Novel cardiovascular protective effects of RhoA signaling and its therapeutic implications

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Abbreviations:

AAA: abdominal aortic aneurysm

AAV: adeno-associated virus

Cdc42: cell division cycle 42

cKO: conditional knockout

Daam1: Dishevelled-associated activator of morphogenesis 1

DCM: dilated cardiomyopathy

eNOS: endothelial nitric oxide synthase

GAP: GTPase-activating protein

GDI: guanine nucleotide dissociation inhibitor

GDP: guanosine diphosphate

GEF: guanine nucleotide exchange factor

GTP: guanosine triphosphate

IL: interleukin

IRS1: insulin receptor substrate-1

KO: knockout

LIMK: Lin11, Isl-1 and Mec-3 kinase

MAPK: mitogen-activated protein kinase

MAP4K4: mitogen-activated protein kinase kinase kinase kinase 4

MLC: myosin light chain

MLCK: myosin light chain kinase

MLCP: myosin light chain phosphatase

MYPT1: myosin phosphatase-targeting subunit 1

NF: nuclear factor

NO: nitric oxide

NSC: neural stem cell

PI3K: phosphatidylinositol-3 kinase

PP2A: protein phosphatase 2A

PTEN: phosphatase and tension homolog

Rac1: Ras-related C3 botulinum toxin substrate 1

RhoA: Ras homolog gene family member A

ROCK: Rho-associated, coiled-coil-containing protein kinase

SERCA2a: sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2a

TNF: tumor necrosis factor

VSM: vascular smooth muscle

VSMC: vascular smooth muscle cell

Abstract

Ras homolog gene family member A (RhoA) belongs to the Rho GTPase superfamily, which was first studied in cancers as one of the essential regulators controlling cellular function. RhoA has long attracted attention as a key molecule involved in cell signaling and gene transcription, through which it affects cellular processes. A series of studies have demonstrated that RhoA plays crucial roles under both physiological states and pathological conditions in cardiovascular diseases. RhoA has been identified as an important regulator in cardiac remodeling by regulating actin stress fiber dynamics and cytoskeleton formation. However, its underlying mechanisms remain poorly understood, preventing definitive conclusions being drawn about its protective role in the cardiovascular system. In this review, we outline the characteristics of RhoA and its related signaling molecules, and present an overview of RhoA classical function and the corresponding cellular responses of RhoA under physiological and pathological conditions. Overall, we provide an update on the novel signaling under RhoA in the cardiovascular system and its potential clinical and therapeutic targets in cardiovascular medicine.

1. Introduction

Ras homolog gene family member A (RhoA) is a small GTPase of the Rho family. In addition to RhoA, this family consists of 20 proteins, including Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division cycle 42 (Cdc42) [1]. RhoA regulates various cellular functions, including cytoskeletal structure, cell proliferation, and cell migration [2]. Rho GTPases cycle between an inactive guanosine diphosphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound state necessary to activate the downstream signaling cascades [1]. This conformational transition relies on GDP/GTP exchange and GTP hydrolysis by some regulatory proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) [1,3]. GEFs activate Rho, in which GDP is released and GTP is bound to it. GAPs function to stimulate the intrinsic GTPase activity for the GTP hydrolysis, inactivating Rho. GDIs prevent Rho activation by inhibiting the dissociation of GDP. These processes are governed by cell surface receptors, such as G protein-coupled receptors, and tyrosine kinase receptors. GTP-bound active RhoA regulates two groups: the first one comprises serine/threonine protein kinases, including Rho-associated, coiled-coil-containing protein kinase (ROCK), and Lin11, Isl-1 and Mec-3 kinase (LIMK); and the second one consists of scaffold proteins, including formin family proteins and rhotekin [4,5]. RhoA is ubiquitously expressed, and its conventional knockout (KO) mice show early embryonic lethality [6]. Thus, mice with the tissue-specific deletion of RhoA have been used to investigate the cell type-specific function of this molecule [7–13]. In this review article, we here briefly introduce the general molecular characteristics of RhoA and its associated molecules, and then, describe our up-to-date findings to illuminate the physiological and pathological effects of RhoA on the cardiovascular system [14,15], followed by its clinical and therapeutic implications.

2. General molecular characteristics of RhoA

RhoA conventionally reorganizes the actin cytoskeleton and governs the cytoskeletal structure, which is mainly mediated by the activation of ROCK [16]. This activation enhances LIMK activity [17]. ROCK also phosphorylates myosin light chain (MLC) element, increasing actomyosin contractility. This is accomplished by the ROCK-mediated suppression of MLC phosphatase (MLCP) via phosphorylation of myosin phosphatase-targeting subunit 1 (MYPT1) [18]. mDia, another RhoA effector, is a formin molecule that initiates actin nucleation and polymerization via the actin-binding protein profilin. ROCK and mDia work together to promote actomyosin assembly and contractility [19].

Elevated RhoA signaling has been extensively studied in carcinogenesis and development of the central nervous system [20,21]. Activated RhoA creates a cleavage furrow by actin polymerization. Then, MLC phosphorylation causes contractile ring contraction via the effector molecules mDia, ROCK, and citron kinase. In a manner comparable to the function of ROCK, citron kinase phosphorylates MLC at Thr18 and Ser19, which induces myosin localization at the cleavage furrow by enhancing nucleation and stability of microtubules in the cells [22].

The RhoA–ROCK signaling plays a role in inflammation by regulating the activity and expression of nuclear factor (NF)-κB, interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor (TNF)-α [23]. RhoA and ROCK promote the degradation of IκB, an inhibitor of NF-κB, thus allowing NF-κB to translocate to the nucleus for the expression of proinflammatory proteins [24]. Furthermore, RhoA regulates the production of α-synuclein, suggesting the involvement of RhoA in the etiology of Parkinson's disease. It was also found that the inhibition of ROCK function by fasudil induces the clearance of α-synuclein through autophagic degradation [25]. These findings show that the RhoA-related

signaling has diverse biological roles and may be a therapeutic target for certain diseases. In the following sections, we describe the functions of several RhoA-associated molecules.

3. RhoA-associated molecules

3.1. *MLCP*

An important target of RhoA is ROCK, which phosphorylates MYPT of MLCP [26]. The activation of ROCK results in the inhibition of MLCP activity either through direct phosphorylation of MYPT or through other indirect mechanisms [18]. The ROCK-mediated MLCP phosphorylation induces dissociation of the catalytic subunit from MYPT, causing MLCP inactivation. This in turn increases the phosphorylation of MLC, which is mainly catalyzed by Ca^{2+}/calmodulin-dependent MLC kinase (MLCK) [27]. Phosphorylated MLC triggers the cross-bridge cycling of actomyosin and increases contractility [26]. ROCK also phosphorylates the cytosolic protein CPI-17 at T38, which inhibits P-MLC as a negative regulatory control [28]. In short, RhoA and ROCK play roles in inhibiting the activity of MLCP and increasing MLC phosphorylation, maintaining cellular contractility.

3.2. *LIMK and cofilin*

LIMK regulates actin dynamics through the phosphorylation of cofilin [29]. For the depolymerization of actin filaments, cofilin, an actin-binding protein, is required [30,31]. ROCK activated by RhoA phosphorylates and inhibits LIMK, resulting in cofilin functioning in the depolymerization and disassembly of actin filaments [32]. Cofilin and ROCK also work together to organize focal adhesions [33].

3.3. *Formin family proteins*

A mammalian homolog of *Drosophila* Diaphanous, mDia, was discovered as a downstream effector of Rho [34]. mDia selectively interacts with active Rho. The three isoforms of the mDia formin family—mDia1, mDia2, and mDia3—are recognized as important RhoA effectors [34,35]. mDia proteins collaborate with ROCK to control the development of actin stress fibers. mDia binds to the barbed ends of actin filaments and stimulates strong actin polymerization activity [36]. Among mDia family members, mDia1 is the most important, and plays a key role in cell polarization and migration [37,38], axonal outgrowth [39], and exocrine vesicle secretion [40]. Structural analysis revealed that Rho switch regions are used for binding to mDia1 [41]. Regarding other mDia family members, mDia2 is involved in the formation of filopodia and endosome trafficking [42,43]. It also contributes to contractile ring formation and cytokinesis during erythroblast cell division [44]. Finally, mDia3 is required for chromosome alignment during cell division via the Aurora B kinase [45].

Another formin family member is Dishevelled-associated activator of morphogenesis 1 (Daam1) [46]. Daam1 has been shown to activate RhoA via a positive feedback loop [47,48]. It has been proposed that a RhoGEF is recruited to active Daam1 to increase GTP-loaded RhoA, or that a RhoGAP is silenced, resulting in less RhoA-GTP hydrolysis. However, another study has reported that Daam1 does not regulate cytoskeletal organization via the Rho family proteins including RhoA [49].

3.4. *Rhotekin*

Rhotekin is an effector of RhoA and consists of a Rho binding domain, a PH domain, two proline-rich regions, and a C-terminal PDZ binding motif [50]. This protein plays a role in neuronal tissues and cancer cells. Its expression has been observed in the brain, kidney, lung, and skeletal muscle [51]. Rhotekin is required for neuron maintenance and survival

by positively regulating differentiation and neurite outgrowth, and is also expressed in neural stem cells (NSCs) to inhibit cell proliferation and promote the differentiation of NSCs into neurons [52]. Rhotekin may be an essential molecule for the development of neuronal tissues, but the precise molecular mechanisms behind its functions remain to be elucidated.

4. Physiological and pathological effects of RhoA in the heart

Physiologically, RhoA is an important regulator of the contractile force of the heart muscle. RhoA and ROCK govern various cellular functions in addition to the actin filament scaffolding in the heart. RhoA expression is increased in response to stress hormones and is associated with the generation of force in cardiac muscle contraction. Sufficient RhoA expression in the heart is believed to be essential in regulating cardiac contraction and to protect cardiac architecture against its remodeling. RhoA and ROCK signaling may have opposite roles in the heart: one is a maladaptive role corresponding to pathological cardiac remodeling and the other is a beneficial adaptive response. ROCK is partly involved in the regulation of agonist-stimulated cardiac contraction via MLC phosphorylation [53]. RhoA and ROCK protect cardiomyocytes through stimulating the focal adhesion kinase/phosphatidylinositol-3 kinase (PI3K)/Akt survival pathway [54]. ROCK has two isoforms: ROCK1 and ROCK2 [55]. Although ROCK1 and ROCK2 are ubiquitously expressed from embryonic development to adulthood, ROCK1 is more abundantly expressed in immunological cells, and ROCK2 is highly expressed in cardiac muscle and VSMCs [56]. Subcellular distribution of ROCK1 and ROCK2 has been reported to be different [57]. Both isoforms cause pathological fibrosis in multiple organs, such as kidney, liver and lung [56], although the level of the contribution of ROCK1 and ROCK2 to the fibrosis is different in each organ. In the heart, several studies using mice with homozygous

or heterozygous KO of ROCK1 have demonstrated an important role of ROCK1 for pathological remodeling, in which this protein causes the onset of cardiac fibrosis rather than hypertrophy [58–60]. It has been shown that ROCK1 deletion, whether partial or complete, does not prevent the development of cardiomyocyte hypertrophy [58,59], but induces a considerable variety of molecular and structural changes resulting from pathological hypertrophic restructuring in the heart. Meanwhile, ROCK2 appears to be involved in cardiomyocyte hypertrophy and death, leading to an increase in cardiac fibrosis during compensatory cardiac hypertrophy. However, further studies are necessary to confirm the contribution of ROCK2 to cardiac decompensation and heart failure.

We have recently identified N-Myc as a novel molecule downstream of RhoA [15]. RhoA and N-Myc are associated with regulating mitochondrial homeostasis in the heart to prevent cardiac dysfunction and failure. ROCK activated by RhoA phosphorylates N-Myc and induces the instability of N-Myc expression, which in turn increases the expression of Parkin, because N-Myc is a transcription factor that negatively regulates expression of the *Parkin* gene [61]. This was certified in cardiomyocytes by using the ROCK inhibitor Y-27632, which inhibits both ROCK1 and ROCK2 isoforms [62]. Parkin is an E3 ubiquitin ligase and is responsible for the pathogenesis of juvenile Parkinson's disease [63]. Parkin facilitates mitophagy for the clearance of superfluous and damaged mitochondria in cardiomyocytes. When RhoA expression is ablated, the level of N-Myc phosphorylation is decreased and its expression is contrarily increased, followed by reduced expression of Parkin in cardiomyocytes and the accumulation of damaged and unnecessary mitochondria. Additional depletion of N-Myc in RhoA-deficient cardiomyocytes rescued Parkin expression with the recovery of mitophagy, confirming that N-Myc functions downstream of RhoA as a negative regulator of Parkin expression. This regulatory system is an underlying mechanism by which RhoA counteracts cardiac senescence and age-related

heart failure (Figure 1). It has been shown that mice with cardiomyocyte-specific conditional KO (cKO) of RhoA have a significantly short lifespan with severely impaired cardiac function, compared with control mice [15]. Results showed that the heart in these cKO mice expresses a high level of senescence marker proteins. Adeno-associated virus (AAV)-mediated supplementation of Parkin expression in the RhoA cKO heart resulted in recovery of the lifespan and cardiac function as well as reduced expression of senescence marker proteins in the cKO mice. Furthermore, RhoA expression was found to be remarkably suppressed in the heart of patients with dilated cardiomyopathy (DCM) in whom known causative gene mutations for DCM were not identified.

Similar to the loss of RhoA in cardiomyocytes, overstimulation of RhoA signaling causes dysfunction of the myocardium, leading to cardiac arrhythmias and heart failure [64,65]. Imbalance between RhoA and its family members, Rac1 and Cdc42, can lead to affect structural and functional remodeling of the myocardium, ultimately resulting in the development of cardiac disease [66–68]. RhoA overactivation is also implicated in ischemia-reperfusion injury, inflammatory damage, cardiomyopathy, and cardiac fibrosis [69–72]. Protein kinase C-induced RhoA activation provokes cardiomyocyte hypertrophy through mitogen-activated protein kinases (MAPKs) [73]. RhoA–ROCK signaling can induce heart failure, and is elevated in patients with heart failure [64,74]. Additionally, a study has shown that ROCK blocks the insulin signaling by phosphorylating insulin receptor substrate-1 (IRS1) [75]. Inactivated IRS1 decouples the insulin receptor from PI3K, and myocardial loss of IRS in mice causes heart failure and death soon after birth [76]. The inhibition of RhoA–ROCK signaling can prevent cardiac hypertrophy and remodeling [77–81]. At the molecular level, the ROCK inhibition attenuates G protein-coupled receptor agonists-induced cardiomyocyte hypertrophy [82–84], suppresses cardiomyocyte apoptosis via Akt activation and Bax inhibition [85,86], reduces vascular

resistance via regulation of Ca^{2+} sensitivity [87], and decreases contraction-mediated sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a) expression [88].

In summary, it is becoming clear that the RhoA–ROCK pathway plays a role in the pathophysiology of heart diseases and that the suppression of this pathway may ameliorate these conditions. As described above, ROCK is known to mediate a wide range of cellular and physiological processes in the heart, and its overactivation tends to be associated with the progression of cardiac pathology. Thus, ROCK may be a promising therapeutic target for suppressing heart diseases. Nonetheless, a better understanding of ROCK activity in the heart is required for the development of ROCK inhibitors with greater efficacy against heart diseases.

5. Physiological and pathological effects of RhoA in the vasculature

RhoA is a small GTPase that is highly expressed in the vasculature, particularly the endothelium and vascular smooth muscle (VSM), and is crucial for regulating contractile structures and homeostatic functions. In endothelial cells, RhoA regulates endothelial barrier integrity and is linked to vascular tone. RhoA–ROCK signaling restricts the generation of nitric oxide (NO) by inhibiting the expression of endothelial NO synthase (eNOS) [89,90]. Following this restriction of NO generation, VSM contractility increases and vascular tone is substantially elevated, because NO-induced elevation of cellular cGMP level and subsequent protein kinase G activation for VSM relaxation are inhibited [91,92]. This results in increased susceptibility to cerebral vasospasm and pulmonary hypertension. Thus, ROCK inhibitors are beneficial for treating patients suffering from cerebral vasospasm and pulmonary hypertension. RhoA in the endothelium also works in conjunction with several other pathways to promote inflammation, which causes decreased NO availability and endothelial dysfunction to initiate atherosclerosis in the vessel walls

[93–95].

The major role of RhoA signaling in the vasculature occurs in VSM cells (VSMCs). Activated RhoA phosphorylates MYPT via ROCK, restraining the phosphatase activity of MLCP. MYPT has many serine and threonine phosphorylation sites that are involved in VSM contractility [96], and the phosphorylation of Thr^{696} and Thr^{850} in MYPT deactivates MLCP [97]. This enhances the phosphorylation level and the activity of MLC in VSMCs, leading to increased vascular contraction [98]. This RhoA-mediated vascular contraction is suppressed by NO- and cGMP-induced activation of protein kinase G [99]. We have recently identified the significant role of VSMC RhoA in maintaining the architecture of the aorta and attenuating the pathogenesis of abdominal aortic aneurysm (AAA) [14]. RhoA expression is markedly reduced in the aneurysm area, compared with that in the normal area, in the aorta of patients with AAA. In mice with VSMC-specific cKO of RhoA, AAA formation occurs more readily than in control mice after pharmacological stimulation. RhoA depletion abnormally activates mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) and its downstream MAPK signaling. This activation suppresses the expression of genes related to VSMC contractility, such as *Mylk*, *Myh11*, and *Acta2*, enhances the vascular inflammation that was confirmed by the accumulation of macrophages, and facilitates vessel wall degradation by increased and decreased expression of matrix metalloproteinases and their tissue inhibitors, respectively. These molecular alterations associated with RhoA deletion in VSMCs make the aortic wall vulnerable to the tension generated by aortic blood flow. Our further investigations have shown how RhoA regulates MAP4K4 activity. In the presence of RhoA in VSMCs, RhoA was found to firmly bind to its binding protein Set, and MAP4K4 can interact with protein phosphatase 2A (PP2A), an inhibitor of MAP4K4, which inhibits the overactivation of MAP4K4. However, in the absence of RhoA, Set strongly binds to PP2A by blocking its

interaction with MAP4K4; subsequently, MAP4K4 activation is upregulated, causing pathological processes in the aorta. On the basis of these results, the inhibition of MAP4K4 activity is considered to be beneficial for the prevention of AAA formation. Actually, the administration of DMX-5804, an inhibitor of MAP4K4, in RhoA cKO mice was found to decrease the formation of AAA. The aforementioned signaling, which occurs in the aortic medial layer, is summarized in Figure 2.

Dysfunction of the RhoA pathway can lead to the development of pathological conditions, such as atherosclerosis, hypertension, and other vascular complications. Hyperactivation of RhoA due to loss of negative regulation is linked to vascular remodeling, increased permeability, VSMC hypertrophy, and vascular constriction [100–103]. In addition, RhoA is involved in the regulation of thrombosis and monocyte–endothelial interaction [104,105]. ROCK is also related to hypoxic pulmonary blood vessel constriction by interacting with phosphatase and tension homolog (PTEN) [106]. ROCK induces the localization of PTEN and the cation channel transient receptor potential canonical 6 at caveolae, allowing Ca^{2+} to flow into pulmonary artery VSMCs and causing vasoconstriction. ROCK activity is elevated at coronary artery regions undergoing spasm, suggesting that ROCK signaling plays a role in the initiation of arterial contraction and vasospasm [107]. This phenomenon can be counteracted by intracoronary injection of the ROCK inhibitor hydroxyfasudil, which greatly decreases the risk of coronary artery spasm. Furthermore, the inhibition of ROCK minimizes vascular inflammation and remodeling by inhibiting several pathways provoked by proinflammatory molecules, including monocyte chemoattractant-1, plasminogen activator inhibitor-1, and transforming growth factor-β, in endothelial cells and VSMCs [108–110]. This inhibition also suppresses the release of ROS by inhibiting the production of NADPH oxidase and cyclophilin A from VSMCs [111,112].

Many other studies have demonstrated that RhoA–ROCK pathway activity increases

in experimental hypertension models and in patients with hypertension [113–116]. The elevated activity may be the outcome of the renin–angiotensin–aldosterone system activation and the production of reactive oxygen species [113,117–119], leading to hypertension-related pathogenesis. ROCK inhibitors have been employed in the *in vivo* investigation of hypertension [78,98,116,120]. Despite the fact that ROCK inhibitors diminish vascular bed remodeling in hypertensive animals, they do not necessarily reduce systemic blood pressure in humans, suggesting the difficulties in translating experimental findings into clinical applications.

6. Clinical and therapeutic implications of RhoA signaling

RhoA signaling is a critical pathway involved in numerous cellular processes, including cell shape changes, migration, proliferation, and gene expression. Aberrant RhoA signaling has been shown to be associated with various diseases, making it an important target for clinical and therapeutic interventions. In cardiovascular diseases, RhoA regulates smooth muscle contraction and endothelial barrier functions in blood vessels and cardiomyocyte homeostasis in the heart. Dysregulation of RhoA signaling can contribute to vascular dysfunction, hypertension, atherosclerosis, and heart failure. Thus, targeting RhoA signaling may be beneficial in treating these cardiovascular diseases. For example, a classical ROCK inhibitor, fasudil, has clinically been approved for the treatment of diseases, such as cerebral vasospasm, brain ischemia, and pulmonary hypertension [121,122]. Recently, the novel ROCK inhibitors ripasudil and netarsudil have been shown to be effective for glaucoma and ocular hypertension [123,124].

In our experimental model, the inhibition of MAP4K4 activated by loss of RhoA in VSMCs reduced the incidence of AAA formation in mice [14]. To further develop an MAP4K4 inhibitor for clinical use, several challenging steps might need to be taken: for

example, searching for a compound that specifically blocks MAP4K4 activity and identifying the delivery system to convey the compound to VSMCs. It is important to confirm its biosafety as well as its pharmacological effect. A selective inhibitor of MAP4K4 is currently undergoing a preclinical trial to examine its cardioprotective role [125]. A PP2A-binding molecule, Set, would also be a beneficial target to suppress MAP4K4 activity, because PP2A can inhibit MAP4K4 when PP2A is released from Set. Small molecules that interfere with Set–PP2A binding have been identified and shown to exert therapeutic effects on acute and chronic myeloid leukemias [126,127].

siRNA-targeting strategies, as a new class of drugs, are now available at the clinical stage. For example, an siRNA drug, vutrisiran, is used to treat polyneuropathy caused by hereditary transthyretin-mediated amyloidosis [128]. This drug exploits the strategy of mRNA silencing targeting the production of mutant transthyretin mRNA and promotes its degradation, thereby decreasing the serum level of mutant transthyretin protein and lowering the amount of amyloid fibril deposits in the patients. Thus, siRNA targeting RhoA signaling holds potential for clinical and therapeutic applications in cardiovascular diseases. Further research is needed to better understand the intricacies of RhoA signaling and develop specific and effective therapeutic strategies.

How to supply or overexpress the target protein by using the AAV system has been widely investigated. This system is currently applicable in the treatment of several diseases. For instance, onasemnogene abeparvovec (Zolgensma™) is a gene therapy administered to infants with spinal muscular atrophy, in whom *SMN1* gene mutations are present. It is created from segments of AAV serotype 9 that can deliver the *SMN1* gene into motor neurons. Then, normal SMN1 protein expression occurs in the neurons to rescue the muscle atrophy in the patients. Other drugs involving a similar approach have also been developed for specific diseases, such as hemophilia and cystic fibrosis [129,130]. As for heart diseases,

clinical trials using AAV1-SERCA2a have been performed in patients with heart failure [131,132]. Although these trials did not find evidence of improved outcomes, no safety concerns were raised. As mentioned above, we have shown that AAV-mediated Parkin expression, which is reduced in the RhoA cKO heart, can improve the failing heart and lifespan in mice [15]. At the cellular level, severe myocardial damage and injured mitochondria in the RhoA cKO heart are restored by AAV-Parkin administration. Moreover, direct AAV-mediated RhoA supplementation may achieve similar efficacy to prevent heart failure due to cardiac senescence. These findings may also be relevant for preventing the age-related transition of cardiac dysfunction in the heart where RhoA expression is decreased. A modified CRISPR/Cas9 system that uses AAV has recently been employed for target gene activation. It enables the activation of endogenous genes *in vivo* by the administration of AAV-mediated guide RNA and the MS2:P65:HSF1 transcriptional activation complex in combination with Cas9 [133]. This new technology has the potential to genetically treat diseases involving a lack of expression of a specific gene.

7. Conclusions

Recent studies have shown the novel functions of RhoA and its related signaling molecules, which are intimately associated with diverse biological processes and various diseases, as summarized in Figure 3. We have recently demonstrated that RhoA prevents AAA formation and cardiac senescence through novel mechanisms [14,15], which are quite different from the classical roles of RhoA, including regulation of the actin cytoskeleton. RhoA signaling appears to be an attractive target for life science research and therapeutic development. Thus, studies focused on this signaling are still being intensively performed. Finally, we should continue to follow achievements in the research field of RhoA signaling,

which may provide us with important perspectives in biomedical science.

Author contribution statement

Joanne Ern Chi Soh: Conceptualization, Writing – original draft, Writing – review & editing, Visualization.

Akio Shimizu: Conceptualization, Writing – original draft, Writing – review & editing, Visualization.

Akira Sato: Writing – original draft, Writing – review & editing.

Hisakazu Ogita: Conceptualization, Writing – original draft, Writing – review & editing, Visualization.

Competing interests

The authors declare no competing interests.

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Figure Legends

Figure 1. Novel function of RhoA in ventricular cardiomyocytes. In the physiological state, the RhoA–ROCK axis induces N-Myc phosphorylation, which leads to N-Myc degradation. Because N-Myc is a negative regulator of Parkin, N-Myc degradation upregulates Parkin expression. Parkin ubiquitinates dysfunctional mitochondria, leading to mitophagy to avoid the accumulation of dysfunctional mitochondria in order to keep cardiomyocytes healthy. In the pathological state associated with the loss of RhoA, N-Myc expression is increased due to loss of RhoA- and ROCK-mediated N-Myc phosphorylation. N-Myc suppresses Parkin expression, resulting in the accumulation of dysfunctional mitochondria and accelerating senescence-related cardiomyopathy.

Figure 2. Novel function of RhoA in VSMCs of the aortic media. In the physiological state, RhoA recruits and binds to Set, an inhibitor of PP2A, and releases Set from PP2A, which interacts with and inhibits (dephosphorylates) MAP4K4. RhoA also activates ROCK to suppress MLCP. This inhibits MLC dephosphorylation and maintains the MLC phosphorylation level. Consequently, vascular contractility is increased. In the pathological state associated with the loss of RhoA, Set firmly interacts with PP2A and makes MAP4K4 free and active (phosphorylated). Activated MAP4K4 phosphorylates MAP kinase and induces vascular inflammation. Loss of RhoA decreases ROCK activity, leading to MLCP activation. Activated MLCP promotes MLC dephosphorylation, which is also supported by MAP4K4-mediated inactivation of MLCK. Thus, vascular contractility is reduced.

Figure 3. Summary of diverse RhoA functions in physiological and pathological states in

the cardiovascular system. FAK: focal adhesion kinase, NOX: NADPH oxidase, ROS: reactive oxygen species.

Figure 1

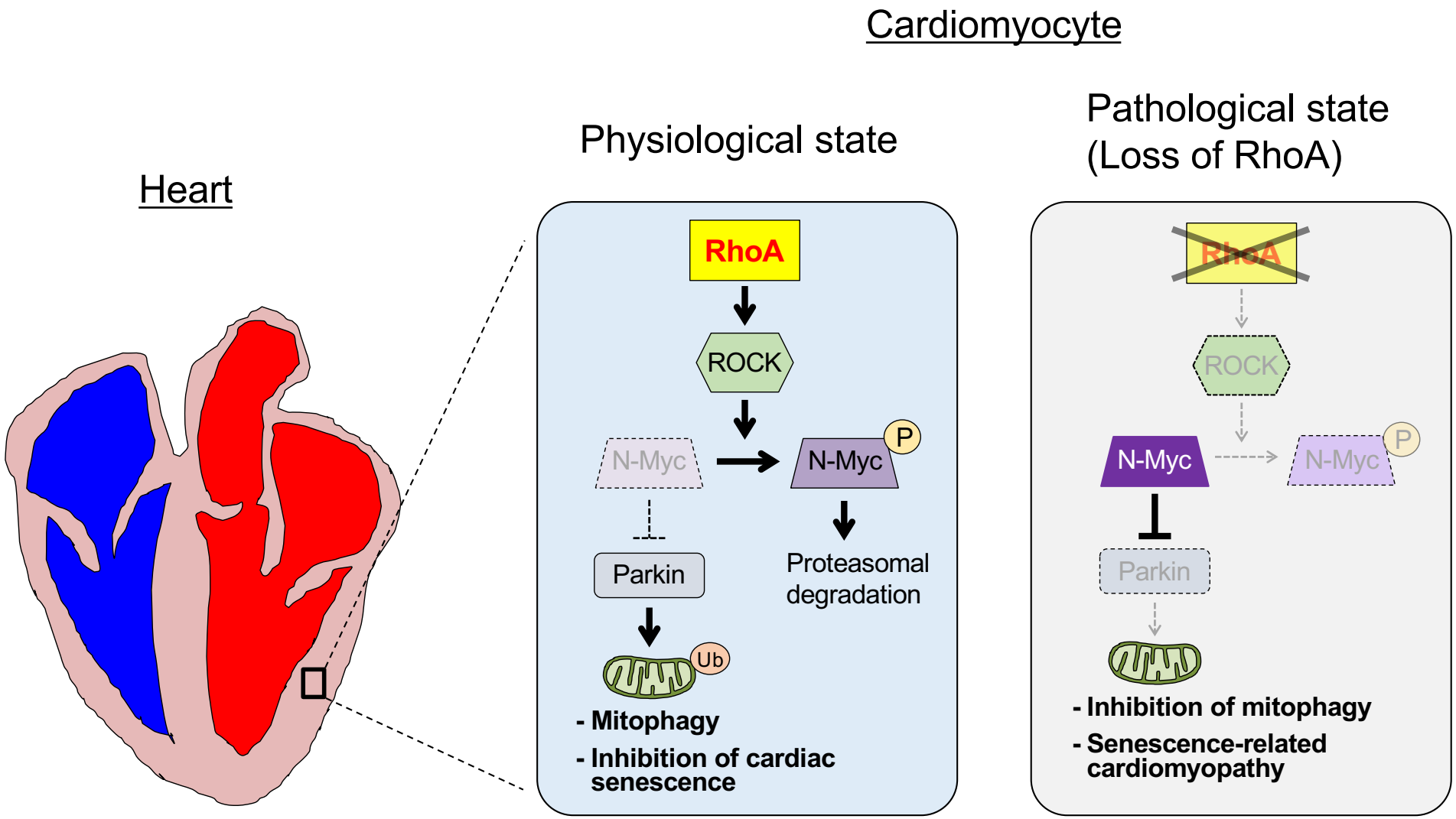

Cardiomyocyte
Heart
Physiological state
RhoA
ROCK
N-Myc
N-Myc
P
Parkin
Proteasomal degradation
Ub
- Mitophagy
- Inhibition of cardiac senescence
Pathological state (Loss of RhoA)
RhoA
ROCK
N-Myc
N-Myc
P
Parkin
- Inhibition of mitophagy
- Senescence-related cardiomyopathy

Figure 2

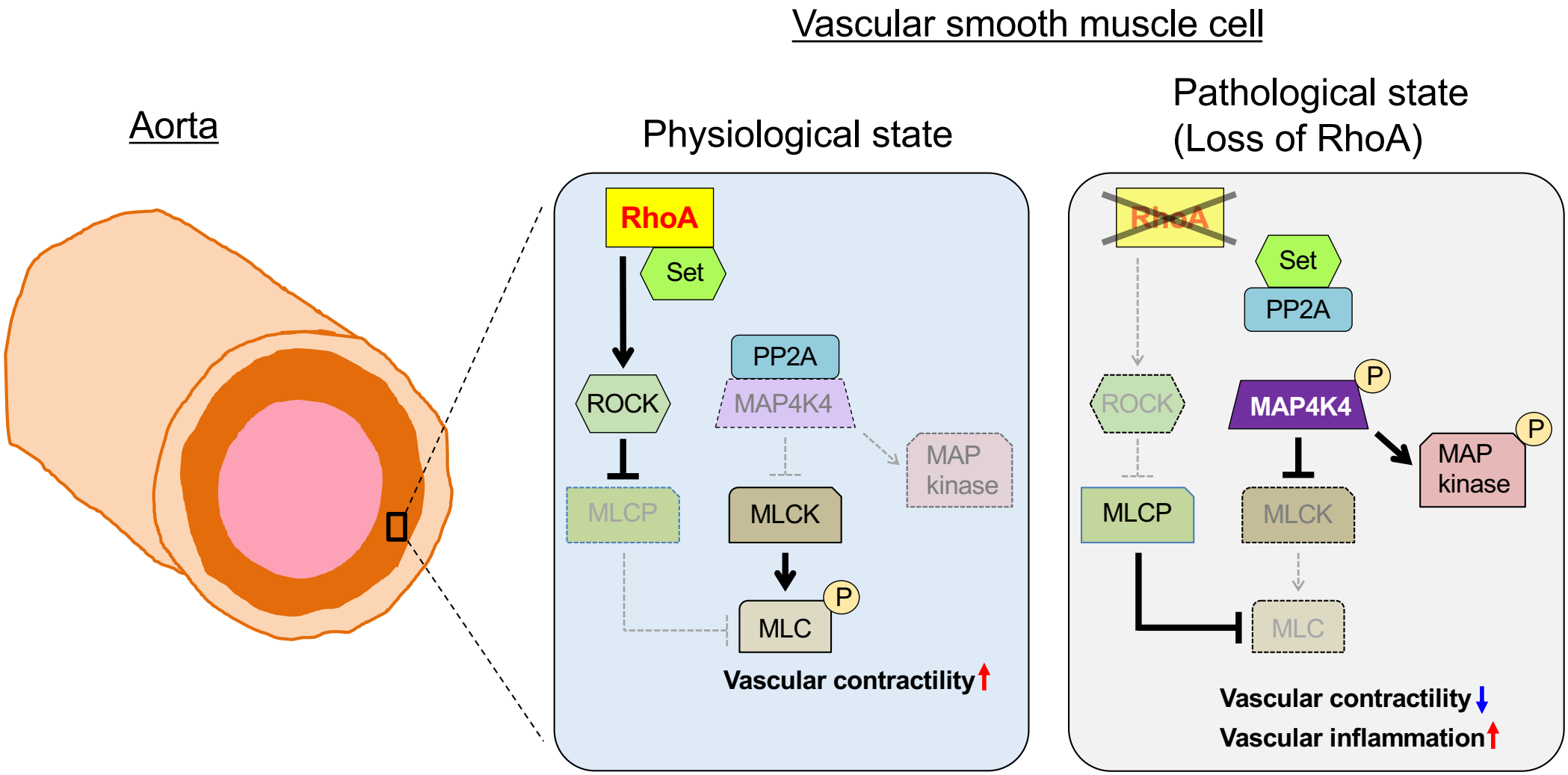
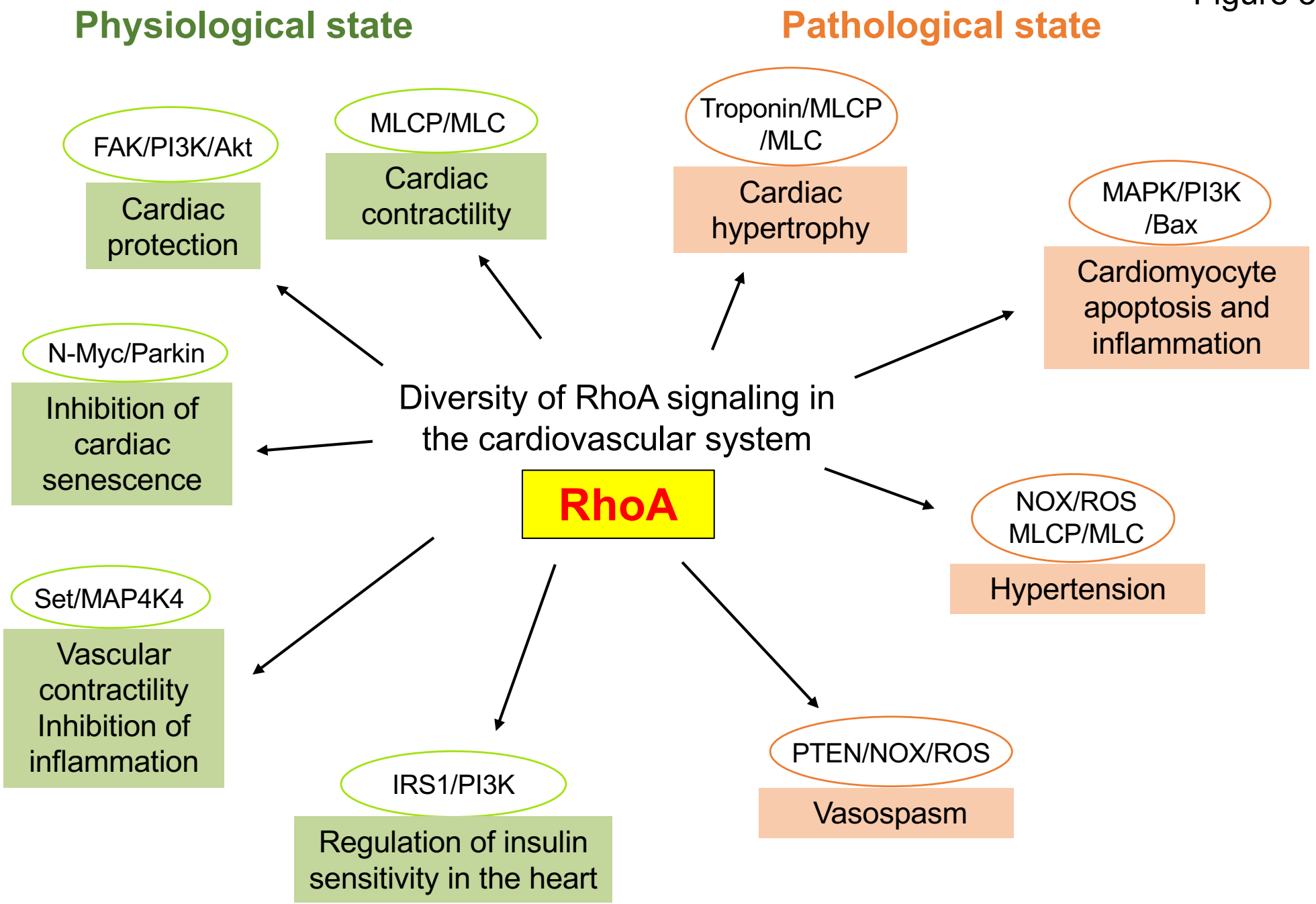

Vascular smooth muscle cell
Aorta
Physiological state
RhoA
Set
PP2A
ROCK
MAP4K4
MAP kinase
MLCP
MLCK
P
MLC
Vascular contractility
Pathological state (Loss of RhoA)
RhoA
Set
PP2A
ROCK
MAP4K4
P
MAP kinase
P
MLCP
MLCK
MLC
Vascular contractility
Vascular inflammation

Figure 3


Physiological state
Pathological state
FAK/PI3K/Akt
Cardiac protection
MLCP/MLC
Cardiac contractility
Troponin/MLCP /MLC
Cardiac hypertrophy
MAPK/PI3K /Bax
Cardiomyocyte apoptosis and inflammation
N-Myc/Parkin
Inhibition of cardiac senescence
Diversity of RhoA signaling in the cardiovascular system
RhoA
NOX/ROS MLCP/MLC
Hypertension
Set/MAP4K4
Vascular contractility Inhibition of inflammation
IRS1/PI3K
Regulation of insulin sensitivity in the heart
PTEN/NOX/ROS
Vasospasm