the balance between proinflammatory and regulatory T cells, selective ROCK2 inhibition could

represent a novel therapeutic strategy for the treatment of autoimmune diseases. This hypothesis is currently being assessed in phase 2 clinical trials in patients with psoriasis (Table 6).

*5. Perspectives for Other Diseases.* Various data showing the effects of ROCK inhibitors in cancer cell lines or in animal cancer models support potential applications of ROCK inhibitors in cancer treatment but they have not yet led to clinical trials (Morgan-Fisher et al., 2013). Both isoform-nonspecific ROCK inhibitors and the specific ROCK2 inhibitor KD-025 delayed tumor growth (Boerma et al., 2008). More data are required, including the use of *Rock1* and *Rock2* knockout mice in cancer studies, to elucidate the usefulness of ROCK inhibitors for the treatment of cancer, prevention of metastasis, and inhibition of tumor growth and vascularization.

On the basis of the potentiating effect of ROCK inhibitors on production and the effect of vascular NO, ROCK signaling has been recognized as a new therapeutic target for the treatment of erectile dysfunction (Sopko et al., 2014; Toque and Caldwell, 2014). A phase 2 clinical trial (ClinicalTrials.gov identifier NCT00914277) comparing SAR407899 to placebo and sildenafil has been completed but no results have been published thus far (Table 5). Further clinical trials are thus required to establish whether ROCK inhibitors are indeed an alternative efficient therapy to current erectile dysfunction treatments.

Chronic kidney disease is a serious public health problem that may also be considered as a relevant indication for the therapeutic use of ROCK inhibitors. Among the major outcomes of chronic kidney disease is the development of cardiovascular disease; conversely, cardiovascular disease induces renal damage and chronic kidney disease. Fasudil has demonstrated renoprotective action in various models of renal disease, owing to combined inhibitory effects on macrophage infiltration, cell proliferation, oxidative stress, expression of extracellular matrix genes, and urinary protein reduction (Kanda et al., 2003; Nishikimi et al., 2004; Ishikawa et al., 2006). A phase 1 study (ClinicalTrials.gov identifier NCT01485900), designed to assess safety and tolerability of repeated multiple doses of SAR407899 in patients with chronic kidney disease treated with angiotensin-converting enzyme inhibitors has been completed, but no results have been reported to date (Table 6).

ROCK inhibitors might also represent interesting therapeutic tools in the treatment of pulmonary and respiratory diseases. In light of recent results in mouse models, the potential beneficial effect of ROCK inhibitors in asthma deserves to be further investigated, particularly for understanding the respective roles of ROCK1 and ROCK2, before considering the use of inhaled ROCK inhibitors for the treatment of allergic and nonallergic airway hyperresponsiveness. Regarding

pulmonary fibrosis, results from animal studies, characterized by short median survival and limited efficient treatment, identified ROCK inhibitors as potential powerful pharmacological agents to halt progression of pulmonary fibrosis (Knipe et al., 2015).

## VII. Conclusions

Major advances emerging from recent research on ROCKs cover three main aspects. The first is the identification of common and divergent roles of ROCK1 and ROCK2 isoforms. Despite their strong homology, both isoforms possess nonredundant functions, which deserve to be more deeply investigated to provide relevant clues that may help to define therapeutic applications of ROCK inhibition and to determine whether isoform-selective inhibitors would be better than nonselective ROCK inhibitors. Generation and analysis of cell- and tissue-specific *Rock1* and *Rock2* knockout mice will likely provide valuable input on this area in the near future. The second aspect, linked to the first, is the development of isoform-selective ROCK inhibitors, with KD-025 as the first ROCK2-specific inhibitor. Development and optimization of new molecules is expected to identify new inhibitors targeting ROCK1, ROCK2, or both. Preclinical studies will be required to address several important questions on the therapeutic use of ROCK inhibitors, including the risk of drug resistance by compensatory mechanisms by other proteins that are involved in the same signaling pathway or are able to play a role similar to ROCKs. An additional question for isoform-selective ROCK inhibitors concerns the risk of cross-compensatory mechanisms.

The third major achievement is the development of translational research and clinical trials to understand the role of ROCKs in human physiology and diseases, as well as to assess not only the usefulness of ROCK inhibitors as new therapeutic tools but also the use of ROCK activity and/or expression as biomarkers of pathologic conditions. From such studies, leukocyte ROCK activity has been identified as a potential biomarker for predicting cardiovascular events, and ripasudil has been approved and launched in Japan for the treatment of glaucoma. It is thus likely that results from current basic and clinical research on ROCKs will continue to improve our knowledge of the role of ROCKs and the clinical indications of ROCK inhibitors in human diseases. Although the blood pressure-lowering effect of ROCK inhibitors makes them attractive candidates as new antihypertensive agents for both pulmonary and systemic hypertension, this can represent an undesirable side effect in uses for other indications. This potential limitation can be more or less easily circumvented depending on the nature and the severity of the targeted disease. For instance, local application of ROCK inhibitors (e.g., intraocular injections for eye diseases, dermal application for skin diseases, or inhalation for lung

diseases) represents an efficient way to avoid systemic side effects. Better knowledge of the differential roles of ROCK1 and ROCK2, together with the development of isoform-specific ROCK inhibitors, is also expected to favorably address this issue.

## Authorship Contributions

Wrote or contributed to the writing of the manuscript: Loirand.

## References

- Abe K, Shimokawa H, Morikawa K, Uwatoku T, Oi K, Matsumoto Y, Hattori T, Nakashima Y, Kaibuchi K, and Sueishi K, et al. (2004) Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ Res* **94**:385–393.
- Akagi EM, Lavorato-Rocha AM, Maia BdeM, Rodrigues IS, Carvalho KC, Stiepcich MM, Baiocchi G, Sato-Kuwabara Y, Rogatto SR, and Soares FA, et al. (2014) ROCK1 as a novel prognostic marker in vulvar cancer. *BMC Cancer* **14**:822.
- Amano M, Chihara K, Nakamura N, Kaneko T, Matsuura Y, and Kaibuchi K (1999) The COOH terminus of Rho-kinase negatively regulates rho-kinase activity. *J Biol Chem* **274**:32418–32424.
- Amano M, Fukata Y, Shimokawa H, and Kaibuchi K (2000) Purification and in vitro activity of Rho-associated kinase. *Methods Enzymol* **325**:149–155.
- Arita R, Hata Y, Nakao S, Kita T, Miura M, Kawahara S, Zandi S, Almulki L, Tayyari F, and Shimokawa H, et al. (2009) Rho kinase inhibition by fasudil ameliorates diabetes-induced microvascular damage. *Diabetes* **58**:215–226.
- Bacharach J, Dubiner HB, Levy B, Kocpczynski CC, and Novack GD; AR-13324-CS202 Study Group (2015) Double-masked, randomized, dose-response study of AR-13324 versus latanoprost in patients with elevated intraocular pressure. *Ophthalmology* **122**:302–307.
- Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLaughlan H, Klevernic I, Arthur JS, Alessi DR, and Cohen P (2007) The selectivity of protein kinase inhibitors: a further update. *Biochem J* **408**:297–315.
- Bhandary L, Whipple RA, Vitolo MI, Charpentier MS, Boggs AE, Chakrabarti KR, Thompson KN, and Martin SS (2015) ROCK inhibition promotes microtubules that enhance reattachment of breast cancer cells. *Oncotarget* **6**:6251–6266.
- Biswas PS, Gupta S, Chang E, Song L, Storzaker RA, Liao JK, Bhagat G, and Pernis AB (2010) Phosphorylation of IRF4 by ROCK2 regulates IL-17 and IL-21 production and the development of autoimmunity in mice. *J Clin Invest* **120**:3280–3295.
- Bivalacqua TJ, Champion HC, Usta MF, Celtek S, Chitaley K, Webb RC, Lewis RL, Mills TM, Hellstrom WJ, and Kadowitz PJ (2004) RhoA/Rho-kinase suppresses endothelial nitric oxide synthase in the penis: a mechanism for diabetes-associated erectile dysfunction. *Proc Natl Acad Sci USA* **101**:9121–9126.
- Boerma M, Fu Q, Wang J, Loose DS, Bartolozzi A, Ellis JL, McGonigle S, Paradise E, Sweetnam P, and Fink LM, et al. (2008) Comparative gene expression profiling in three primary human cell lines after treatment with a novel inhibitor of Rho kinase or atorvastatin. *Blood Coagul Fibrinolysis* **19**:709–718.
- Bottino J, Gelaleti GB, Maschio LB, Jardim-Perassi BV, and de Campos Zuccari DAP (2014) Immunoreexpression of ROCK-1 and MMP-9 as prognostic markers in breast cancer. *Acta Histochem* **116**:1367–1373.
- Brown JH, Del Re DP, and Sussman MA (2006) The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. *Circ Res* **98**:730–742.
- Chang J, Xie M, Shah VR, Schneider MD, Entman ML, Wei L, and Schwartz RJ (2006) Activation of Rho-associated coiled-coil protein kinase 1 (ROCK-1) by caspase-3 cleavage plays an essential role in cardiac myocyte apoptosis. *Proc Natl Acad Sci USA* **103**:14495–14500.
- Chen CP, Chen X, Qiao YN, Wang P, He WQ, Zhang CH, Zhao W, Gao YQ, Chen C, and Tao T, et al. (2015) In vivo roles for myosin phosphatase targeting subunit-1 phosphorylation sites T694 and T852 in bladder smooth muscle contraction. *J Physiol* **593**:681–700.
- Chen XQ, Tan I, Ng CH, Hall C, Lim L, and Leung T (2002) Characterization of RhoA-binding kinase ROKalpha implication of the pleckstrin homology domain in ROKalpha function using region-specific antibodies. *J Biol Chem* **277**:12680–12688.
- Cheng CI, Lin YC, Tsai TH, Lin HS, Liou CW, Chang WN, Lu CH, Yuen CM, and Yip HK (2014) The prognostic values of leukocyte Rho kinase activity in acute ischemic stroke. *BioMed Res Int* **2014**:214587.
- Chevrier V, Piel M, Collomb N, Saoudi Y, Frank R, Paintrand M, Narumiya S, Bornens M, and Job D (2002) The Rho-associated protein kinase p160ROCK is required for centrosome positioning. *J Cell Biol* **157**:807–817.
- Chuang HH, Yang CH, Tsay YG, Hsu CY, Tseng LM, Chang ZF, and Lee HH (2012) ROCK1 Ser1366 phosphorylation reflects the activation status. *Biochem J* **443**:145–151.
- Cicek FA, Kandilci HB, and Turan B (2013) Role of ROCK upregulation in endothelial and smooth muscle vascular functions in diabetic rat aorta. *Cardiovasc Diabetol* **12**:51.
- Couch BA, DeMarco GJ, Gourley SL, and Koleske AJ (2010) Increased dendrite branching in AbetaPP/PS1 mice and elongation of dendrite arbors by fasudil administration. *J Alzheimers Dis* **20**:1003–1008.
- Creager MA, Lüscher TF, Cosentino F, and Beckman JA (2003) Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. *Circulation* **108**:1527–1532.
- Davis GE, Stratman AN, Sacharidou A, and Koh W (2011) Molecular basis for endothelial lumen formation and tubulogenesis during vasculogenesis and angiogenic sprouting. *Int Rev Cell Mol Biol* **288**:101–165.

- Demiryürek S, Okumus S, Bozgeyik İ, Oztuzcu S, Coskun E, Mat E, Durucu E, Tatar MG, Erbagci İ, and Gürlü B, et al. (2015) Investigation of the Rho-kinase gene polymorphism in primary open-angle glaucoma. *Ophthalmic Genet* DOI: 10.3109/13816810.2014.895016 [published ahead of print].
- Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, and McKerracher L (2002) Rho signaling pathway targeted to promote spinal cord repair. *J Neurosci* **22**:6570–6577.
- Do e Z, Fukumoto Y, Sugimura K, Miura Y, Tabe S, Yamamoto S, Aoki T, Nochioka K, Nergui S, and Yaoita N, et al. (2013) Rho-kinase activation in patients with heart failure. *Circ J* **77**:2542–2550.
- Do e Z, Fukumoto Y, Takaki A, Tawara S, Ohashi J, Nakano M, Tada T, Saji K, Sugimura K, and Fujita H, et al. (2009) Evidence for Rho-kinase activation in patients with pulmonary arterial hypertension. *Circ J* **73**:1731–1739.
- Doe C, Bentley R, Behm DJ, Lafferty R, Stavenger R, Jung D, Bamford M, Panchal T, Grygielko E, and Wright LL, et al. (2007) Novel Rho kinase inhibitors with anti-inflammatory and vasodilatory activities. *J Pharmacol Exp Ther* **320**:89–98.
- Dong M, Jiang X, Liao JK, and Yan BP (2013) Elevated rho-kinase activity as a marker indicating atherosclerosis and inflammation burden in polyvascular disease patients with concomitant coronary and peripheral arterial disease. *Clin Cardiol* **36**:347–351.
- Dong M, Liao JK, Fang F, Lee APW, Yan BPY, Liu M, and Yu CM (2012) Increased Rho kinase activity in congestive heart failure. *Eur J Heart Fail* **14**:965–973.
- Doran JD, Liu X, Taslimi P, Saadat A, and Fox T (2004) New insights into the structure-function relationships of Rho-associated kinase: a thermodynamic and hydrodynamic study of the dimer-to-monomer transition and its kinetic implications. *Biochem J* **384**:255–262.
- Fagan KA, Oka M, Bauer NR, Gebb SA, Ivy DD, Morris KG, and McMurtry IF (2004) Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase. *Am J Physiol Lung Cell Mol Physiol* **287**:L656–L664.
- Farah S, Agazie Y, Ohan N, Ngsee JK, and Liu XJ (1998) A rho-associated protein kinase, ROKalpha, binds insulin receptor substrate-1 and modulates insulin signaling. *J Biol Chem* **273**:4740–4746.
- Fava A, Wung PK, Wigley FM, Hummers LK, Daya NR, Ghazarian SR, and Boin F (2012) Efficacy of Rho kinase inhibitor fasudil in secondary Raynaud's phenomenon. *Arthritis Care Res (Hoboken)* **64**:925–929.
- Fehlings M, Theodore N, Harrop J, Maurais G, Kuntz C, Shaffrey CI, Kwon BK, Chapman J, Yee A, and Tighe A, et al. (2011) A phase I/IIa clinical trial of a recombinant Rho protein antagonist in acute spinal cord injury. *J Neurotrauma* **28**:787–796.
- Feng J, Ito M, Kureishi Y, Ichikawa K, Amano M, Isaka N, Okawa K, Iwamoto A, Kaibuchi K, and Hartshorne DJ, et al. (1999) Rho-associated kinase of chicken gizzard smooth muscle. *J Biol Chem* **274**:3744–3752.
- Feng Y and LoGrasso PV (2014) Rho kinase inhibitors: a patent review (2012–2013). *Expert Opin Ther Pat* **24**:295–307.
- Feske SK, Sorond FA, Henderson GV, Seto M, Hitomi A, Kawasaki K, Sasaki Y, Asano T, and Liao JK (2009) Increased leukocyte ROCK activity in patients after acute ischemic stroke. *Brain Res* **1257**:89–93.
- Fournier AE, Takizawa BT, and Strittmatter SM (2003) Rho kinase inhibition enhances axonal regeneration in the injured CNS. *J Neurosci* **23**:1416–1423.
- Fu P, Liu F, Su S, Wang W, Huang XR, Entman ML, Schwartz RJ, Wei L, and Lan HY (2006) Signaling mechanism of renal fibrosis in unilateral ureteral obstructive kidney disease in ROCK1 knockout mice. *J Am Soc Nephrol* **17**:3105–3114.
- Fujita H, Fukumoto Y, Saji K, Sugimura K, Demachi J, Nawata J, and Shimokawa H (2010) Acute vasodilator effects of inhaled fasudil, a specific Rho-kinase inhibitor, in patients with pulmonary arterial hypertension. *Heart Vessels* **25**:144–149.
- Fujita Y and Yamashita T (2014) Axon growth inhibition by RhoA/ROCK in the central nervous system. *Front Neurosci* **8**:338.
- Fukumoto Y, Matoba T, Ito A, Tanaka H, Kishi T, Hayashidani S, Abe K, Takeshita A, and Shimokawa H (2005) Acute vasodilator effects of a Rho-kinase inhibitor, fasudil, in patients with severe pulmonary hypertension. *Heart* **91**:391–392.
- Fukumoto Y, Yamada N, Matsubara H, Mizoguchi M, Uchino K, Yao A, Kihara Y, Kawano M, Watanabe H, and Takeda Y, et al. (2013) Double-blind, placebo-controlled clinical trial with a rho-kinase inhibitor in pulmonary arterial hypertension. *Circ J* **77**:2619–2625.
- Furukawa N, Ongusaha P, Jahng WJ, Araki K, Choi CS, Kim HJ, Lee YH, Kaibuchi K, Kahn BB, and Masuzaki H, et al. (2005) Role of Rho-kinase in regulation of insulin action and glucose homeostasis. *Cell Metab* **2**:119–129.
- Greenman C, Stephens P, Smith R, Dalgleish GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, and Stevens C, et al. (2007) Patterns of somatic mutation in human cancer genomes. *Nature* **446**:153–158.
- Guan R, Xu X, Chen M, Hu H, Ge H, Wen S, Zhou S, and Pi R (2013) Advances in the studies of roles of Rho/Rho-kinase in diseases and the development of its inhibitors. *Eur J Med Chem* **70**:613–622.
- Guérin P, Sauzeau V, Rolli-Derkinden M, Al Habbash O, Scalbert E, Crochet D, Picaud P, and Loirand G (2005) Stent implantation activates RhoA in human arteries: inhibitory effect of rapamycin. *J Vasc Res* **42**:21–28.
- Gutjahr MC, Rossy J, and Niggli V (2005) Role of Rho, Rac, and Rho-kinase in phosphorylation of myosin light chain, development of polarity, and spontaneous migration of Walker 256 carcinosarcoma cells. *Exp Cell Res* **308**:422–438.
- Hara M, Takayasu M, Watanabe K, Noda A, Takagi T, Suzuki Y, and Yoshida J (2000) Protein kinase inhibition by fasudil hydrochloride promotes neurological recovery after spinal cord injury in rats. *J Neurosurg* **93**(1, Suppl):94–101.
- Hata T, Goto C, Soga J, Hidaka T, Fujii Y, Idei N, Fujimura N, Maruhashi T, Mikami S, and Kihara Y, et al. (2011a) Measurement of Rho-associated kinase (ROCK) activity in humans: validity of leukocyte p-MBS/t-MBS in comparison with vascular response to fasudil. *Atherosclerosis* **214**:117–121.
- Hata T, Soga J, Hidaka T, Idei N, Fujii Y, Fujimura N, Mikami S, Maruhashi T, Kihara Y, and Chayama K, et al.; ROCK Study Group (2011b) Calcium channel blocker and Rho-associated kinase activity in patients with hypertension. *J Hypertens* **29**:373–379.
- Hattori T, Shimokawa H, Higashi M, Hiroki J, Mukai Y, Tsutsui H, Kaibuchi K, and Takeshita A (2004) Long-term inhibition of Rho-kinase suppresses left ventricular remodeling after myocardial infarction in mice. *Circulation* **109**:2234–2239.
- Hayashi K, Wakino S, Kanda T, Homma K, Sugano N, and Saruta T (2006) Molecular mechanisms and therapeutic strategies of chronic renal injury: role of rho-kinase in the development of renal injury. *J Pharmacol Sci* **100**:29–33.
- He Y, Xu H, Liang L, Zhan Z, Yang X, Yu X, Ye Y, and Sun L (2008) Anti-inflammatory effect of Rho kinase blockade via inhibition of NF-kappaB activation in rheumatoid arthritis. *Arthritis Rheum* **58**:3366–3376.
- Heemskerk N, van Rijssel J, and van Buul JD (2014) Rho-GTPase signaling in leukocyte extravasation: an endothelial point of view. *Cell Adhes Migr* **8**:67–75.
- Herskowitz JH, Feng Y, Mattheyses AL, Hales CM, Higginbotham LA, Duong DM, Montine TJ, Troncoso JC, Thambisetty M, and Seyfried NT, et al. (2013) Pharmacologic inhibition of ROCK2 suppresses amyloid- $\beta$  production in an Alzheimer's disease mouse model. *J Neurosci* **33**:19086–19098.
- Higashi M, Shimokawa H, Hattori T, Hiroki J, Mukai Y, Morikawa K, Ichiki T, Takahashi S, and Takeshita A (2003) Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: effect on endothelial NAD(P)H oxidase system. *Circ Res* **93**:767–775.
- Horowitz S, Binion DG, Nelson VM, Kanaa Y, Javadi P, Lazarova Z, Andreopoulos C, Kalyanaraman B, Otterson MF, and Rafiee P (2007) Increased arginase activity and endothelial dysfunction in human inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol* **292**:G1323–G1336.
- Hoshijima M, Sah VP, Wang Y, Chien KR, and Brown JH (1998) The low molecular weight GTPase Rho regulates myofibril formation and organization in neonatal rat ventricular myocytes. Involvement of Rho kinase. *J Biol Chem* **273**:7725–7730.
- Hou Y, Zhou L, Yang QD, Du XP, Li M, Yuan M, and Zhou ZW (2012) Changes in hippocampal synapses and learning-memory abilities in a streptozotocin-treated rat model and intervention by using fasudil hydrochloride. *Neuroscience* **200**:120–129.
- Hung MJ, Cherng WJ, Hung MY, Kuo LT, Cheng CW, Wang CH, Yang NI, and Liao JK (2012) Increased leukocyte Rho-associated coiled-coil containing protein kinase activity predicts the presence and severity of coronary vasospastic angina. *Atherosclerosis* **221**:521–526.
- Inoue T and Tanihara H (2013) Rho-associated kinase inhibitors: a novel glaucoma therapy. *Prog Retin Eye Res* **37**:1–12.
- Isagro J, Gupta S, Jacek E, Pavri T, Duculan R, Kim M, Kirou KA, Salmon JE, and Pernis AB (2013) Enhanced Rho-associated protein kinase activation in patients with systemic lupus erythematosus. *Arthritis Rheum* **65**:1592–1602.
- Ishikawa Y, Nishikimi T, Akimoto K, Ishimura K, Ono H, and Matsuoka H (2006) Long-term administration of rho-kinase inhibitor ameliorates renal damage in malignant hypertensive rats. *Hypertension* **47**:1075–1083.
- Ishikura K, Yamada N, Ito M, Ota S, Nakamura M, Isaka N, and Nakano T (2006) Beneficial acute effects of rho-kinase inhibitor in patients with pulmonary arterial hypertension. *Circ J* **70**:174–178.
- Isobe T, Mizuno K, Kaneko Y, Ohta M, Koide T, and Tanabe S (2014) Effects of K-115, a rho-kinase inhibitor, on aqueous humor dynamics in rabbits. *Curr Eye Res* **39**:813–822.
- Kahles T, Luedike P, Endres M, Galla H-J, Steinmetz H, Busse R, Neumann-Haefelin T, and Brandes RP (2007) NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. *Stroke* **38**:3000–3006.
- Kajikawa M, Noma K, Maruhashi T, Mikami S, Iwamoto Y, Iwamoto A, Matsumoto T, Hidaka T, Kihara Y, and Chayama K, et al. (2014) Rho-associated kinase activity is a predictor of cardiovascular outcomes. *Hypertension* **63**:856–864.
- Kalender ME, Demiryürek S, Oztuzcu S, Kizilyer A, Demiryürek AT, Sevinc A, Dikilitas M, Yildiz R, and Camci C (2010) Association between the Thr431Asn polymorphism of the ROCK2 gene and risk of developing metastases of breast cancer. *Oncol Res* **18**:583–591.
- Kamai T, Tsujii T, Arai K, Takagi K, Asami H, Ito Y, and Oshima H (2003) Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer. *Clin Cancer Res* **9**:2632–2641.
- Kamijo H, Matsumura Y, Thumkeo D, Koike S, Masu M, Shimizu Y, Ishizaki T, and Narumiya S (2011) Impaired vascular remodeling in the yolk sac of embryos deficient in ROCK-I and ROCK-II. *Genes Cells* **16**:1012–1021.
- Kanda T, Wakino S, Hayashi K, Homma K, Ozawa Y, and Saruta T (2003) Effect of fasudil on Rho-kinase and nephropathy in subtotally nephrectomized spontaneously hypertensive rats. *Kidney Int* **64**:2009–2019.
- Kanda T, Wakino S, Homma K, Yoshioka K, Tatamatsu S, Hasegawa K, Takamatsu I, Sugano N, Hayashi K, and Saruta T (2006) Rho-kinase as a molecular target for insulin resistance and hypertension. *FASEB J* **20**:169–171.
- Kasahara DI, Ninin FM, Wurmbbrand AP, Liao JK, and Shore SA (2015) Abrogation of airway hyperresponsiveness but not inflammation by rho kinase insufficiency. *Clin Exp Allergy* **45**:457–470.
- Kikuchi Y, Yamada M, Imakiire T, Kushiya M, Higashi K, Hyodo N, Yamamoto K, Oda T, Suzuki S, and Miura S (2007) A Rho-kinase inhibitor, fasudil, prevents development of diabetes and nephropathy in insulin-resistant diabetic rats. *J Endocrinol* **192**:595–603.
- Kikuchi Y, Yasuda S, Aizawa K, Tsuburaya R, Ito Y, Takeda M, Nakayama M, Ito K, Takahashi J, and Shimokawa H (2011) Enhanced Rho-kinase activity in circulating neutrophils of patients with vasospastic angina: a possible biomarker for diagnosis and disease activity assessment. *J Am Coll Cardiol* **58**:1231–1237.
- Knipe RS, Tager AM, and Liao JK (2015) The Rho kinases: critical mediators of multiple profibrotic processes and rational targets for new therapies for pulmonary fibrosis. *Pharmacol Rev* **67**:103–117.
- Kobayashi N, Horinaka S, Mita S, Nakano S, Honda T, Yoshida K, Kobayashi T, and Matsuoka H (2002) Critical role of Rho-kinase pathway for cardiac performance and remodeling in failing rat hearts. *Cardiovasc Res* **55**:757–767.

- Kolavennu V, Zeng L, Peng H, Wang Y, and Danesh FR (2008) Targeting of RhoA/ROCK signaling ameliorates progression of diabetic nephropathy independent of glucose control. *Diabetes* **57**:714–723.
- Kuwahara K, Kinoshita H, Kuwabara Y, Nakagawa Y, Usami S, Minami T, Yamada Y, Fujiwara M, and Nakao K (2010) Myocardin-related transcription factor A is a common mediator of mechanical stress- and neurohumoral stimulation-induced cardiac hypertrophic signaling leading to activation of brain natriuretic peptide gene expression. *Mol Cell Biol* **30**:4134–4148.
- Lane J, Martin TA, Watkins G, Mansel RE, and Jiang WG (2008) The expression and prognostic value of ROCK I and ROCK II and their role in human breast cancer. *Int J Oncol* **33**:585–593.
- Laufs U and Liao JK (1998) Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem* **273**:24266–24271.
- Lee DH, Shi J, Jeoung NH, Kim MS, Zabolotny JM, Lee SW, White MF, Wei L, and Kim YB (2009) Targeted disruption of ROCK1 causes insulin resistance in vivo. *J Biol Chem* **284**:11776–11780.
- Lee HH, Tien SC, Jou TS, Chang YC, Zhong JG, and Chang ZF (2010) Src-dependent phosphorylation of ROCK participates in regulation of focal adhesion dynamics. *J Cell Sci* **123**:3368–3377.
- Lee JH, Zheng Y, von Bornstadt D, Wei Y, Balcioglu A, Daneshmand A, Yalcin N, Yu E, Herisson F, and Atalay YB, et al. (2014a) Selective ROCK2 inhibition in focal cerebral ischemia. *Ann Clin Transl Neurol* **1**:2–14.
- Lee SH, Huang H, Choi K, Lee DH, Shi J, Liu T, Chun KH, Seo JA, Lima IS, and Zabolotny JM, et al. (2014b) ROCK1 isoform-specific deletion reveals a role for diet-induced insulin resistance. *Am J Physiol Endocrinol Metab* **306**:E332–E343.
- Leung T, Chen XQ, Manser E, and Lim L (1996) The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol Cell Biol* **16**:5313–5327.
- Leung T, Manser E, Tan L, and Lim L (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J Biol Chem* **270**:29051–29054.
- Li M, Huang Y, Ma AA, Lin E, and Diamond MI (2009) Y-27632 improves rotarod performance and reduces huntingtin levels in R6/2 mice. *Neurobiol Dis* **36**:413–420.
- Li Q, Xu Y, Li X, Guo Y, and Liu G (2012a) Inhibition of Rho-kinase ameliorates myocardial remodeling and fibrosis in pressure overload and myocardial infarction: role of TGF- $\beta$ 1-TAK1. *Toxicol Lett* **211**:91–97.
- Li R, Martin MP, Liu Y, Wang B, Patel RA, Zhu JY, Sun N, Pireddu R, Lawrence NJ, and Li J, et al. (2012b) Fragment-based and structure-guided discovery and optimization of Rho kinase inhibitors. *J Med Chem* **55**:2474–2478.
- Li Y, Harada T, Juang YT, Kyttaris VC, Wang Y, Zidanic M, Tung K, and Tsokos GC (2007) Phosphorylated ERM is responsible for increased T cell polarization, adhesion, and migration in patients with systemic lupus erythematosus. *J Immunol* **178**:1938–1947.
- Liao YC, Liu PY, Lin HF, Lin WY, Liao JK, and Juo SH (2015) Two functional polymorphisms of ROCK2 enhance arterial stiffening through inhibiting its activity and expression. *J Mol Cell Cardiol* **79**:180–186.
- Liu GJ, Wang ZJ, Wang YF, Xu LL, Wang XL, Liu Y, Luo GJ, He GH, and Zeng YJ (2012) Systematic assessment and meta-analysis of the efficacy and safety of fasudil in the treatment of cerebral vasospasm in patients with subarachnoid hemorrhage. *Eur J Clin Pharmacol* **68**:131–139.
- Liu L, Cao Y, Cui G, Li Z, Sun J, Zhang L, Chen C, Wang Y, Wang P, and Ding H, et al. (2013) Association analysis of polymorphisms in ROCK2 with cardiovascular disease in a Chinese population. *PLoS One* **8**:e53905.
- Liu PY, Chen JH, Lin LJ, and Liao JK (2007) Increased Rho kinase activity in a Taiwanese population with metabolic syndrome. *J Am Coll Cardiol* **49**:1619–1624.
- Liu PY, Liu YW, Lin LJ, Chen JH, and Liao JK (2009a) Evidence for statin pleiotropy in humans: differential effects of statins and ezetimibe on rho-associated coiled-coil containing protein kinase activity, endothelial function, and inflammation. *Circulation* **119**:131–138.
- Liu S, Goldstein RH, Scepanky EM, and Rosenblatt M (2009b) Inhibition of rho-associated kinase signaling prevents breast cancer metastasis to human bone. *Cancer Res* **69**:8742–8751.
- Liu X, Choy E, Hornicek FJ, Yang S, Yang C, Harmon D, Mankin H, and Duan Z (2011) ROCK1 as a potential therapeutic target in osteosarcoma. *J Orthop Res* **29**:1259–1266.
- Lochhead PA, Wickman G, Mezna M, and Olson MF (2010) Activating ROCK1 somatic mutations in human cancer. *Oncogene* **29**:2591–2598.
- Löhn M, Plettenburg O, Ivashchenko Y, Kannat A, Hofmeister A, Kadereit D, Schaefer M, Linz W, Kohlmann M, and Herbert JM, et al. (2009) Pharmacological characterization of SAR407899, a novel rho-kinase inhibitor. *Hypertension* **54**:676–683.
- Loirand G, Guérin P, and Pacaud P (2006) Rho kinases in cardiovascular physiology and pathophysiology. *Circ Res* **98**:322–334.
- Loirand G and Pacaud P (2010) The role of Rho protein signaling in hypertension. *Nat Rev Cardiol* **7**:637–647.
- Loirand G, Sauzeau V, and Pacaud P (2013) Small G proteins in the cardiovascular system: physiological and pathological aspects. *Physiol Rev* **93**:1659–1720.
- Lowery DM, Clauser KR, Hjerrild M, Lim D, Alexander J, Kishi K, Ong SE, Gammeltoft S, Carr SA, and Yaffe MB (2007) Proteomic screen defines the Polo-box domain interactome and identifies Rock2 as a Plk1 substrate. *EMBO J* **26**:2262–2273.
- Mallat Z, Gojova A, Sauzeau V, Brun V, Silvestre JS, Esposito B, Merval R, Groux H, Loirand G, and Tedgui A (2003) Rho-associated protein kinase contributes to early atherosclerotic lesion formation in mice. *Circ Res* **93**:884–888.
- Manser E, Leung T, Saliuddin H, Zhao ZS, and Lim L (1994) A brain serine/threonine kinase activated by Cdc42 and Rac1. *Nature* **367**:40–46.
- Masumoto A, Hirooka Y, Shimokawa H, Hironaga K, Setoguchi S, and Takeshita A (2001) Possible involvement of Rho-kinase in the pathogenesis of hypertension in humans. *Hypertension* **38**:1307–1310.
- Masumoto A, Mohri M, Shimokawa H, Urakami L, Usui M, and Takeshita A (2002) Suppression of coronary artery spasm by the Rho-kinase inhibitor fasudil in patients with vasospastic angina. *Circulation* **105**:1545–1547.
- Matsumoto Y, Uwatoku T, Oi K, Abe K, Hattori T, Morishige K, Eto Y, Fukumoto Y, Nakamura K, and Shibata Y, et al. (2004) Long-term inhibition of Rho-kinase suppresses neointimal formation after stent implantation in porcine coronary arteries: involvement of multiple mechanisms. *Arterioscler Thromb Vasc Biol* **24**:181–186.
- Ming XF, Barandier C, Viswambharan H, Kwak BR, Mach F, Mazzolai L, Hayoz D, Ruffieux J, Rusconi S, and Montani JP, et al. (2004) Thrombin stimulates human endothelial arginase enzymatic activity via RhoA/ROCK pathway: implications for atherosclerotic endothelial dysfunction. *Circulation* **110**:3708–3714.
- Ming XF, Viswambharan H, Barandier C, Ruffieux J, Kaibuchi K, Rusconi S, and Yang Z (2002) Rho GTPase/Rho kinase negatively regulates endothelial nitric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. *Mol Cell Biol* **22**:8467–8477.
- Mita S, Kobayashi N, Yoshida K, Nakano S, and Matsuoka H (2005) Cardioprotective mechanisms of Rho-kinase inhibition associated with eNOS and oxidative stress-LOX-1 pathway in Dahl salt-sensitive hypertensive rats. *J Hypertens* **23**:87–96.
- Morgan-Fisher M, Wewer UM, and Yoneda A (2013) Regulation of ROCK activity in cancer. *J Histochem Cytochem* **61**:185–198.
- Mueller BK, Mack H, and Teusch N (2005) Rho kinase, a promising drug target for neurological disorders. *Nat Rev Drug Discov* **4**:387–398.
- Nagaoka T, Fagan KA, Gebb SA, Morris KG, Suzuki T, Shimokawa H, McMurtry IF, and Oka M (2005) Inhaled Rho kinase inhibitors are potent and selective vasodilators in rat pulmonary hypertension. *Am J Respir Crit Care Med* **171**:494–499.
- Nagaoka T, Morio Y, Casanova N, Bauer N, Gebb S, McMurtry I, and Oka M (2004) Rho/Rho kinase signaling mediates increased basal pulmonary vascular tone in chronically hypoxic rats. *Am J Physiol Lung Cell Mol Physiol* **287**:L665–L672.
- Nagatoya K, Moriyama T, Kawada N, Takeji M, Oseto S, Murozono T, Ando A, Imai E, and Hori M (2002) Y-27632 prevents tubulointerstitial fibrosis in mouse kidneys with unilateral ureteral obstruction. *Kidney Int* **61**:1684–1695.
- Nishikimi T, Akimoto K, Wang X, Mori Y, Tadokoro K, Ishikawa Y, Shimokawa H, Ono H, and Matsuoka H (2004) Fasudil, a Rho-kinase inhibitor, attenuates glomerulosclerosis in Dahl salt-sensitive rats. *J Hypertens* **22**:1787–1796.
- Nishimura T and Takeichi M (2008) Shroom3-mediated recruitment of Rho kinases to the apical cell junctions regulates epithelial and neuroepithelial planar remodeling. *Development* **135**:1493–1502.
- Noma K, Rikitake Y, Oyama N, Yan G, Alcaide P, Liu PY, Wang H, Ahl D, Sawada N, and Okamoto R, et al. (2008) ROCK1 mediates leukocyte recruitment and neointima formation following vascular injury. *J Clin Invest* **118**:1632–1644.
- Nuno DW and Lamping KG (2010) The role of rho kinase in sex-dependent vascular dysfunction in type 1 diabetes. *Exp Diabetes Res* **2010**:176361.
- Ocaranza MP, Giarelli L, Mora I, Garcia L, McNab P, Godoy I, Braun S, Córdova S, Castro P, and Novoa U, et al. (2011) Markedly increased Rho-kinase activity in circulating leukocytes in patients with chronic heart failure. *Am Heart J* **161**:931–937.
- Oguz E, Alasehirli B, Pehlivan Y, Onat AM, Oztuzcu S, Ozkara E, Kısacik B, Camci C, and Demiryurek AT (2012) Association between Rho-kinase (ROCK2) gene polymorphisms and Behçet's disease. *Transl Res* **160**:428–434.
- Okamoto R, Li Y, Noma K, Hiroi Y, Liu PY, Taniguchi M, Ito M, and Liao JK (2013) FHL2 prevents cardiac hypertrophy in mice with cardiac-specific deletion of ROCK2. *FASEB J* **27**:1439–1449.
- Paintlia AS, Paintlia MK, Hollis BW, Singh AK, and Singh I (2012) Interference with RhoA-ROCK signaling mechanism in autoreactive CD4+ T cells enhances the bioavailability of 1,25-dihydroxyvitamin D3 in experimental autoimmune encephalomyelitis. *Am J Pathol* **181**:993–1006.
- Palomino Doza J, Topf A, Benthaz J, Bhattacharya S, Cosgrove C, Brook JD, Granados-Riveron J, Bu'Lock FA, O'Sullivan J, and Stuart AG, et al. (2013) Low-frequency intermediate penetrance variants in the ROCK1 gene predispose to Tetralogy of Fallot. *BMC Genet* **14**:57.
- Pan P, Shen M, Yu H, Li Y, Li D, and Hou T (2013) Advances in the development of Rho-associated protein kinase (ROCK) inhibitors. *Drug Discov Today* **18**:1323–1333.
- Patel RA, Liu Y, Wang B, Li R, and Sebt SM (2014) Identification of novel ROCK inhibitors with anti-migratory and anti-invasive activities. *Oncogene* **33**:550–555.
- Pinner S and Sahai E (2008) PDK1 regulates cancer cell motility by antagonising inhibition of ROCK1 by RhoE. *Nat Cell Biol* **10**:127–137.
- Rankinen T, Church T, Rice T, Markward N, Blair SN, and Bouchard C (2008) A major haplotype block at the rho-associated kinase 2 locus is associated with a lower risk of hypertension in a recessive manner: the HYPGENE study. *Hypertens Res* **31**:1651–1657.
- Rath N and Olson MF (2012) Rho-associated kinases in tumorigenesis: re-considering ROCK inhibition for cancer therapy. *EMBO Rep* **13**:900–908.
- Ray P, Wright J, Adam J, Bennett J, Boucharens S, Black D, Cook A, Brown AR, Epemolu O, and Fletcher D, et al. (2011) Fragment-based discovery of 6-substituted isouquinolin-1-amine based ROCK-I inhibitors. *Bioorg Med Chem Lett* **21**:97–101.
- Reedquist KA and Tak PP (2012) Signal transduction pathways in chronic inflammatory autoimmune disease: small GTPases. *Open Rheumatol J* **6**:259–272.
- Riento K and Ridley AJ (2003) Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* **4**:446–456.
- Rikitake Y and Liao JK (2005) Rho GTPases, statins, and nitric oxide. *Circ Res* **97**:1232–1235.
- Rikitake Y, Oyama N, Wang CY, Noma K, Satoh M, Kim HH, and Liao JK (2005) Decreased perivascular fibrosis but not cardiac hypertrophy in ROCK1<sup>-/-</sup> haploinsufficient mice. *Circulation* **112**:2959–2965.
- Salminen A, Suuronen T, and Kaarimäntä K (2008) ROCK, PAK, and Toll of synapses in Alzheimer's disease. *Biochem Biophys Res Commun* **371**:587–590.

- Sapet C, Simoncini S, Loriod B, Puthier D, Sampol J, Nguyen C, Dignat-George F, and Anfoso F (2006) Thrombin-induced endothelial microparticle generation: identification of a novel pathway involving ROCK-II activation by caspase-2. *Blood* **108**:1868–1876.
- Sari I, Berberoglu B, Ozkara E, Oztuzu S, Camci C, and Demiryurek AT (2013) Role of rho-kinase gene polymorphisms and protein expressions in colorectal cancer development. *Pathobiology* **80**:138–145.
- Sasaki Y, Suzuki M, and Hidaka H (2002) The novel and specific Rho-kinase inhibitor (S)-(+)-2-methyl-1-[4-methyl-5-isoquinoline)sulfonyl]-homopiperazine as a probing molecule for Rho-kinase-involved pathway. *Pharmacol Ther* **93**: 225–232.
- Satoh S, Ueda Y, Koyanagi M, Kadokami T, Sugano M, Yoshikawa Y, and Makino N (2003) Chronic inhibition of Rho kinase blunts the process of left ventricular hypertrophy leading to cardiac contractile dysfunction in hypertension-induced heart failure. *J Mol Cell Cardiol* **35**:59–70.
- Satoh S, Utsunomiya T, Tsurui K, Kobayashi T, Ikegaki I, Sasaki Y, and Asano T (2001) Pharmacological profile of hydroxy fasudil as a selective rho kinase inhibitor on ischemic brain damage. *Life Sci* **69**:1441–1453.
- Satoh S, Yamaguchi T, Hitomi A, Sato N, Shiraiwa K, Ikegaki I, Asano T, and Shimokawa H (2002) Fasudil attenuates interstitial fibrosis in rat kidneys with unilateral ureteral obstruction. *Eur J Pharmacol* **455**:169–174.
- Sawada N, Itoh H, Ueyama K, Yamashita J, Doi K, Chun TH, Inoue M, Masatsugu K, Saito T, and Fukunaga Y, et al. (2000) Inhibition of rho-associated kinase results in suppression of neointimal formation of balloon-injured arteries. *Circulation* **101**:2030–2033.
- Schaafsma D, Bos IS, Zuidhof AB, Zaagsma J, and Meurs H (2006) Inhalation of the Rho-kinase inhibitor Y-27632 reverses allergen-induced airway hyperresponsiveness after the early and late asthmatic reaction. *Respir Res* **7**:121.
- Schaafsma D, Bos IS, Zuidhof AB, Zaagsma J, and Meurs H (2008) The inhaled Rho kinase inhibitor Y-27632 protects against allergen-induced acute bronchoconstriction, airway hyperresponsiveness, and inflammation. *Am J Physiol Lung Cell Mol Physiol* **295**:L214–L219.
- Schofield AV and Bernard O (2013) Rho-associated coiled-coil kinase (ROCK) signaling and disease. *Crit Rev Biochem Mol Biol* **48**:301–316.
- Seasholtz TM, Wessel J, Rao F, Rana BK, Khandrika S, Kennedy BP, Lillie EO, Ziegler MG, Smith DW, and Schork NJ, et al. (2006) Rho kinase polymorphism influences blood pressure and systemic vascular resistance in human twins: role of heredity. *Hypertension* **47**:937–947.
- Sebbagh M, Hamelin J, Bertoglio J, Solary E, and Bréard J (2005) Direct cleavage of ROCK II by granzyme B induces target cell membrane blebbing in a caspase-independent manner. *J Exp Med* **201**:465–471.
- Sebbagh M, Renvoizé C, Hamelin J, Riché N, Bertoglio J, and Bréard J (2001) Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat Cell Biol* **3**:346–352.
- Shah DI and Singh M (2006a) Effect of fasudil on macrovascular disorder-induced endothelial dysfunction. *Can J Physiol Pharmacol* **84**:835–845.
- Shah DI and Singh M (2006b) Involvement of Rho-kinase in experimental vascular endothelial dysfunction. *Mol Cell Biochem* **283**:191–199.
- Shi J, Zhang YW, Summers LJ, Dorn GW 2nd, and Wei L (2008) Disruption of ROCK1 gene attenuates cardiac dilation and improves contractile function in pathological cardiac hypertrophy. *J Mol Cell Cardiol* **44**:551–560.
- Shi J, Zhang YW, Yang Y, Zhang L, and Wei L (2010) ROCK1 plays an essential role in the transition from cardiac hypertrophy to failure in mice. *J Mol Cell Cardiol* **49**: 819–828.
- Shibuya M, Hirai S, Seto M, Satoh S, and Ohtomo E; Fasudil Ischemic Stroke Study Group (2005) Effects of fasudil in acute ischemic stroke: results of a prospective placebo-controlled double-blind trial. *J Neurol Sci* **238**:31–39.
- Shibuya M, Suzuki Y, Sugita K, Saito I, Sasaki T, Takakura K, Nagata I, Kikuchi H, Takemae T, and Hidaka H, et al. (1992) Effect of AT877 on cerebral vasospasm after aneurysmal subarachnoid hemorrhage. Results of a prospective placebo-controlled double-blind trial. *J Neurosurg* **76**:571–577.
- Shimizu T, Fukumoto Y, Tanaka S, Satoh K, Ikeda S, and Shimokawa H (2013) Crucial role of ROCK2 in vascular smooth muscle cells for hypoxia-induced pulmonary hypertension in mice. *Arterioscler Thromb Vasc Biol* **33**:2780–2791.
- Shimizu Y, Thumkeo D, Keel J, Ishizaki T, Oshima H, Oshima M, Noda Y, Matsumura F, Taketo MM, and Narumiya S (2005) ROCK-I regulates closure of the eyelids and ventral body wall by inducing assembly of actomyosin bundles. *J Cell Biol* **168**: 941–953.
- Shimokawa H, Hiramori K, Inuma H, Hosoda S, Kishida H, Osada H, Katagiri T, Yamauchi K, Yui Y, and Minamoto T, et al. (2002) Anti-anginal effect of fasudil, a Rho-kinase inhibitor, in patients with stable effort angina: a multicenter study. *J Cardiovasc Pharmacol* **40**:751–761.
- Shin HK, Salomone S, Potts EM, Lee S-W, Millican E, Noma K, Huang PL, Boas DA, Liao JK, and Moskowitz MA, et al. (2007) Rho-kinase inhibition acutely augments blood flow in focal cerebral ischemia via endothelial mechanisms. *J Cereb Blood Flow Metab* **27**:998–1009.
- Sin WC, Chen XQ, Leung T, and Lim L (1998) RhoA-binding kinase alpha translocation is facilitated by the collapse of the vimentin intermediate filament network. *Mol Cell Biol* **18**:6325–6339.
- Smith CJ, Santhanam L, and Alexander LM (2013) Rho-Kinase activity and cutaneous vasoconstriction is upregulated in essential hypertensive humans. *Microvasc Res* **87**:58–64.
- Soga J, Noma K, Hata T, Hidaka T, Fujii Y, Idei N, Fujimura N, Mikami S, Maruhashi T, and Kihara Y, et al.; ROCK Study Group (2011) Rho-associated kinase activity, endothelial function, and cardiovascular risk factors. *Arterioscler Thromb Vasc Biol* **31**:2353–2359.
- Song Y, Chen X, Wang LY, Gao W, and Zhu MJ (2013) Rho kinase inhibitor fasudil protects against  $\beta$ -amyloid-induced hippocampal neurodegeneration in rats. *CNS Neurosci Ther* **19**:603–610.
- Sopko NA, Hannan JL, and Bivalacqua TJ (2014) Understanding and targeting the Rho kinase pathway in erectile dysfunction. *Nat Rev Urol* **11**:622–628.
- Stirzaker RA, Biswas PS, Gupta S, Song L, Bhagat G, and Pernis AB (2012) Administration of fasudil, a ROCK inhibitor, attenuates disease in lupus-prone NZB/W F1 female mice. *Lupus* **21**:656–661.
- Stockton RA, Shenkar R, Awad LA, and Ginsberg MH (2010) Cerebral cavernous malformations proteins inhibit Rho kinase to stabilize vascular integrity. *J Exp Med* **207**:881–896.
- Sung JK, Miao L, Calvert JW, Huang L, Louis Harkey H, and Zhang JH (2003) A possible role of RhoA/Rho-kinase in experimental spinal cord injury in rat. *Brain Res* **959**:29–38.
- Suzuki Y, Shibuya M, Satoh S, Sugimoto Y, and Takakura K (2007) A postmarketing surveillance study of fasudil treatment after aneurysmal subarachnoid hemorrhage. *Surg Neurol* **68**:126–131; discussion 131–132.
- Takata M, Tanaka H, Kimura M, Nagahara Y, Tanaka K, Kawasaki K, Seto M, Tsuruma K, Shimazawa M, and Hara H (2013) Fasudil, a rho kinase inhibitor, limits motor neuron loss in experimental models of amyotrophic lateral sclerosis. *Br J Pharmacol* **170**:341–351.
- Tanihara H, Inoue T, Yamamoto T, Kuwayama Y, Abe H, and Araie M; K-115 Clinical Study Group (2013) Phase 2 randomized clinical study of a Rho kinase inhibitor, K-115, in primary open-angle glaucoma and ocular hypertension. *Am J Ophthalmol* **156**:731–736.
- Tanihara H, Inoue T, Yamamoto T, Kuwayama Y, Abe H, Suginami H, and Araie M; K-115 Clinical Study Group (2015) Intra-ocular pressure-lowering effects of a Rho kinase inhibitor, ripasudil (K-115), over 24 hours in primary open-angle glaucoma and ocular hypertension: a randomized, open-label, crossover study. *Acta Ophthalmol* **93**:e254–e260.
- Thumkeo D, Keel J, Ishizaki T, Hirose M, Nonomura K, Oshima M, Oshima M, Taketo MM, and Narumiya S (2003) Targeted disruption of the mouse rho-associated kinase 2 gene results in intrauterine growth retardation and fetal death. *Mol Cell Biol* **23**:5043–5055.
- Thumkeo D, Shimizu Y, Sakamoto S, Yamada S, and Narumiya S (2005) ROCK-I and ROCK-II cooperatively regulate closure of eyelid and ventral body wall in mouse embryo. *Genes Cells* **10**:825–834.
- Thumkeo D, Watanabe S, and Narumiya S (2013) Physiological roles of Rho and Rho effectors in mammals. *Eur J Cell Biol* **92**:303–315.
- Tokushige H, Inatani M, Nemoto S, Sakaki H, Katayama K, Uehata M, and Tanihara H (2007) Effects of topical administration of y-39983, a selective rho-associated protein kinase inhibitor, on ocular tissues in rabbits and monkeys. *Invest Ophthalmol Vis Sci* **48**:3216–3222.
- Tonges L, Frank T, Tatenhorst L, Saal KA, Koch JC, Szego EM, Bahr M, Weishaupt JH, and Lingor P (2012) Inhibition of rho kinase enhances survival of dopaminergic neurons and attenuates axonal loss in a mouse model of Parkinson's disease. *Brain* **135**:3355–3370.
- Toque HA and Caldwell RW (2014) New approaches to the design and discovery of therapies to prevent erectile dysfunction. *Expert Opin Drug Discov* **9**: 1447–1469.
- Torka R, Thuma F, Herzog V, and Kirfel G (2006) ROCK signaling mediates the adoption of different modes of migration and invasion in human mammary epithelial tumor cells. *Exp Cell Res* **312**:3857–3871.
- Tsounapi P, Saito M, Kitatani K, Dimitriadis F, Ohmasa F, Shimizu S, Kinoshita Y, Takenaka A, and Satoh K (2012) Fasudil improves the endothelial dysfunction in the aorta of spontaneously hypertensive rats. *Eur J Pharmacol* **691**:182–189.
- Van de Velde S, Van Bergen T, Sijnave D, Hollanders K, Castermans K, Defert O, Leysen D, Vandewalle E, Moons L, and Stalmans I (2014) AMA0076, a novel, locally acting Rho kinase inhibitor, potentially lowers intraocular pressure in New Zealand white rabbits with minimal hyperemia. *Invest Ophthalmol Vis Sci* **55**: 1006–1016.
- van Nieuw Amerongen GP, Beckers CM, Achekar ID, Zeeman S, Musters RJ, and van Hinsbergh VW (2007) Involvement of Rho kinase in endothelial barrier maintenance. *Arterioscler Thromb Vasc Biol* **27**:2332–2339.
- Vesterinen HM, Currie GL, Carter S, Mee S, Watzlawick R, Egan KJ, Macleod MR, and Sena ES (2013) Systematic review and stratified meta-analysis of the efficacy of RhoA and Rho kinase inhibitors in animal models of ischaemic stroke. *Syst Rev* **2**:33.
- Vicari RM, Chaitman B, Keefe D, Smith WB, Chrysant SG, Tonkon MJ, Bittar N, Weiss RJ, Morales-Ballejo H, and Thadani U; Fasudil Study Group (2005) Efficacy and safety of fasudil in patients with stable angina: a double-blind, placebo-controlled, phase 2 trial. *J Am Coll Cardiol* **46**:1803–1811.
- Wang HW, Liu PY, Oyama N, Rikitake Y, Kitamoto S, Gitlin J, Liao JK, and Boisvert WA (2008) Deficiency of ROCK1 in bone marrow-derived cells protects against atherosclerosis in LDLR<sup>-/-</sup> mice. *FASEB J* **22**:3561–3570.
- Wang J, Liu X, and Zhong Y (2013) Rho/Rho-associated kinase pathway in glaucoma (Review). *Int J Oncol* **43**:1357–1367.
- Wang Y, Zheng XR, Riddick N, Bryden B, Baur W, Zhang X, and Surks HK (2009) ROCK isoform regulation of myosin phosphatase and contractility in vascular smooth muscle cells. *Circ Res* **104**:531–540.
- Wang YX, da Cunha V, Martin-McNulty B, Vincelette J, Li W, Choy DF, Halks-Miller M, Mahmoudi M, Schroeder M, and Johns A, et al. (2005) Inhibition of Rho-kinase by fasudil attenuated angiotensin II-induced cardiac hypertrophy in apolipoprotein E deficient mice. *Eur J Pharmacol* **512**:215–222.
- Ward Y, Yap SF, Ravichandran V, Matsumura F, Ito M, Spinelli B, and Kelly K (2002) The GTP binding proteins Gem and Rad are negative regulators of the Rho-Rho kinase pathway. *J Cell Biol* **157**:291–302.
- Watzlawick R, Sena ES, Dirnagl U, Brommer B, Kopp MA, Macleod MR, Howells DW, and Schwab JM (2014) Effect and reporting bias of RhoA/ROCK-blockade intervention on locomotor recovery after spinal cord injury: a systematic review and meta-analysis. *JAMA Neurol* **71**:91–99.
- Wei L, Roberts W, Wang L, Yamada M, Zhang S, Zhao Z, Rivkees SA, Schwartz RJ, and Imanaka-Yoshida K (2001) Rho kinases play an obligatory role in vertebrate embryonic organogenesis. *Development* **128**:2953–2962.

- Wen W, Liu W, Yan J, and Zhang M (2008) Structure basis and unconventional lipid membrane binding properties of the PH-C1 tandem of rho kinases. *J Biol Chem* **283**:26263–26273.
- Whitehead KJ, Chan AC, Navankasattusas S, Koh W, London NR, Ling J, Mayo AH, Drakos SG, Jones CA, and Zhu W, et al. (2009) The cerebral cavernous malformation signaling pathway promotes vascular integrity via Rho GTPases. *Nat Med* **15**:177–184.
- Wolfrum S, Dendorfer A, Rikitake Y, Stalker TJ, Gong Y, Scalia R, Dominiak P, and Liao JK (2004) Inhibition of Rho-kinase leads to rapid activation of phosphatidylinositol 3-kinase/protein kinase Akt and cardiovascular protection. *Arterioscler Thromb Vasc Biol* **24**:1842–1847.
- Wong CCL, Wong CM, Tung EKK, Man K, and Ng IOL (2009) Rho-kinase 2 is frequently overexpressed in hepatocellular carcinoma and involved in tumor invasion. *Hepatology* **49**:1583–1594.
- Wu J, Li J, Hu H, Liu P, Fang Y, and Wu D (2012) Rho-kinase inhibitor, fasudil, prevents neuronal apoptosis via the Akt activation and PTEN inactivation in the ischemic penumbra of rat brain. *Cell Mol Neurobiol* **32**:1187–1197.
- Yanazume T, Hasegawa K, Wada H, Morimoto T, Abe M, Kawamura T, and Sasayama S (2002) Rho/ROCK pathway contributes to the activation of extracellular signal-regulated kinase/GATA-4 during myocardial cell hypertrophy. *J Biol Chem* **277**:8618–8625.
- Yang X, Li Q, Lin X, Ma Y, Yue X, Tao Z, Wang F, Mckeehan WL, Wei L, and Schwartz RJ, et al. (2012) Mechanism of fibrotic cardiomyopathy in mice expressing truncated Rho-associated coiled-coil protein kinase 1. *FASEB J* **26**:2105–2116.
- Yao L, Chandra S, Toque HA, Bhatta A, Rojas M, Caldwell RB, and Caldwell RW (2013) Prevention of diabetes-induced arginase activation and vascular dysfunction by Rho kinase (ROCK) knockout. *Cardiovasc Res* **97**:509–519.
- Yoneda A, Multhaupt HA, and Couchman JR (2005) The Rho kinases I and II regulate different aspects of myosin II activity. *J Cell Biol* **170**:443–453.
- Yoo SY, Kim J, Cheong S, Shin DH, Jang J, Lee C, Tahk SJ, Shin JH, Choi SY, and Yoon MH (2012) Rho-associated kinase 2 polymorphism in patients with vasospastic angina. *Korean Circ J* **42**:406–413.
- Zanin-Zhorov A, Weiss JM, Nyuydzefe MS, Chen W, Scher JU, Mo R, Depoil D, Rao N, Liu B, and Wei J, et al. (2014) Selective oral ROCK2 inhibitor down-regulates IL-21 and IL-17 secretion in human T cells via STAT3-dependent mechanism. *Proc Natl Acad Sci USA* **111**:16814–16819.
- Zee RY, Wang QM, Chasman DI, Ridker PM, and Liao JK (2014) Gene variations of ROCKs and risk of ischaemic stroke: the Women's Genome Health Study. *Clin Sci (Lond)* **126**:829–835.
- Zhang YM, Bo J, Taffet GE, Chang J, Shi J, Reddy AK, Michael LH, Schneider MD, Entman ML, and Schwartz RJ, et al. (2006) Targeted deletion of ROCK1 protects the heart against pressure overload by inhibiting reactive fibrosis. *FASEB J* **20**:916–925.
- Zhou L, Liu F, Huang XR, Liu F, Chen H, Chung AC, Shi J, Wei L, Lan HY, and Fu P (2011) Amelioration of albuminuria in ROCK1 knockout mice with streptozotocin-induced diabetic kidney disease. *Am J Nephrol* **34**:468–475.
- Zhou Q and Liao JK (2009) Rho kinase: an important mediator of atherosclerosis and vascular disease. *Curr Pharm Des* **15**:3108–3115.
- Zhou Q, Mei Y, Shoji T, Han X, Kaminski K, Oh GT, Ongusaha PP, Zhang K, Schmitt H, and Moser M, et al. (2012) Rho-associated coiled-coil-containing kinase 2 deficiency in bone marrow-derived cells leads to increased cholesterol efflux and decreased atherosclerosis. *Circulation* **126**:2236–2247.
- Zhou Y, Su Y, Li B, Liu F, Ryder JW, Wu X, Gonzalez-DeWhitt PA, Gelfanova V, Hale JE, and May PC, et al. (2003) Nonsteroidal anti-inflammatory drugs can lower amyloidogenic Abeta42 by inhibiting Rho. *Science* **302**:1215–1217.
- Zhu M, Liu PY, Kasahara DI, Williams AS, Verbout NG, Halayko AJ, Fedulov A, Shoji T, Williams ES, and Noma K, et al. (2011) Role of Rho kinase isoforms in murine allergic airway responses. *Eur Respir J* **38**:841–850.