Review article

Role of RhoA and Rho-associated kinase in phenotypic switching of vascular smooth muscle cells: Implications for vascular function

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ABSTRACT

Cardiovascular disease (CVD) continues to be the primary cause of global mortality. Vascular smooth muscle cells (VSMCs) are integral components of vascular structure and function, evident by their vital roles in modulating blood flow and pressure. Such roles exist due to the differentiated contractile phenotype of VSMCs. However, VSMCs may switch to a dedifferentiated, proliferative synthetic phenotype in a phenomenon known as phenotypic switching. This switch involves dramatic changes in VSMC migration, proliferation, gene expression programs, differentiation, cellular stiffness and extracellular matrix (ECM) deposition. In this review, we explore the role of the small GTPase Rho and its effector, Rho-associated kinase (ROCK), in phenotypic switching as well as apoptotic pathways in VSMCs. We critically dissect how RhoA promotes cell migration and proliferation as well as its role in modulating the expression of a battery of VSMC marker proteins. We also discuss how RhoA modulates apoptosis, induces dedifferentiation, increases vascular stiffness, or modifies ECM accumulation. These alterations in VSMC phenotypes contribute to multiple vascular dysfunctions, including hypertension and atherosclerosis. Understanding the molecular underpinnings and the signaling pathways involved in these altered phenotypes may provide novel avenues of drug design and other therapeutic interventions for the management of CVDs.

1. Introduction

Proper vascular smooth muscle cell (VSMC) activity is essential for physiologic functioning of the vasculature, such as regulation of vasotone and blood flow. In healthy vasculature, VSMCs are contractile, stationary, and quiescent. In addition, VSMCs are considered differentiated and express contractile proteins, like smooth muscle α-actin (SM-α-actin), SM myosin heavy chain, calponin, SM 22α, smoothelin, and SM myosin light chain (MLC), among others [1–3]. Despite the differentiated phenotype of VSMCs, they still show plasticity. VSMCs may shift from the contractile phenotype in response to stimuli in the interest of maintaining homeostasis of the vasculature. Importantly, VSMC phenotype switching is necessary during development and vascular remodeling for the formation and maturation of vessels [2]. While this phenotype switching is primarily physiological, it may, when dysregulated, contribute to vascular disease processes. For instance, intimal hyperplasia, a process underlying the pathogenesis of several diseases such as atherosclerosis, aortic aneurysm, and hypertension, involves VSMC dysfunctional phenotypic shifts [1,2,4–6]. Notably, the process of VSMC phenotypic switching is often one of the earliest manifestations of vascular diseases. Small conditional RNA (scRNA) studies have been useful in examining the transcriptome of VSMCs that undergo

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phenotypic transformation, as well as the factors that contribute to this change [7]. During a diseased vascular state and upon phenotype switching, VSMCs dedifferentiate, exhibit abnormal gene expression profiles, assume a synthetic and proliferative phenotype. In addition, they become more migratory - especially to areas of cellular injury, prone to apoptosis, and prone to clustering as per scRNA studies [3, 8–10]. Contextually, scRNA sequencing reveals that VSMC-derived foam cells display a propensity to undergo multiple forms of cell death, such as apoptosis, autophagy, necroptosis, and pyroptosis, all of which contribute to the structure and physio-pathology of atherosclerotic plaques [11].

Given the involvement of VSMC phenotypic switching in the initial stages of vascular disease, it is prudent to elucidate the molecular pathways that regulate this process. A better understanding of the molecular mechanisms of this phenotypic switching may help shed light on potentially new therapeutic targets in the management of CVD. In context, this review analyzes the involvement of the small GTPase RhoA and its effector kinase, Rho-associated kinase (ROCK), in the various aspects of VSMC phenotypic switching.

2. RhoA and ROCK regulate VSMC contractility

VSMC relaxation and contraction are determined by the dephosphorylation and phosphorylation of MLC by myosin light chain phosphatase (MLCP) and myosin light chain kinase (MLCK), respectively. Initially, contraction is mediated by an elevation of intracellular calcium (Ca^{2+}), which leads to calmodulin-mediated activation of MLCK. Vascular contraction is then maintained by mechanisms that sensitize MLCK to Ca^{2+} signaling (Fig. 1).

Calcium or upstream vasoactive signals can activate MLCK leading to MLC phosphorylation, which then induces polymerized F-actin to form actomyosin, the machinery needed for VSMC contraction. Vasoconstrictors can activate Rho/ROCK signaling which then increases MLC phosphorylation and the consequent VSMC. ROCK can phosphorylate and inactivate MLCP, thus increasing levels of phosphorylated MLC and thereby actomyosin-mediated VSMC contraction. Active ROCK can phosphorylate CPI-17, thus inhibiting MLCP leading to contraction. ROCK: Rho-associated kinase; MLCK: myosin light chain kinase; MLC: myosin light chain; MLCP: myosin light chain phosphatase.

RhoA is a member of the small GTPase family that includes RhoA, Rac1, and cdc42. RhoA acts as a molecular switch due to its GTPase activity, which cycles RhoA between an active GTP-bound state and an inactive GDP-bound state (Fig. 1). RhoA activity is also modulated by activating guanine nucleotide exchange factors (GEFs), as well as inactivating GTPase activating proteins (GAPs). Principal effectors of RhoA include Rho-associated kinases or ROCKs [12,13], which robustly induce VSMC contraction by directly phosphorylating and inhibiting the activity of MLCP, an enzyme that attenuates VSMC contraction by dephosphorylating MLC [14]. Active ROCK can also indirectly inactivate MLCP through CPI-17, a phosphorylation-dependent regulatory protein that, when phosphorylated, inhibits the phosphatase subunit of MLCP. This leads to heightened MLC activation, VSMC contraction, and stress fiber formation (Fig. 1) [15,16].

ROCK can also enhance VSMC contractility through direct action on MLC. Indeed, ROCK phosphorylates, and consequently sensitizes, MLC to Ca^{2+} signaling, which eventually results in a potentiated and more sustained VSMC contractile response (Fig. 1) [6,13,14,17,18]. Thus, RhoA/ROCK-mediated prolongation of MLC action underlies the increased contraction, and potentially heightened vasoconstriction [6, 14].

Modulation of RhoA/ROCK-mediated VSMC contraction process is an integral part of VSMC phenotype switching. Indeed, VSMC contraction and the involved actin polymerization have been intrinsically shown to modulate VSMC migration and stiffness. Of prime importance to this review is the signaling which, downstream of ROCK, contributes to pathological VSMC phenotypic switching. We henceforth dissect the signaling molecules associated with RhoA/ROCK signaling in the context of pathological phenotypic switching, specifically in relation to VSMC migration, proliferation, gene expression, apoptosis, dedifferentiation, ECM deposition, and vascular stiffness.

3. Mechanisms of RhoA/ROCK-mediated VSMC migration

VSMC migration involves remodeling of the cytoskeleton in response to signals that emanate from cell surface receptors. During VSMC migration, reorganization of the actin cytoskeleton occurs and causes protrusion of the leading edge, along either a path of variable adhesion to the ECM or in the direction of a chemotactic cue [19]. Instantly, new focal adhesions form in the vicinity immediate to the leading edge, resulting in enhanced adhesion of the cell membrane to the ECM [19]. Actomyosin molecules within the cytoplasm cause contraction that, in addition to remodeling of the cytoskeleton and the disassembly of focal adhesions at the trailing edge, moves the cell toward its destination [19].

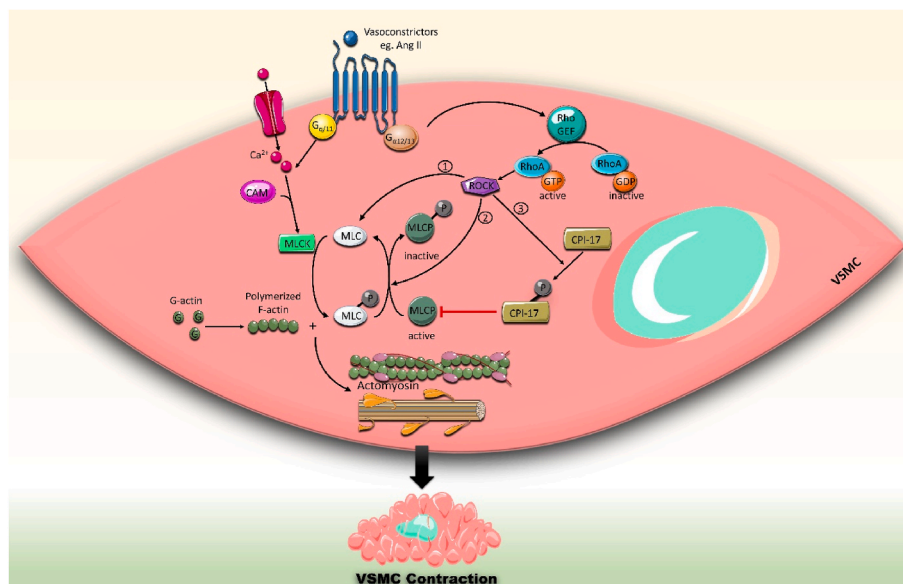Migration of VSMCs is important for many physiological processes,

Fig. 1. Activation of RhoA/ROCK pathway sensitizes VSMCs to calcium-mediated contraction.

prime of which are vascular development and the maintenance of vascular integrity [19]. Dysregulation of VSMC migration is equally as important in the pathogenesis of vascular diseases, such as the response to vascular injury or atherogenesis [19]. For instance, migration of VSMCs from the medial to intimal layers of vessels is at the heart of atherosclerosis and vascular injury or restenosis [2,20].

The upstream signals that activate the process of VSMC migration, as well as the downstream effectors, have been extensively studied. Chemotactic factors involved include platelet-derived growth factor-BB (PDGF-BB), angiotensin II (Ang II), thrombin, and tumor necrosis factor-α (TNF-α), among others [2,20]. These molecules can stimulate VSMC migration not only during development, but also under physiological and pathological states. The Rho/ROCK pathway is a major player in the signaling implicated in these states. In order to reasonably limit the scope of this review, the discussion will be limited to upstream signaling by Ang II, PDGF-BB, and asymmetric dimethyl arginine (ADMA) that culminate in RhoA/ROCK-mediated VSMC migration.

Angiotensin II is a vasoactive peptide involved in the homeostasis of vascular volume and pressure. Upon binding to and activating its receptor (AT1 receptor), Ang II triggers the activation of several downstream effectors, including Rho/ROCK (Fig. 2), which then results in polymerization of G (globular)-actin into F (fiber)-actin. Paradoxically, this polymerization will downregulate contractile proteins [21]. Notably, contractile protein downregulation induces VSMC remodeling, an indispensable step in the initiation of VSMC migration. In this context, inhibitors of AT1 receptor, RhoA, or ROCKs have been shown to suppress migration [21].

ERK and JNK MAPKs can be activated by ROCK. These kinases can translocate to the nucleus to activate transcription factors like c-jun (activated by JNK), thereby modulating expression of specific genes implicated in cell migration. Ang II binding to the AT1R receptor also activates RhoA/ROCK which then activates the p38-Syk-Src kinase cascade, eventually promoting migration. In addition, RhoA/ROCK can act by phosphorylating and thus inhibiting MLCP. This renders MLC active, and facilitates actin polymerization and actomyosin assembly necessary for VSMC contraction and migration. In the urokinase

pathway, uPA binds to uPAR at the leading fronts forming a complex along with TYK2 and PI3K. This complex then recruits RhoA to the leading fronts resulting in its activation. RhoA will then activate ROCK which, through MLCP inhibition, results eventually in actin polymerization and consequent migration. Finally, RhoA, independently of ROCK, can activate mDia1, leading to polymerization of actin filaments and filopodia formation, an initial step in VSMC migration. Ang II: angiotensin II; PDGF-BB: platelet derived growth factor-BB; ADMA: asymmetric dimethyl arginine; ROCK: Rho-associated kinase; ERK: extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase; MAPK: mitogen activated protein kinase; AT1R: angiotensin II receptor type 1; Syk: spleen tyrosine kinase, uPA: urokinase-type plasminogen activator; uPAR: uPA receptor; MLC: myosin light chain; MLCP: myosin light chain phosphatase; mDia 1: mammalian diaphanous protein 1.

Ang II can also stimulate VSMC migration by a different mechanism. Ang II can activate p38 MAPK, a downstream target of RhoA/ROCK and a member of the MAPK family of kinases [22]. The involvement of RhoA/ROCK/p38 in VSMC migration warrants further investigation, and the idea currently established is still an evolving model. For example, accumulating evidence points to RhoA/ROCK activation of p38 as one driver of VSMC migration. Current evidence for potential p38 involvement in VSMCs migration includes the fact that activated p38 can activate c-Src protein production through Syk (spleen tyrosine kinase), leading to cell migration (Fig. 2) [23]. Another piece of evidence shows that p38 activates Mitogen Activated Protein Kinase 2/3 (MAPKAPK 2.3), which then phosphorylates heat-shock protein 27 (HSP 27) leading eventually to VSMC migration [24] (Fig. 3). In addition, p38 can phosphorylate and activate paxillin, a key protein involved in focal adhesion dynamics [25]. These pathways could possibly be Rho/ROCK dependent, since p38 activation is a direct downstream event of Rho/ROCK activation [22]. Given that blocking p38 inhibits VSMC migration [20,26], by association, we can postulate that activation of p38 by Ang II, via the RhoA pathway, could promote migration of VSMCs. Nevertheless, this notion remains to be fully elucidated.

Evidence suggests that p38-mediated phosphorylation of caldesmon, a thin filament binding protein, plays a role in urokinase-stimulated
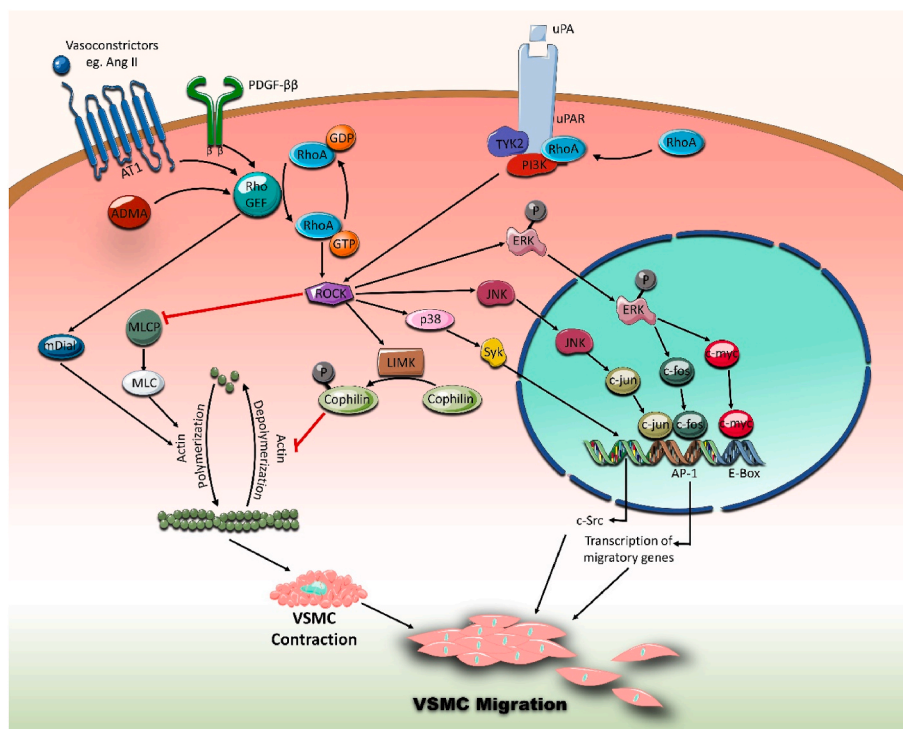

Fig. 2. Upstream signals that promote VSMC migration like include Ang II, PDGF-BB or ADMA, which act by stimulating the RhoA/ROCK pathway.
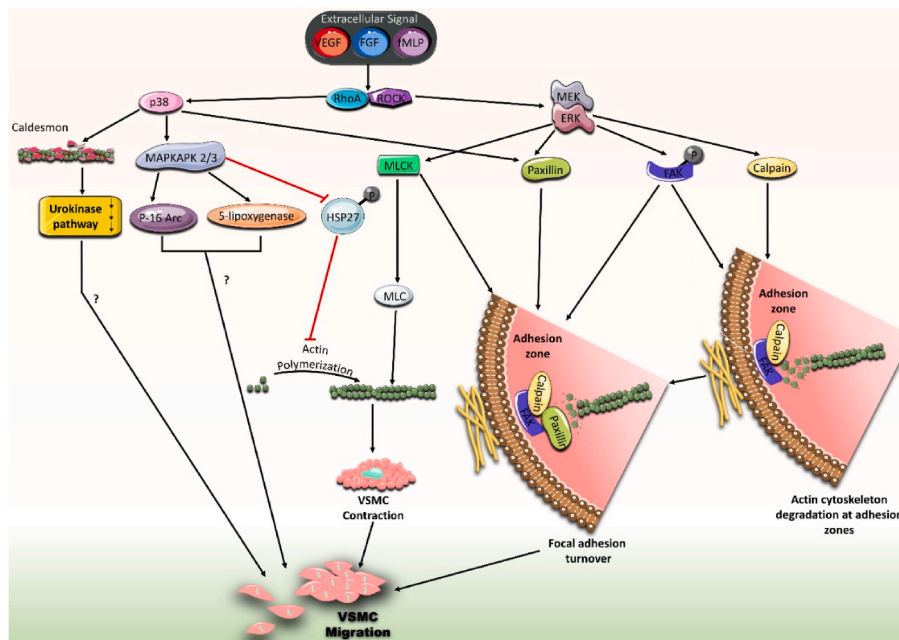
Fig. 3. Extracellular signals such as VEGF, FGF and fMLP can activate p38. RhoA/ROCK may possibly mediate this activation in VSMCs. Once activated, p38 can mediate cell migration via three different pathways.

VSMC migration (Fig. 3) [27]. Importantly, urokinase-type plasminogen activator (uPA) itself can lead to the activation of RhoA, leading to VSMC migration. Interestingly, this uPA-mediated migratory pathway is unique to VSMCs. uPA stimulation of cell migration is done *via* binding and activation of uPA receptor (uPAR) signaling (Fig. 2). Active uPAR complexes tend to form at the leading fronts of VSMC membranes and contain the Janus kinase Tyk2 and phosphatidylinositol 3-kinase (PI3K) [28]. The active form of RhoA is recruited to VSMC leading fronts as well, where it associates with the uPAR complex [29]. RhoA then activates ROCK, which phosphorylates MLC, leading to VSMC migration. However, the mechanism by which RhoA becomes recruited and activated by the uPAR complex remains poorly understood.

p38 can activate MAPKAPK 2/3 which then phosphorylates and inactivates HSP27, a heat-shock protein needed for cell migration. Phosphorylated HSP27 can no longer inhibit actin polymerization, thus blocking VSMC migration. MAPKAPK 2/3 may also activate p-16 Arc and 5 lipoxygenase leading to cell migration. In addition, p38 can also activate paxillin, a protein that is involved in focal adhesion dynamics facilitating the process of cell migration. Finally, p38 can activate caldesmon, which induces the urokinase pathway allowing VSMC migration. VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor; fMLP: N-formyl-methionyl-leucyl-phenylalanine; ROCK: Rho-associated kinase; HSP27: heat shock protein 27; MAPKAP 2/3: mitogen activated protein kinase activated protein kinase 2/3; p-16 Arc: Arp2/3 complex 16 kDa subunit. RhoA/ROCK also activates the MEK/ERK pathway in VSMCs. Such activation may lead to the stimulation of four important mediators: MLCK, calpain, FAK and paxillin. Activated MLCK phosphorylates and activates MLC leading to actin polymerization, an important step in VSMC migration. MLCK is also directly involved in the process of focal adhesion turnover. FAK is phosphorylated and activated by MEK/ERK. Activated FAK can interact with activated paxillin, an interaction implicated in focal adhesion turnover. Also, activated FAK can interact with activated calpain. This will allow calpain to degrade cytoskeletal proteins at the focal adhesion facilitating for focal adhesion turnover. This continuous turnover is essential for appropriate cell migration. ROCK: Rho-associated kinase; FAK: focal adhesion kinase; ERK: extracellular signal-regulated kinase; MEK: MAPK/ERK kinase; MAPK: mitogen activated protein kinase; MLC: myosin light chain; MLCK: myosin light chain kinase.

p38 can also mediate VSMC migration induced by several upstream effectors. For example, pathologically elevated cyclic strain can induce VSMC migration and proliferation by suppressing Rho-GDIα (Rho GDP dissociation inhibitor alpha)- a negative regulator of several Rho family GTPases. This subsequently induces phosphorylation of Rac1 and p38, which, we hypothesize could possibly be due to increased activity of RhoA [30]. Consistent with this, pharmacological inhibition of p38, RhoA, or ROCK suppressed sphingosylphosphorylcholine-induced VSMC migration [31,32].

PDGF-BB is another upstream ligand that can trigger RhoA/ROCK-mediated VSMC migration. PDGF-BB is a growth factor known to regulate growth and proliferation in cells of mesenchymal origin, including glial cells, fibroblasts and VSMCs [33]. It is a potent mitogen and chemoattractant of VSMCs and was shown to be secreted in detectable quantities during atherogenesis and, as a result, was suggested to mediate this pathology by virtue of its effects on VSMC migration and proliferation [34]. This is especially since PDGF-BB activates ROCK and MLCK, both of which can then regulate MLC activity (Fig. 1) [35].

Binding of PDGF-BB to its surface receptors on VSMCs can activate both ROCK isotypes, ROCK 1 and ROCK 2 [36]. However, only ROCK 1 stimulates the nuclear translocation of p42/44 MAPK, also known as ERK1/2, a key MAPK involved in cellular proliferation and migration (Fig. 2) [37]. Nucleus-localized ERK1/2 can phosphorylate a set of transcription factors that include c-fos and c-myc leading to the down-regulation of the expression of numerous differentiation-specific contractile proteins. This ERK-mediated cytoskeletal remodeling eventually precipitates VSMC migration [37].

ERK1/2 can mediate cell migration through other pathways [25]. For instance, ERK can phosphorylate and activate MLCK, a key enzyme in the actomoysin dynamics (Fig. 3) [25,38–41]. It can also phosphorylate m-calpains, a family of Ca^{2+}-activated proteolytic enzymes (Fig. 3) [42,43], that are needed for focal adhesion turnover and, consequently, cell migration [44]. ERK1/2 also activates FAK and paxillin (Fig. 3), whose assembly/disassembly is involved in focal adhesion turnover and, as such, migration [25]. These ERK-mediated cell migration pathways are well-established in many cell types. However, much remains to be understood about the extent of their contribution to ROCK-mediated VSMC migration.

PDGF-BB also regulates VSMC migration by impacting the actin superstructure, an event critical to cell migration. Actin cytoskeleton remodeling involves a myriad of proteins like Arp2/3 (actin-related proteins 2 and 3), mammalian diaphanous proteins (mDia), cofilin, Wiscott–Aldrich syndrome protein (WASP), and profilin. These proteins regulate the polymerization/depolymerization of the actin cytoskeleton in response to several upstream signals, including PDGF-BB. PDGF-BB and other VSMC chemotactic signals can induce RhoA/ROCK activation, leading to cytoskeletal remodeling and VSMC migration by a unique mechanism [45–50]. The details of this process include ROCK phosphorylation and activation of LIM kinase (LIMK), which then phosphorylates and inactivates cofilin (Fig. 2). Since cofilin is needed for actin filament severing and depolymerization, the inhibition of its activity blocks lamellipodium formation and directional VSMC migration [51,52]. Notably, RhoA, independently of ROCK, can activate mDia1, an actin-nucleating protein involved in the polymerization of actin filaments, resulting in filopodia formation, an initial step in the process of cell migration (Fig. 2) [53].

ADMA is another upstream signal that can activate Rho/ROCK, leading to VSMC migration. In the VSMC cytoplasm, ADMA activates ROCK, which then stimulates ERK to downregulate contractile protein expression and precipitate a migratory phenotype (Fig. 2) [54]. In addition, ADMA can facilitate Rho/ROCK-mediated VSMC migration *via* endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO). This NO can deactivate the Rho/ROCK axis in VSMCs by inhibiting the Gα (12/13) subunit of heterotrimeric G-proteins, which, in turn, suppresses Ang II-induced vascular migration [55]. ADMA, being an endogenous inhibitor of eNOS, can relieve the repressive effect of eNOS on Rho/R-OCK, allowing migration of VSMCs [54].

Rac1 is another member of the family of small GTPases that is largely implicated in VSMC migration [56]. Rac1 is involved in lamellipodia formation by activating Arp 2/3, a complex required for the nucleation of actin and its polymerization [50]. Rac1 can also activate p21 activated kinase (PAK), a serine/threonine kinase that stabilizes microtubules and regulates cell polarity. Interestingly, RhoA and Rac1 exhibit an antagonistic relationship that largely coordinates the process of cytoskeletal remodeling and cell migration. This is evident through Rac1-inibhition of RhoA via multiple mechanisms, including negative feedback by p63 RhoGEF, a GEF responsible for activating RhoA [57], activation of RhoGAP [58,59], or by direct RhoA degradation [60,61]. Reciprocal negative control of Rac1 activity by RhoA has also been described, and includes the stimulation of FilGAP [62]. Apparently, this shared antagonism between Rac1 and RhoA is essential for achieving VSMC polarity and regulating the formation of membrane ruffles, leading edge protrusion, and cellular spreading and retraction. Furthermore, it has been reported in a human cellular model that Rac1 is inhibited by azathioprine [63]. Since RhoA and Rac1 have a mutually inhibitory relationship and usually promote opposing cellular functions [64], a Rac1 inhibitor could, in some sense, be viewed or even utilized as a possible RhoA activator. This means that azathioprine can be used in scenarios where RhoA upregulation is desired. However, selectivity remains an important issue that warrants further experimentation, both from biochemical and pharmacological perspectives.

Various non-coding RNAs, like microRNAs (miRNA), also regulate Rho/ROCK-stimulated VSMC migration. The original set of miRNA that was shown to modulate VSMC functions were called myomiRs and initially included miR-1, miR-133, miR-206 and miR-208. Later, myomiRs were expanded to include many other miRNAs [65–67]. miRNAs are abundantly expressed in the vascular wall, where they contribute to numerous VSMC functions. For instance, miR-26, miR-29a, miR31, miR133, among several others, regulate VSMC proliferation [65,68]. Emerging evidence shows an important contributory role of miRNAs in the pathogenesis of several CVDs including atherosclerosis. Recently, miRNAs have been documented to be involved in VSMC phenotypic switching [69]. In addition, miRNAs have been found to regulate VSMC migration. For example, miR15b/16, miR22, miR24, miR34a, miR214,

miR362-3p, miR379, miR638, and others were reported to regulate VSMC migration as well as proliferation by silencing the mRNA levels of an array of VSMC phenotype switching-related genes [65,70]. Importantly, miRNAs are involved in pathological VSMC migration and proliferation, such as in the case of atherosclerosis [70]. In regards to the signaling role of Rho/ROCK in VSMC migration, stimulation of ROCK or MLCK was found to activate Stat3 (signal transducer and activator of transcription), a transcription factor associated with cellular growth. Activated STAT3 can upregulate miR-92a, which targets Kruppel-like factor 4 (KLF4), a transcription factor that regulates cell cycle genes and contractile genes. When KLF4 is downregulated due to miR-92a action, cell migration increases (Fig. 4) [35]. Additionally, the pro-atherosclerotic activity of KLF4 on VSMC phenotypic transformation was found to vary according to phosphorylation, cellular state, as well as other microenvironmental factors. Compelling evidence shows that KLF4 can indeed have a marked impact on VSMC phenotype [71,72]. So much so that KLF4 was suggested as a possible therapeutic target in disease management, and as a biomarker for monitoring the progression of atherosclerosis [71]. More importantly, KLF4 was shown to be a major determining factor in the switching of VSMCs between the six different phenotypes, namely osteogenic, contractile, mesenchymal, fibroblast, macrophage, and adipocyte, all based on scRNA-seq studies [73].

Other miRNAs can also play other roles in VSMC phenotypes. For instance, preliminary evidence shows that Shp2-mediated increase in miR204 levels downregulates ROCK 1 levels in pulmonary artery VSMCs (Fig. 4) [74]. Likewise, miR-145 negatively regulates RhoA/ROCK-mediated VSMC proliferation and migration, partly by decreasing mRNA levels of ROCK 1. This occurs through directly binding ROCK 1 mRNA 3′UTR (Fig. 4) [75]. In fact, miR143/miR145 are regarded as molecular switches of the phenotype of VSMCs since these miRNAs target many genes that regulate VSMC function, including different components of the Rho/ROCK cascade as well as other regulators of actin dynamics. In this way, miR143/miR145 are regarded as global regulators of VSMC phenotype switching, including the switches geared towards cellular migration and proliferation [76].

Shp2 signaling increases the level of miR-204. miR204 then blocks RhoA/ROCK by binding to its corresponding mRNA in the cytoplasm. miR-145 can also block the activity of RhoA/ROCK via the same mechanism and can as well interfere with actin dynamics by downregulating the translation of the corresponding mRNAs. LncRNA SMILR blocks the expression of miR-141 in the cytoplasm, preventing the suppression of RhoA/ROCK. Lnc RNA430945 increases the expression of ROR2 which in turn increases the activity of RhoA/ROCK. Increased activity of RhoA/ROCK manifests as increased migration. RhoA/ROCK itself activates STAT3, a nuclear transcription factor, which then increases the level of miR-92a expression. miR-92a blocks Klf-4, a transcription factor that regulates cell cycle and contractile genes.

In a similar manner, miR-223 suppresses RhoB, leading to reduced RhoB/ROCK signaling as well as decreased migration and proliferation in VSMCs [77]. Likewise, miR141 downregulates RhoA, causing blunted RhoA/ROCK signaling and decreased migration and proliferation [78, 79]. A reversal of this inhibition is seen with the long non-coding RNA (LncRNA)-SMILR, which directly binds to miR141 and represses its expression, thus leading to an increase in cell proliferation and migration. Concordant with this, silencing of LncRNA-SMILR robustly inhibits vascular remodeling and mitigation of pulmonary arterial hypertension in an *in vivo* rat model (Fig. 4) [79]. Furthermore, lncRNA430945 promotes the expression of receptor tyrosine kinase-like orphan receptor 2 (ROR2). ROR2 subsequently activates RhoA/ROCK, which eventually stimulates VSMC migration and proliferation (Fig. 4) [35].

One important pathological manifestation of migratory dysregulation that warrants further elaboration is what occurs in atherosclerosis. Indeed, new studies focus on the role of RhoA/ROCK in trapping macrophages within atherosclerotic lesions and its contribution to atherosclerosis progression [80]. As is well-known, atherosclerosis is a chronic
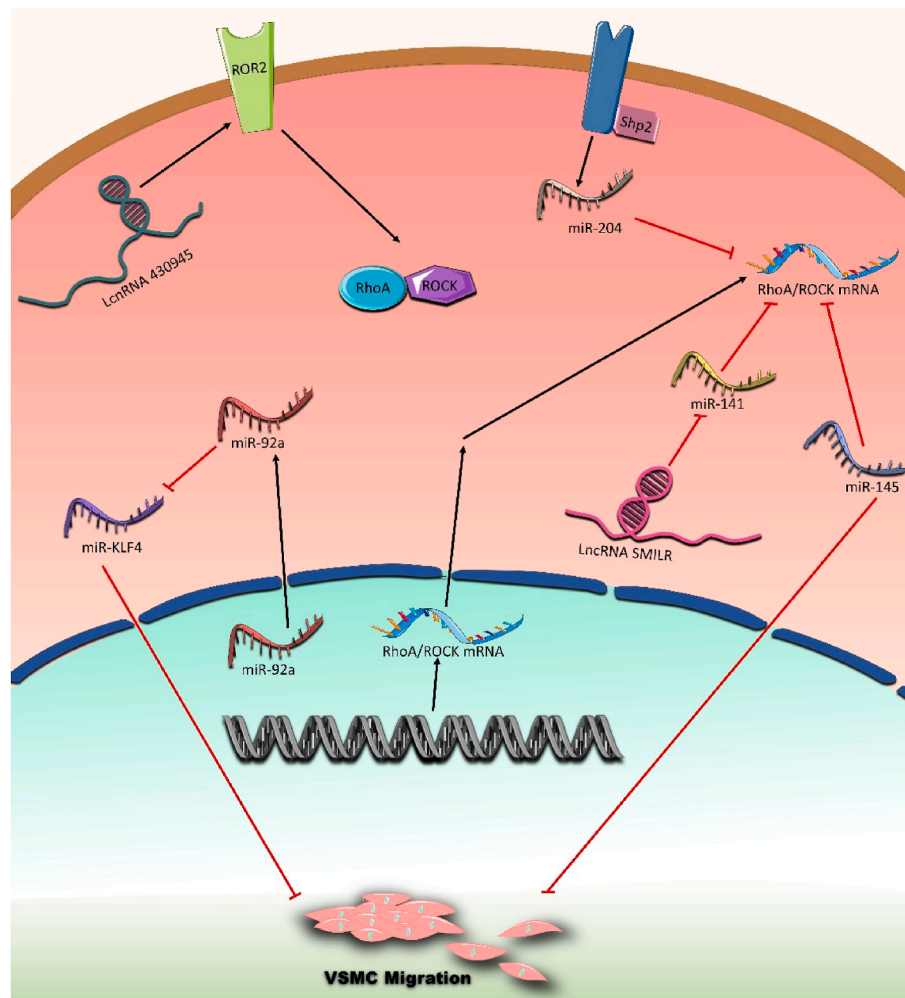
Fig. 4. RhoA/ROCK involvement in VSMC migration can be regulated by an array of miRNAs.

inflammatory disorder that involves the progressive accumulation of lipids within arterial walls. The pathogenesis of this disease involves a multi-step process that occurs over the course of several years or even decades. The initial stage lesions start as small lipid deposits in the intimal layers. These lesions evolve into fatty streaks consisting mainly of foam cells in the subendothelial space. Further accumulation of lipids, foam cells, and immune cells leads to the disorganization and thickening of the intima, media, and adventitia and the proceeding deformation of the artery wall. More advanced atherosclerotic lesions are characterized by extensive lipid deposition, fibrous connective tissue formation and calcification. Macrophages, have been shown to play a pivotal role in the progression of atherosclerotic lesions, as they first infiltrate the damaged endothelium in the form of monocytes. Monocytes then differentiate into naïve M0 macrophages which can then be channeled into M1 (pro-inflammatory) or M2 (anti-inflammatory) or Mox (oxidate stress phenotype) [81]. Macrophages phagocytize ox-LDL becoming foam cells which constitute the lipidic necrotic core of atherosclerotic plaques. M2 macrophages internalize more lipids than M1 macrophages, due to a lower expression of cholesterol efflux proteins [82].

In healthy arteries, VSMCs are located in the tunica media and are well differentiated. In response to vascular injury and inflammatory triggers such as what happens in atherosclerosis, they undergo dedifferentiation and take on a proliferative, and migratory phenotype which is greatly implicated in the pathogenesis of atherosclerosis [83]. Importantly, dedifferentiated VSMCs can express macrophagic markers. They also can internalize lipids and become foam cells [84].

RhoA/ROCK is believed to be involved in the entrapment process of

lipid-laden macrophages in atherosclerotic plaques. Cholesterol-laden macrophages have been shown to have lower levels of the active GTP-bound RhoA, lower level of MLC phosphorylation and decreased motility [85]. Studies also demonstrated that constitutively active RhoA prevent cholesterol-mediated inhibition of macrophage motility. This shows that the increase in cholesterol content prevents RhoA activation and subsequently compromise macrophage migratory abilities leaving them trapped within atherosclerotic lesions as foam cells [85]. Iterestingly, undifferentiated VSMCs under inflammatory conditions, display many macrophagic features. As such, one might envision a scenario whereby RhoA/ROCK deactivation promotes lipid-laden VSMC entrapment in atherosclerotic lesions in the form of foam cells.

4. Mechanisms of RhoA/ROCK mediated VSMC proliferation

VSMC phenotypic switching is heavily implicated in plaque formation and the pathogenesis of atherosclerosis. RhoA/ROCK promotes VSMC proliferation during neointima formation after balloon injury, and its inhibition suppresses neointima formation [86]. Indeed, compelling evidence shows that RhoA or ROCK inhibitors halt VSMC proliferation, leading to a blockade of neointima formation during atherosclerosis [87]. Moreover, in CADASIL, a cerebral arteriopathy caused by a mutation that amplifies the *NOTCH3* (neurogenic locus notch homolog protein 3) gene, there are high levels of NOX5 leading to overproduction of reactive oxygen species (ROS). This pro-oxidant milieu precipitates endoplasmic reticulum stress, followed by enhanced RhoA/ROCK expression, resulting in pathological increase of

VSMCs proliferation [87]. It is also well known that ROS play critical roles in phenotypic switch [88].

There is an interplay between Rho/ROCK and MEK/ERK, a major driver of proliferative phenotype. Indeed, active RhoA/ROCK stimulates the MEK/ERK signaling axis, which then triggers MEK/ERK to promote VSMC proliferation [54,89–91]. Contextually, ERK inhibitors or knock down of ROCK1 and ROCK2 by siRNA causes inhibition of VSMC proliferation [89]. Furthermore, inhibition of ERK1/2 is known to inhibit hyper-proliferation of VSMCs from spontaneously hypertensive rats [92]. Likewise, RhoA/ROCK-induced ERK activation mediates mechanical stretch-induced VSMC contractility and proliferation [93]. Similarly, RhoA/ROCK signaling, in combination with ERK and PI3K activation, is critical for stretch-induced saphenous vein VSMC proliferation [94,95]. When activated, ERK can translocate to the nucleus to activate transcription factors that upregulate the expression of cyclin D1 and PCNA (proliferation cell nuclear antigen), both known to be potent cell cycle regulators (Fig. 5). Cyclin D1 and PCNA can activate VSMC cell cycle, resulting in the eventual proliferation of VSMCs [89].

RhoA/ROCK activates MLCK which then phosphorylates and activates STAT3. In the nucleus, STAT3 induces the transcription of miR-92a, which in turn silences the expression of KLF4, a protein that arrests the cell cycle. This culminates in increased VSMC proliferation. Moreover, RhoA/ROCK activates MEK1/ERK leading to ERK phosphorylation and its translocation to the nucleus to activate transcription factors like c-fos or c-myc. The activated transcription factors can enhance the transcription of proliferation genes like Cyclin D and PCNA, leading to VSMC growth. RhoA/ROCK can also activate JNK which

induces c-jun to transcribe cyclinD1, CDK2, and CDK4, leading to VSMC proliferation. On the other hand, ROCK itself is negatively regulated by miR-143/145 and by p63RhoGEF. miR-143/145 silences ROCK1 leading to a decrease in cell migration and proliferation. MLK 3 binds and makes p63RhoGEF less available for RhoA activation, causing a reduced RhoA/ROCK signaling and leading to a decrease in VSMC proliferation. ROCK: Rho-associated kinase; MLCK: myosin light-chain Kinase; STAT3: signal transducer and activator of transcription 3; KLF4: krüppel-like factor 4; MEK1: MAPK/ERK kinase 1; ERK: extracellular signal-regulated kinase; PCNA: proliferating cell nuclear antigen; JNK: cJun N-terminal kinase; CDK: cyclin dependent kinase.

In addition to its role in migration, ADMA is a major contributor to Rho/ROCK/ERK-mediated VSMC proliferation (Fig. 2) [90]. Similarly, JNK is involved in Rho/ROCK-induced VSMC proliferation [96]. In PDGF-BB-stimulated VSMC proliferation, JNK appears to activate the oncogenic transcription factor c-jun, which upregulates cell cycle regulators Cyclin D1, CDK2, and CDK4 leading to increased proliferation (Fig. 5). This JNK-mediated VSMC proliferation (and also migration) promotes neointima formation [97,98]. Interestingly, inhibition of RhoA suppresses PDGF-induced DNA synthesis, phosphorylation of retinoblastoma protein, and levels of p27 cell cycle inhibitor. This further confirms the role of Rho/ROCK signaling in modulating cell cycle genes and the consequent phenotypic changes [99].

Neuron-derived orphan receptor (NOR-1), a transcription factor, also impacts the role of Rho/ROCK. Indeed, higher levels of NOR-1 are associated with increasing levels of CREB (cAMP response element binding protein), which then amplifies the effect of RhoA/ROCK
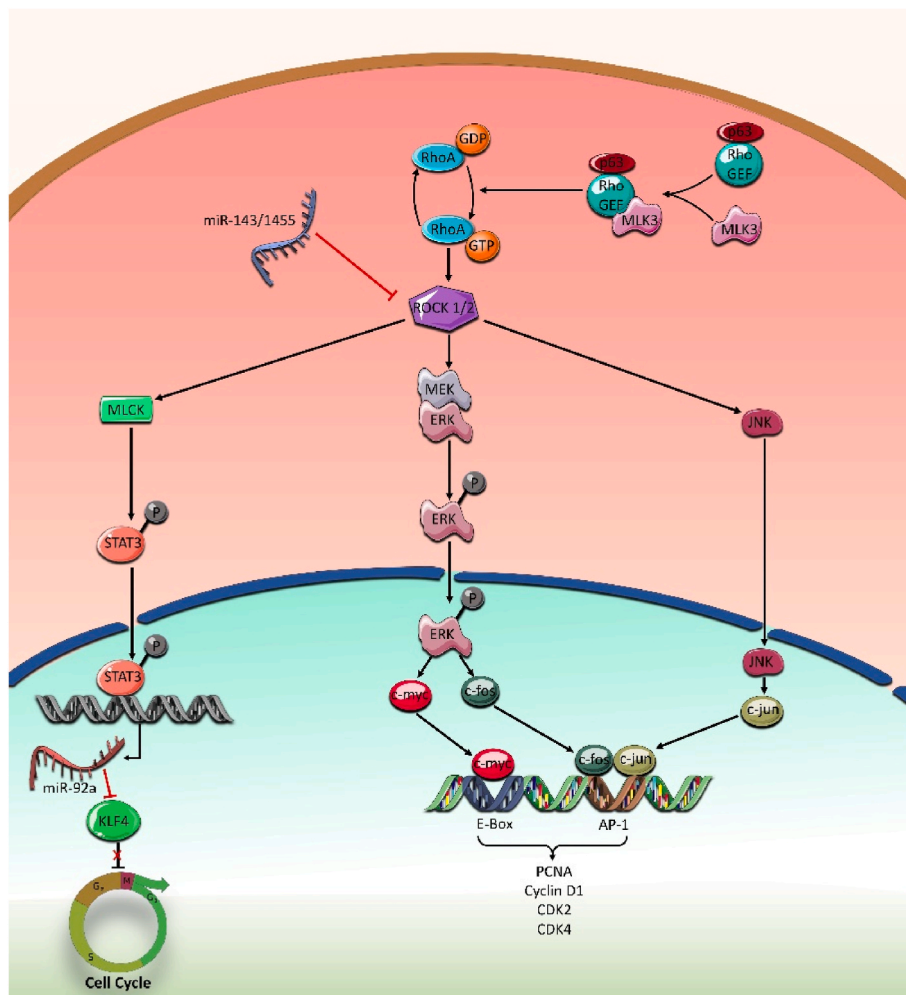

Fig. 5. RhoA/ROCK signaling modulates several downstream pathways implicated in cellular proliferation.

activation, leading to enhanced proliferation of aortic VSMCs [90]. In addition to its role in vascular disease [100–109], cAMP can also inhibit VSMC proliferation in a rather intriguing proposed hypothesis. Increased levels of cAMP can inhibit RhoA, leading to actin depolymerization, which in turn induces the phosphorylation and nuclear export of YAP/TAZ (Yes Associated Protein/Transcriptional Coactivator With PDZ-Binding Motif). This then diminishes the activity of TEAD (TEA domain) transcription factors, leading to reduced VSMC proliferation (Fig. 6) [110,111]. Other reports show that RhoA/ROCK activation induce nuclear import of ERK1/2, resulting in phosphorylation of Elk1 (ETS domain-containing protein 1) (Fig. 6), a coactivator of the transcription factor SRF (serum response factor). In turn, phospho-ELK1/SRF mediates the transcription of proliferation-related genes, like Cyclin D1 and the transcription factor Egr1 (early growth response 1), an immediate early response gene that controls cell proliferation. RhoA/ROCK-stimulated ERK1/2 can also enhance the binding of Egr1 to its DNA binding site leading to enhanced VSMC proliferation [112].

Normally, G-actin monomers bind and sequester MTRF-A in the cytoplasm. Activated Rho/ROCK induce G-actin polymerization into F-actin fibers; MTRF-A is released from the G-actin and translocate to the nucleus. In the nucleus, MTRF-A coactivates SRF, which in turn modulates the transcription of contractile and cytoskeletal genes. In a different mechanism, RhoA/ROCK signaling induces the YAP/TAZ/TEAD transcriptional program. RhoA/ROCK stimulates YAP activation by dephosphorylation of YAP which translocates to the nucleus where it coactivates TEAD transcription factors resulting in enhanced transcription of VSMC growth-related genes. Interestingly, there is an interaction between the MTRF-A/SRF and the YAP/TAZ/TEAD pathways. RhoA/ROCK can activate MEK/ERK causing the translocation of phosphorylated ERK to the nucleus. In the nucleus, phosphorylated ERK can activate ELK1, which then competes for binding to a common site on SRF and can replace the myocardin/MRTF-A that is already bound to SRF. This leads to a downregulation of the expression of contractile proteins. KLF4 is a transcription factor that can induce VSMC dedifferentiation. KLF4 binds to G/C repressor elements that are usually close to CArG boxes, SRF DNA binding sites. KLF-4 can attenuate SRF-mediated

expression of contractile proteins through three different routes. 1) KLF4 disrupts myocardin/SRF interaction, 2) KLF4 can inhibit myocardin expression, and 3) KLF4 can recruit chromatin modifying enzymes to epigenetically repress the expression of CArG/SRF-mediated expression of contractile VSMC proteins. ROCK: Rho-associated kinase; MTRF-A: myocardin related transcription factor A; SRF: serum response factor; YAP: Yes associated protein; TAZ: transcriptional coactivator with PDZ-binding motif; TEAD: TEA domain; ERK: extracellular signal-regulated kinase; MEK: MAPK/ERK kinase; ELK1: ETS domain-containing protein 1; KLF4: krüppel-like factor 4.

5. Mechanisms of RhoA/ROCK-induced VSMC dedifferentiation

Dedifferentiation of VSMCs during phenotypic switching involves loss or downregulation of contractility-related phenotypes and genes as well as the acquisition of an epithelioid phenotype [113]. Dedifferentiated synthetic VSMCs release matrix-digesting enzymes, migrate to injury sites, elevate their rate of proliferation, and synthesize new ECM. When the injury is resolved and a physiologic status is regained, synthetic VSMCs re-assume the contractile phenotype and resume their contractile function [114]. This phenotype shift, despite being physiological, is a critical event in vascular pathologies.

VSMC dedifferentiation is an initial step in the process of atherosclerotic plaque formation, where it enables VSMCs to migrate into the intima to form the fibrous cap [115,116]. Differentiated contractile VSMCs express transcription factors like myocardin, contractile proteins such as smooth muscle alpha actin (α-SMA) and SM myosin heavy chain, intermediate filaments like desmin and vimentin as well as matrix adhesion proteins such as α1 and β1 integrin. Synthetic dedifferentiated VSMCs, however, express calcification genes, like osteopontin or osteocalcin, gap junction proteins like connexin 43, transcription factors like KLF4, collagenase enzymes like collagenase IV, and different matrix metalloproteinases (MMPs) isoforms [3]. The precise molecular mechanisms underlying this event are still not well understood. However, evidence suggests that this process is not only limited to downregulation of smooth muscle specific genes in contractile VSMCs, but rather that drastic cytoskeletal modifications take place due to a global
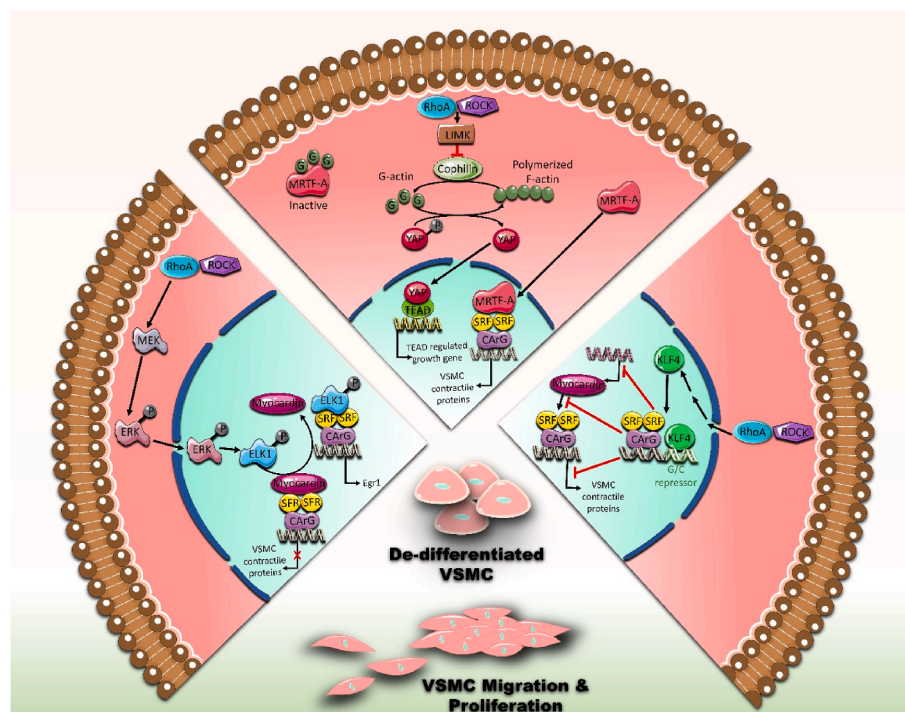

Fig. 6. Pathways of transcription factor-mediated VSMC dedifferentiation. Rho/ROCK signaling triggers the nuclear localization of myocardin/MTRF-A.

modification of VSMC gene expression. Rho/ROCK signaling, being a prominent regulator of cytoskeletal dynamics in VSMCs, is positioned to play a key role during VSMC dedifferentiation.

Rho/ROCK signaling can modulate the expression of a set of smooth muscle specific genes, leading to the modulation of VSMC differentiation status through two important pathways. First, Rho/ROCK signaling is crucial for stabilization and remodeling of the actin cytoskeleton, by both inhibiting MLCP in addition to increasing the sensitivity of MLC to Ca^{2+} (Fig. 1). In addition, several RhoA/ROCK direct and indirect effectors like cofilin, mDia, Arp 2/3 proteins, or WASP are regulators of actin polymerization. Contextually, inhibiting Rho/ROCK disrupts the actin cytoskeleton, indicative of the need for Rho activity in the early phase of VSMC remodeling/dedifferentiation [117]. Paradoxically, Ang II-induced Rho/ROCK-mediated actin polymerization suppresses the expression of VSMC contractile proteins, an integral step for VSMC dedifferentiation, which is, in turn, a prerequisite for VSMC migration [21]. This discrepancy can be explained by the fact that Ang II effects seem to be concentration-dependent. A concentration of 100 nM Ang II suppressed the expression of contractile muscle genes whereas a concentration of 1000 nM had the opposite effect [21]. However, more attention is needed to better elucidate this discordance.

Second, the remarkable effects of activated Rho/ROCK pathway on cytoskeletal dynamics not only regulate VSMC contraction, adhesion, and migration, but also include transcriptional regulation. RhoA/ROCK-induced actin polymerization can stimulate serum response factor (SRF)-mediated gene transcription *via* different ways. Rho/ROCK signaling, for instance, triggers the nuclear localization of the myocardin family of transcriptional coactivators, including, myocardin-related transcription factor (MTRF)-A, and MTRF-B. Indeed, G-actin monomers bind and sequester MTRF-A in the cytoplasm. However, upon Rho/ROCK activation and G-actin polymerization into F-actin fibers, MTRF-A is released from the G-actin monomers and becomes free to translocate to the nucleus. In the nucleus, MTRF-A coactivates SRF by forming a ternary complex with it at the CArG site, the SRF DNA binding site (Fig. 6) [118,119]. MTRFA-coactivated SRF can modulate the transcription of a repertoire of target genes most of which are muscle-specific contractile and cytoskeletal genes. For example, active Rho/ROCK induces the nuclear translocation of myocardin, leading to a new expression profile in VSMCs, which then contributes to vascular pathologies [120]. Moreover, ROCK-mediated phosphorylation of protein phosphatase 1 regulatory subunit 14A (CPI 17), a phosphorylation-dependent inhibitor of smooth muscle protein phosphatase 1α (PP1α), will activate CPI-17 [15, 121]. Activated CPI 17 will inhibit PP1α, which usually represses the transcription factor MEF2C (myocyte specific enhancer factor 2C). The now-active MEF2C triggers the transcription of myocardin, whose increased protein levels will increase binding and activation of SRF, leading to a gene expression pattern characteristic of differentiated VSMCs [121,122]. Interestingly, dedifferentiated VSMCs have low levels of myocardin and SRF [117]. Altogether, it seems that Rho/ROCK signaling can modify the differentiated state of VSMCs through a myocardin/SRF-mediated mechanism.

KLF4, a transcription factor that can induce VSMC dedifferentiation, is expressed by synthetic but absent in contractile VSMCs. KLF4 binds to G/C repressor elements that are usually close to CArG boxes, and can interact with SRF, leading to disruption of myocardin/MRTF-A interaction with SRF (Fig. 6). KLF4 involvement in VSMC dedifferentiation can be specifically attributed to disruption of myocardin-MRTF-A/SRF interaction, KLF4 inhibition of myocardin expression, or KLF4 recruitment of repressive epigenetic remodeling activities to VSMC genes [123]. In addition, other transcription factors, like phospho-Elk-1, can contribute to the differentiation/dedifferentiation of VSMCs by modifying the interaction of myocardin/MRTF-A with SRF [124,125]. Indeed, myocardin and Elk-1 compete for binding to a common site on SRF, which creates a "binary switch" through which signaling pathways can modify the VSMCs phenotypes. For example, PDGF stimulation of VSMCs leads to Elk1 phosphorylation, by ERK1/2, resulting in its

binding to SRF by replacing myocardin/MRTF-A (Fig. 6) [125].

Another transcription program elicited by RhoA/ROCK signaling is the YAP/TAZ/TEAD transcriptional program. RhoA/ROCK-transduced mechanical cues or alterations of actin dynamics can provoke YAP activation (Fig. 6) [126,127]. In this case, RhoA/ROCK mediates dephosphorylation of YAP, which translocates to the nucleus [126–128]. Nuclear YAP binds and coactivates TEAD transcription factors, many of which are VSMC growth-related genes (Fig. 6) [110,111]. Some of the Rho/ROCK/YAP/TAZ/TEAD-regulated genes are, remarkably, also regulated by RhoA/ROCK/MTRF-A/SRF, demonstrating that cytoskeletal dynamics can mediate an interaction between these pathways [111,129].

Epigenetic regulation of the aforementioned pathways and others have also been shown to play a role in VSMC phenotype. There are many reports about epigenetic modifications taking place in the chromatin structure of several smooth muscle specific contractile proteins like SM-α-actin, SM myosin heavy chain, and SM 22α upon differentiation/dedifferentiation. For example, reduced acetylation of histone H4 was reported in PDGF-BB-stimulated VSMCs and in rat carotid artery after balloon injury [124]. Similarly, disruption of telomeric silencing 1-like (DOT1L), the only known methylation writer at histone 3 lysine 79 (H3K79), has been shown to contribute to VSMC proliferation and consequently intimal hyperplasia [130]. Evidence supporting this notion is provided by a study that looked into genome-wide DNA methylation changes in atherosclerotic aorta [131]. This study showed that hypermethylation of differentially methylated regions at aorta enhancer chromatin were noted in several genes that play critical roles in VSMC phenotype and in atherosclerosis [131]. These proteins include elastin, myocardin, α2 smooth muscle actin as well as smooth muscle myosin, among others [131].

Post transcriptionally, VSMC differentiation/dedifferentiation can be regulated by miRNAs. In VSMCs, miR-1, miR-21, miR −23, miR −125b, miR-143/145 and miR-155 were found to be involved in the different aspects of VSMC phenotype switching [66,67]. The miR143/145 cluster is a particularly potent promoter of the VSMC contractile phenotype, while other miRNAs like miR21, miR221, or miR222 favor a dedifferentiated and proliferative VSMC phenotype. Many of these miRNA can target the transcription factors that mediate differentiation/dedifferentiation of VSMC. For example, miR143/145 can target KLF4, Elk1 and myocardin [65]. A recent study found that RhoA/ROCK can induce miR-145 expression to silence KLF4 and induce the differentiation of mesenchymal stem cells into functional VSMCs [66].

While the early stage of dedifferentiation involves destabilization of actin cytoskeleton, later stages encompass an intricate network of signaling events, most notable of which are the MAPKs, ERK1/2 and p38 signaling [132]. For example, MAPK activation, in a Rho/ROCK dependent manner, contributes to the late stage of dedifferentiation [22, 132]. Likewise, ADMA contributes to VSMC dedifferentiation in a process that involves the nuclear translocation of ERK2 [54]. Nevertheless, the mechanism of late stage VSMC dedifferentiation downstream of MAPKs is still not fully understood and warrants future investigation.

6. Effect of RhoA on VSMC apoptosis

Apoptosis is an intricately regulated but only recently studied component of VSMC behavior. It is integral to multiple critical events, such as vascular remodeling, thrombosis and atherosclerosis, among others [133]. During these processes, apoptotic VSMCs usually undergo extensive cytoskeletal rearrangements. Since Rho/ROCK signaling contributes to the actin cytoskeleton stability and remodeling, its involvement in VSMC apoptosis warrants discussion. For instance, Ang II–induced abdominal aortic aneurysm (AAA) in apoE-knockout mice is associated with a significant increase in apoptosis in aortic wall VSMCs. Inhibition of ROCK by fasudil blunted Ang II–induced apoptosis of cells of the aortic wall and reduced AAA [134]. Contextually, Rho/ROCK

inactivation can partly account for the elaborate alterations that occur at the level of the actin cytoskeleton during VSMC apoptosis. Inhibition of RhoA/Rac-1 disrupts the integrity of the actin cytoskeleton of rat VSMCs *in vivo* and *in vitro* [135]. Concurrently, bcl-2-modifying factor (Bmf), a pro-apoptotic factor that binds to actin filaments, dissociated and moved into the mitochondria. Concomitantly, dissipation of mitochondrial membrane potential, caspase-3 activation and eventually apoptosis ensue (Fig. 7) [135]. As such, administration of actin stabilizing agents can rescue these cells from contractile dysfunction as well as apoptosis, which both confirms the integrity of the previous findings and also suggests that disruption of RhoA/Rac-1 signaling leads to disruption of cytoskeletal integrity, which in turn can induce apoptosis [135].

Apart from its direct effect on the actin cytoskeleton, Rho/ROCK can mediate apoptosis through a variety of pathways in different cell types [136]. For example, in rat cardiomyocytes, the Rho/ROCK pathway induces apoptosis by activation of MAPKs p38 and JNK [137]. Activation of JNK was documented to induce apoptosis in VSMCs (Fig. 7) [138, 139]. It is noteworthy to mention that Rho/ROCK signaling was found to elicit JNK activation in VSMCs [140]. As such, one could envisage a mechanism whereby Rho/ROCK activates JNK in VSMCs in order to induce apoptosis; however, this requires further investigation.

Concomitant with disruption of the actin cytoskeleton integrity, Bmf, a pro-apoptotic factor attached to actin filaments, dissociates and moves into the mitochondria, where dissipation of mitochondrial membrane potential, caspase-3 activation and ultimately apoptosis ensue. Separately, Rho/ROCK can independently activate JNK in many cell types resulting in cell apoptosis. Finally, RhoA/ROCK activation can disrupt the vimentin cytoskeleton resulting in eventual VSMC apoptosis. ROCK: Rho-associated kinase; Bmf: Bcl-2-modifying factor; JNK: c-Jun N-terminal kinase.

Rho/ROCK can negatively regulate apoptosis in VSMCs. For example, inhibition of ROCK led to inhibition of VSMC migration and an enhancement of apoptosis of VSMCs, causing a reduction of neointima formation after vascular injury [141]. In another study, the same group confirmed that ROCK inhibition enhances apoptosis by increasing Bax expression and thereby reducing neointima formation [142]. Others also reported that shear stress downregulates the expression of Rho-GDIα and Rac1. Since Rho-GDIα downregulation promotes VSMC migration and apoptosis, this suggests that RhoA or Rac1 act to enhance apoptosis of VSMCs during shear stress [143]. Nonetheless, the evidence for the involvement of Rho/ROCK signaling in VSMC apoptosis is evolving and awaits further validation.

7. Mechanisms of RhoA/ROCK-mediated modulation of VSMC extracellular matrix deposition

Within the vascular system, the ECM has structural and regulatory roles. ECM is involved in relaying mechanical signals to cells and providing enough elasticity for blood flow, all while maintaining the tensile strength needed to handle changes in pressure [144]. Major producers of ECM within the vessel wall are VSMCs. As a result, pathological states, most notable of which were atherosclerotic plaque formation and arterial hypertension, were reported to emerge when synthesis of ECM proteins by VSMCs was dysregulated [145]. Similar to other aspects of VSMC phenotypic switching, dysregulated ECM deposition seems to engage extensive cytoskeletal modifications. Rho/ROCK signaling, being a chief modifier of actin filaments stability, is suggested to contribute to regulation of the process of VSMC ECM deposition [146].

RhoA/ROCK can mediate the process of ECM deposition through multiple downstream signaling pathways that mostly emanate from TGF-β as an upstream signal [147–149]. TGF-β signaling via the Smad pathway has prominent roles in VSMCs stiffness and fibrosis. It is involved in the regulation of expression of most VSMC ECM-related proteins, including the pro-fibrotic mediator CTGF (connective tissue growth factor), the main ECM component Type I Collagen (Col-1), and the plasminogen activator inhibitor-type 1 (PAI-1; SERPINE1) [147–149].

Initially, links between RhoA/ROCK and ECM deposition were reported while studying the effect of statins on TGF-β/SMAD pathway in vascular cells and also during atherosclerosis [150]. It was found that blockade of RhoA with either statins or RhoA specific inhibitors will exacerbate TGF-β-induced increase of Smad3 phosphorylation and ECM deposition [150]. Murine transcriptome studies have yielded an
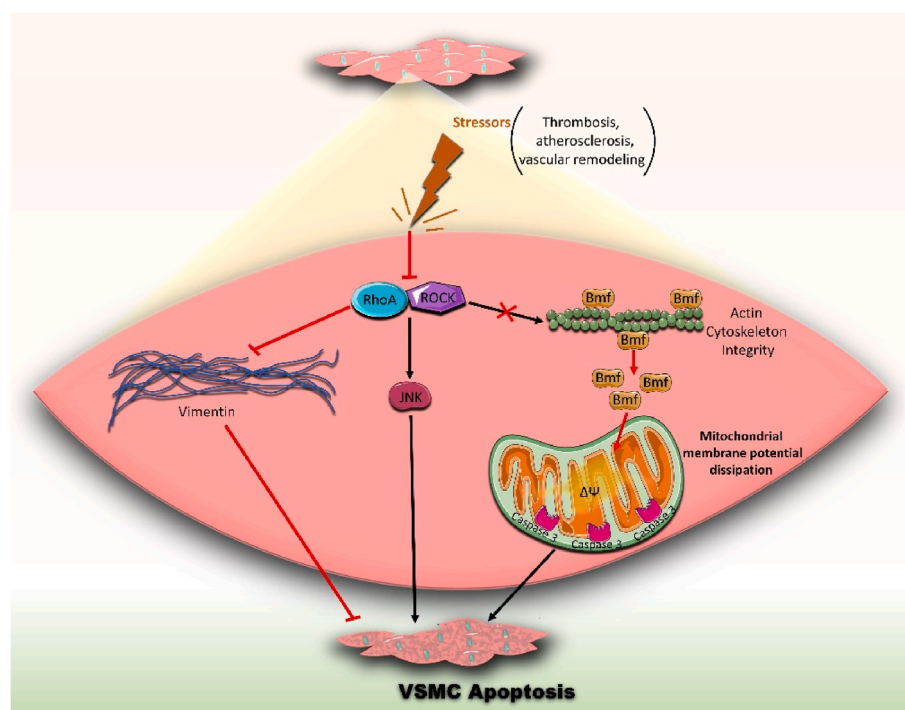
Fig. 7. Numerous stressors inhibit the RhoA/ROCK pathway leading to alteration of cytoskeletal stability and other phenomena like apoptosis.

independent significant connection between Smad3 and aortic dilatation pathology in the setting of hereditary thoracic aortic aneurysm; loss of contact between VSMC and ECM, which is implicated in aneurysm pathogenesis, was linked to Smad3 activity and VSMC response to microenvironmental factors [151]. Moreover, inhibition of RhoA/ROCK pathway can increase the expression of TGF-β and its receptor TGF-β receptor type II (TRII). Overall, RhoA/ROCK signaling serves to reduce TGF-β levels. These signaling activities suggest that hyperactivation of RhoA may impinge on this pathway during pathological conditions, leading to unstable plaque formation or a modulation of elastic ECM deposition by VSMCs [150]. Aneurysmal formation, due to its link with abnormal ECM deposition, may also be influenced by RhoA, as its correlation with Smad3 activity has already been established by transcriptome analysis, albeit in animal models [151].

PAI-1 is involved in the modulation of VSMC stiffness [149]. PAI-1 inhibits tissue plasminogen activator, preventing the cleavage of plasminogen into active plasmin, which in turn can activate MMPs. PAI-1 blocks plasmin-mediated degradation of fibrin and hence ECM degradation [149]. Activated ROCK is an activator of PAI-1 expression, since inhibition of RhoA/ROCK inhibited Ang II-induced PAI-1 activation [152]. In addition, ERK1/2 was implicated in RhoA/ROCK-mediated activation of PAI-1 expression [152].

MMPs mediate remodeling of the ECM under physiological and pathological states. MMPs degrade the ECM to allow for cell migration and their proteolytic activity is also needed to control the availability of growth factors in close proximity with cells. During phenotypic switching, synthetic VSMCs elevate the expression of several MMPs like MMP-2, MMP-9, and other matrix digesting enzymes, possibly as a part of the dedifferentiation process [153]. As such, MMPs contribute to formation and remodeling of the vasculature. In addition, remodeling of the ECM is a requirement for VSMC migration, and upregulated MMP activation induces VSMC migration and proliferation [153,154]. Consistent with this assumption is the failure of *in vitro* migration of VSMCs from MMP-2 knock out mice [155]. Likewise, MMP-9 is necessary for VSMC migration and proliferation [156]. Moreover, dysregulated MMP activity can lead to vascular pathologies like hypertension-induced arterial changes and persistent hypertension [154].

The interplay between Rho and MMPs is becoming more lucid. MMPs, which can destabilize the atherosclerotic plaque, are themselves regulated by RhoA/ROCK signaling [153]. For instance, PDGF-BB-induced MMP-2 expression in rat VSMCs is mediated via ROCK, ERK and p38 MAPKs [157]. Also, Rho/ROCK mediates PDGF-induced secretion of MMP-9 in saphenous vein VSMCs via ERK, p38, and NFκB [158]. Taken together, regulation of MMP expression by RhoA/ROCK appears to be integral to VSMC-mediated ECM deposition during phenotypic switching.

Src homology region 2 domain-containing phosphatase-2 (SHP2) is a protein tyrosine phosphatase that acts upstream of TGFβ1-SMAD2 pathway and contributes to VSMC ECM stiffness [159]. It was found that Ang II activates SHP2, which deactivates RhoGAP p190A, causing RhoA to be mainly present as the active RhoA-GTP form. This activates ROCK, which then inhibits the TGFβ/Smad pathway [160]. This Ang II-mediated signaling via SHP2/RhoGAP p190A/RhoA/TGFβ/SMAD appears to be also an important player in phenotypic regulation. Remarkably, an opposing observation was reported in the case of Marfan Syndrome aortic aneurysms, where high levels of active RhoA-GTP paradoxically favor ECM deposition [161]. It was proposed that this phenomenon may be indirectly related to chronic activation of TGF-β and overexpression of myocardin in Marfan syndrome vascular cells [161]. Yet, the underlying mechanism remains to be elucidated.

There are other important roles that VSMCs play in pathological states. For example, during atherosclerosis and when phosphate concentration is high, VSMCs actively drive the deposition of calcified matrix within the vessel wall [162,163]. Deposition of mineralized ECM is a characteristic of dedifferentiated synthetic VSMCs during

pathological phenotype switching [162]. Pathological dedifferentiation of VSMCs can cause them to follow osteogenic, chondrogenic, or even macrophage cell lineages that can form foam cells. Differentiation into osteoblast-like cells entails secretion of mineralized ECM components like osteocalcin, leading to vascular calcification [163,164]. Despite this understanding, the mechanisms underlying the osteogenic differentiation of VSMCs are still poorly understood [163]. A recent study reported that inhibition of ROCK causes a reduction in VSMC calcification [165]. Another recent study reported that RhoA/ROCK positively regulates VSMC calcification, where Wnt3a, a pleiotropic cellular function signal transducer protein, mediates calcification of VSMCs *via* the Rho/ROCK/JNK pathway [166]. Conversely, Rho/ROCK has also been shown to be a negative regulator of bovine VSMC deposition of calcified matrix [167]. Collectively, more research is needed to unravel the role of RhoA/ROCK in VSMC deposition of calcified matrix.

8. Mechanisms of RhoA/ROCK-induced vascular stiffness

Vascular stiffness is correlated with the rigidity of the vessel wall. An increase in vascular stiffness is implicated in a number of cardiovascular disorders since it can lead to increased vascular impedance and impairment of the elastic component of vessel structure [168]. In addition, increased arterial stiffness is associated with end-organ remodeling, such as arterial intima-media thickening in adults [169]. Vascular stiffness is modified by an alteration of the composition of the main components of the vessel wall *viz*: elastin, collagen, and smooth muscle cells. Stiffness of VSMCs *per se* has a major contribution to arterial stiffness [170,171]. In this context, Rho/ROCK signaling contributes to both VSMC and arterial stiffness.

The transcriptional program that takes place due to the action of myocardin/SRF transcriptional program is the main driver behind VSMC stiffness that manifests in response to hypertension, among other cues [172]. Rho/ROCK signaling can directly induce myocardin nuclear translocation, the subsequent activation of myocardin/SRF transcriptional program, and eventually the switch to a stiffer VSMC phenotype (Fig. 8). This can culminate in the stiffness of the whole vascular wall and the alteration of the hemodynamic properties of the blood vessel [173].

Transforming growth factor beta (TGF-β), is a pleiotropic cytokine involved in many cellular processes including cell proliferation, differentiation, apoptosis, and migration. VSMCs obtained from Marfan Syndrome patients exhibit overactive RhoA/ROCK pathway owing to an overactive TGF-β signaling. Such cells, as discussed above, exhibit increased expression of contractile protein markers, indicative of increased cellular stiffness. In addition, higher levels of nuclear-translocated myosin-related transcription factor A were observed. These findings propose a pathway that involves increased TGF-β signaling as the trigger of eventual RhoA/ROCK activation, leading to increased stiffness. This pathway may increase nuclear localization of myosin related transcription factor A, which can start a new transcription program leading to phenotypic switching of VSMCs [161]. However, due to the paradoxical findings for RhoA/ROCK in Marfan syndrome patient VSMCs, this particular finding warrants verification.

RhoA/ROCK signaling can contribute to VSMC stiffness by other mechanisms involving cullin-3, polo-like kinase (Plk1), or neurolipin-2 (NLP-2). Cullin-3 is a component of the machinery responsible for RhoA degradation [174]. A cullin-3 loss-of-function mutation (CUL3Δ9) leads to accumulation of RhoA, and as a result increased arterial stiffness (Fig. 8) [174]. Polo-like kinase 1 (Plk1), a serine/threonine kinase normally expressed during cellular division, regulates Ang II-mediated activation of RhoA/ROCK in VSMCs, causing stress fiber formation which effectively increases cellular stiffness (Fig. 8). Specific deletion of Plk1 in VSMCs causes aberrant arrangement of cellular stress fibers and decreased arterial elasticity [175]. In addition, Plk1 modulates Ang II–dependent activation of RhoA, placing Plk1 upstream of RhoA in the regulation of VSMC stiffness [175]. In bladder smooth muscle cells,
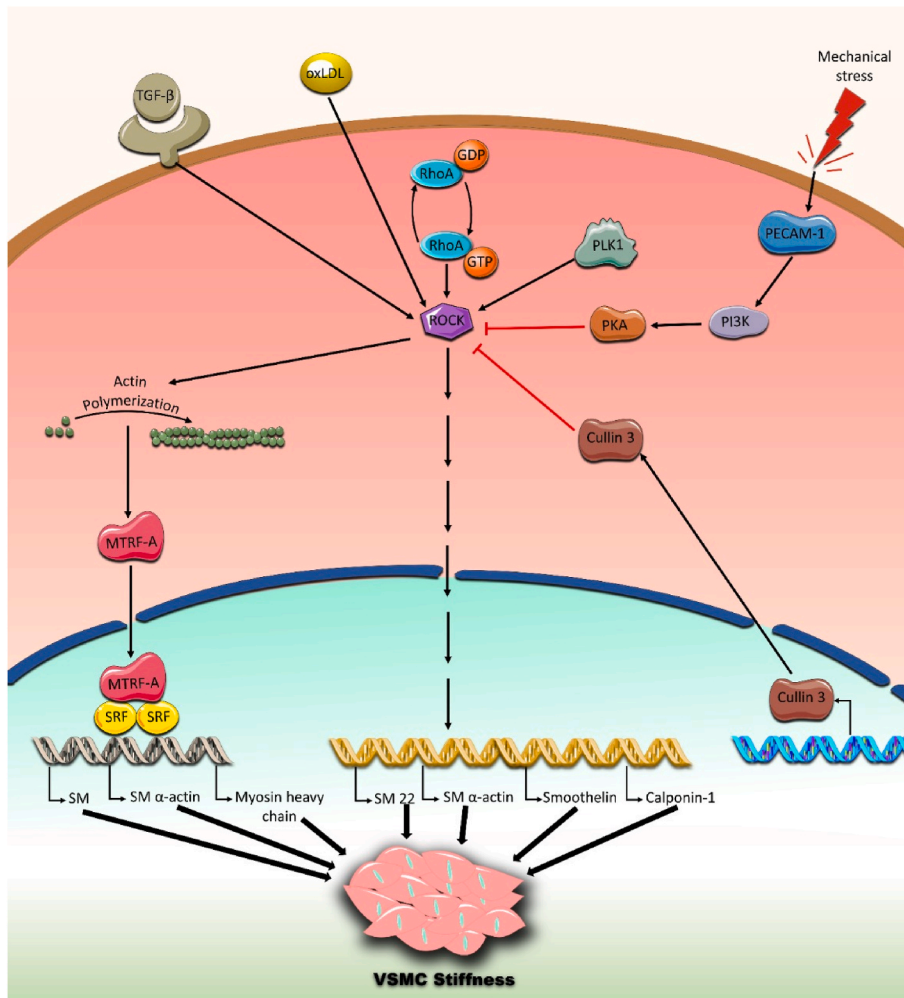
Fig. 8. Mechanisms of RhoA/ROCK-induced VSMC stiffness.

TGF-β, PLK1, or oxidized low-density lipoprotein (oxLDL), can induce ROCK to activate RhoA, which then triggers myocardin family transcription factors like MFTR-A to co-activate serum response factor (SRF). This then initiates expression of target genes like Myosin-related transcription factor, SMA, smoothelin, SM22, and calponin-1, proteins that heighten VSMC stiffness. In addition, Cullin-3 can inhibit ROCK and modulate the expression of these genes. In addition, stress signals can induce PECAM-1 to activate PI3K, which then inhibits ROCK via PKA. TGF-β: transforming growth factor β; PLK1: polo like kinase 1; oxLDL: oxidized low-density lipoprotein; ROCK: Rho-associated kinase; MRTF-A: myocardin-related transcription factor; SRF: serum response factor; SMA: smooth muscle actin; MYH: smooth muscle myosin heavy chain; SMTN: smoothelin, α-SMA: α-smooth muscle actin, SM22: smooth muscle protein 22; CUL3: cullin-3; PECAM-1: platelet endothelial cell adhesion molecule 1; PI3K: phosphoinositide 3-kinase; PKA: protein kinase A.

activation of NRP2, a cellular receptor protein, downregulates RhoA/ROCK [176]. This leads to a reduction of cellular stiffness due to reduced phosphorylation of MLC [176]. While this pathway is active in bladder smooth muscle, testing the potential effect of NRP2 signaling on VSMC phenotype switching and cellular stiffness in particular is worthy of investigation [176].

9. Rho/ROCK-mediated phenotypic switching in "venous" SMCs

While this review focuses mostly on Rho/ROCK-related pathways that govern phenotypic behavior in arterial territories, it is important to note that phenotypic switching is not limited to arterial tree. Indeed, it was shown that venous SMCs can also undergo phenotypic changes that are implicated in multiple physiological and pathological processes [177–179]. For instance, arteriovenous fistula failure as well as coronary artery venous graft disease are well-known vascular pathological entities where dysfunctional SMC phenotypes lead to maladaptive remodeling at the venous side culminating in thrombosis, stenosis or obstruction [177–181]. It is worth mentioning here that in AV fistulas, Rho/ROCK alters the eNOS pathway, a major regulator of endothelial line properties and a known contributor to the arterialization of veins [182,183].

Despite some similarities in the endpoint of phenotypic switching in arteries and veins, there are many differences at the cellular aspect in the behavior of venous versus arterial VSMCs. Indeed, the molecular pathways dictating these phenotypic behaviors are different and their alteration account for the arterial adaption of veins in the case of

arteriovenous fistulas as well as venous grafts. In vein grafts, Rho/ROCK is induced under pulsatile stretch conditions in venous tissues and is shown to trigger venous SMCs phenotypic changes, inducing a transition from the quiescent, or contractile, phenotype, into a synthetic, migratory, and proliferative phenotype [184]. One translational aspect of this difference is noticeable when statins are part of a therapeutic regimen, as these drugs are known to prevent pulsatile stretch-induced proliferation of human saphenous vein smooth muscle cells via inhibition of Rho/Rho-kinase pathway [95,184]. In particular, simvastatin suppresses the RhoA/ROCK pathway leading to inhibition of MMP-9, a matrix metalloproteinase associated with intimal hyperplasia in saphenous vein (SV) bypass grafts [185].

Likewise, SMCs were found to show distinct metabolic patterns when subjected to venous versus arterial mechanical stretches [186]. Indeed, mechanical stretch activates the Rho/ROCK signaling in venous SMCs, which is then followed by phosphorylation and activation of JNK. JNK then phosphorylates and inactivates specific protein 1 (SP1), a transcription factor involved in mitofusin-2 (MFN2) expression. MFN2 is a mechanoresponsive protein that interacts with Phosphofructokinase 1 (PFK1) to mediate PFK1 degradation and therefore suppresses glycolysis in VSMCs. As such, when SP1 is suppressed by active JNK, venous SMCs switch to the glycolytic pathway favoring cellular proliferation and migration [186].

One factor believed to play a prominent role in defining venous identity is Ephrin type-B receptor 4 (Eph-B4). This receptor, a member of the Eph receptor tyrosine kinase (RTK) family, has been described not only as a marker of venous endothelial cells but also as player in the

behavior of venous smooth muscle cells [184]. Importantly, Eph-B4 signaling in venous graft SMCs is believed to limit proper venous adaptation to the arterial environment. In this context, vein graft adaptation is associated with intimal thickening, largely comprised of mature SMCs that spatially localize with decreased Eph-B4 expression [187]. Apparently, Rho/ROCK can act as a downstream effector of EphB4 in multiple cell lines. Activation of EphB4 receptor, by its ligand ephrin-B2-Fc, stimulates RhoA and induces cell migration via actin cytoskeleton reorganization [188]. In other cells, EphrinB2/EphB4 signaling was implicated in junctional stability of the endothelial line via Rac1/Rho-mediated regulation of cytoskeletal contractility [189]. Given this interaction between ephrinB2/EphB4 and Rho/ROCK, and since both elements are expressed in venous SMCs, it would be tempting to speculate that this interaction modulates some phenotypic behavior of such cells. Nonetheless, this requires further investigation to elucidate the major components of this newly proposed pathway.

10. Current and potential therapeutic uses

Fasudil hydrochloride is a RhoA/ROCK inhibitor that was approved for cerebrovascular vasospasm since 1994 [190]. This may suggest some level of clinical safety for RhoA/ROCK inhibitors despite the theoretical complication raised by the pleiotropic action of Rho/ROCK signaling on a multitude of cell types. Fasudil is also used in the treatment of glaucoma [191], pulmonary hypertension [190], and coronary vasospam [190]. Recently, other RhoA/ROCK inhibitors have been proposed as treatments for sepsis cardiovascular complications [192] and diabetic macular edema [193]. It has also been suggested to be beneficial in the management of Raynaud's disease [100,105,106,194] and multiple sclerosis [195]. Besides the possibly extensive side effect profile imposed by the activity of RhoA/ROCK in several tissues, limitations for the use of RhoA/ROCK inhibitors include their teratogenicity, as it has been shown to participate in fetal cardiac development [87]. The selectivity of the inhibition exerted by the inhibitors also affects the possible clinical use, especially that RhoA/ROCK inhibitors might reduce the activity of other kinases, increasing the risk of undesirable side effects [196] with long-term or high dose use. In addition to that, the selectivity of RhoA/ROCK inhibitors to ROCK1 versus ROCK2 isoforms might have some pharmacological implications.

Over the past 15 years, three dimensional molecular design technologies have become robust approaches for discovering new chemical entities against multiple molecular targets and, among them, structure-based virtual screening is the most prominent. Briefly, thousands of structures from different chemical databases are screened *in silico*, and a defined number of candidates are examined for biological potency, sensitivity and specificity through experimental trials. In the context of Rho/ROCKs inhibitors, multiple novel drugs are the manifestation of that computational technology [197]. Such advancement has allowed the modeling of new Rho/ROCK inhibitory agents with high specificity and sensitivity compared to old drugs such as fasudil. For instance, many of these drugs showed superior potency and selectivity against ROCK kinases than towards other kinases like protein kinase A and C [198]. They also showed high selectivity for ROCK 2 isoforms [198,199]. These include drugs of the 2,3-diaminopyrazines derivatives, benzodioxane derivatives and benzyl pyridinethiazole-based amide derivatives among many others. Recently, a long-acting hypoxia -activated prodrug of fasudil was designed for the purpose of minimizing the systemic side effects of the parent drug. This prodrug has shown exceptional selectivity against cancer cells as well as hypoxic regions of the lungs in patients with pulmonary hypertension. Consequently, the clinical implications of this novel agent might be promising [200].

To utilize these advances for mitigating vein graft failure, stenosis and neointimal hyperplasia, a certain degree a vasospecificity is needed in order to load Rho/ROCK inhibitors in the desired location while minimizing the systemic side effects of these agents. To achieve such purpose, multiple mechanistic strategies for drug delivery have been

designed. Early testing with a rhosin (RhoA inhibitor)-eluting stent in a leporine carotid model yielded attenuation of neointima formation as compared to a bare metal stent [201]. Additionally, intimal hyperplasia after balloon injury was reduced upon administration of anagliptin in a murine model, through the action of the SOD-1/RhoA/JNK pathway [202]. These preliminary results suggested, at the very least, a potential use for RhoA/ROCK inhibitors for preventing predictable, procedural, VSMC pathology mediated vascular injury events.

One recent strategy for dealing with venous graft failure involves a novel complex electro-spun external sheath which can slowly/gradually and time-dependently release loaded fasudil dihydrochloride, everolimus and simvastatin at the level of a vein graft [203]. The combined effect of mechanical restriction exerted by the sheath and loaded fasudil can alleviate the damage on the endothelial line and trigger endothelial repair through Rho/ROCK activation to prevent early phase graft failure [204,205]. Middle phase graft failure due to SMC proliferation is efficiently inhibited by the loaded everolimus. Late stage graft failure principally caused by atherosclerosis is prevented by slow-released simvastatin [206,207]. It is expected that, in the near future, these and other targeted delivery approaches take the front seat in the management of several cardiovascular diseases.

11. Conclusion

While important in various physiological processes, VSMC phenotypic switching, when dysregulated, can contribute to multiple vascular pathologies like atherosclerosis, intimal hyperplasia and hypertension. The relationship between dysregulated VSMC phenotypes and Rho/ROCK signaling demands extensive future investigation, and is a requisite for novel therapeutic approaches as well as for understanding of vascular function. Despite possible challenges relating to non-selectivity, teratogenicity, and pleiotropy, the development of "vaso-specific" RhoA/ROCK inhibitors offers a valuable prospective tool for the management of vascular disease.

CRediT authorship contribution statement

Tedy Sawma: Writing – original draft. **Abdullah Shaito:** Writing – original draft. **Nicolas Najm:** Writing – original draft. **Munir Sidani:** Writing – original draft. **Alexander Orekhov:** Writing – review & editing. **Ahmed F. El-Yazbi:** Writing – review & editing. **Rabah Iratni:** Writing – review & editing. **Ali H. Eid:** Conceptualization, Writing – review & editing, Resources, Supervision, Project administration.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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