The Ins and Outs of small GTPase Rac1 in the Vasculature

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Nonstandard abbreviations:

GTPase, protein that can hydrolyze guanosine triphosphate (GTP); Ras, protein superfamily of small GTPases; RhoA, Ras homolog gene family, member A; Cdc42, Cell division control protein 42 homolog; Rac1, Ras-related C3 botulinum toxin substrate 1, GDP, Guanosine diphosphate; GTP, Guanosine-5'-triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; GEF, Guanine nucleotide Exchange Factor; GAP, GTPase Activating Proteins; GDI, Guanine nucleotide Dissociation Inhibitor; NOX, NADPH oxidase; ROS, reactive oxygen species; AT1R, Angiotensin-II receptor type; JNK, c-Jun N-terminal kinase; ACE, angiotensin converting enzyme; eNOS, endothelial nitric oxide synthase expression; NO, nitric oxide.
ABSTRACT

The Rho family of small GTPases forms a 20-member family within the Ras superfamily of GTP-dependent enzymes that are activated by a variety of extracellular signals. The most well-known Rho family members are RhoA, Cdc42 and Rac1, which affect intracellular signaling pathways that regulate a plethora of critical cellular functions such as oxidative stress, cellular contacts, migration and proliferation. In this review, we describe the current knowledge on the role of GTPase Rac1 in the vasculature. While most recent reviews focus on the role of vascular Rac1 in endothelial cells, in the present review we also highlight the functional involvement of Rac1 in other vascular cells types; namely smooth muscle cells present in the media and fibroblasts located in the adventitia of the vessel wall. Collectively, this overview shows that Rac1 activity is involved in various functions within one cell type at distinct locations within the cell, and that there are overlapping but also cell type-specific functions in the vasculature. Chronically enhanced Rac1 activity seems to contribute to vascular pathology, however, Rac1 is essential to vascular homeostasis, which makes Rac1 inhibition as therapeutic option a delicate balancing act.
INTRODUCTION

The Rho GTPases RhoA, Cdc42 and Rac1 have been implicated in various vascular diseases, such as atherosclerosis, hypertension, diabetes, vascular leakage, angiogenesis, and aneurysm formation (Boissier and Huynh-Do, 2014; Ferri, et al., 2013; Jin, et al., 2013; Khan, et al., 2013; Koyama, et al., 2013; Liu, et al., 2014; Marinkovic, et al., 2013; Nagase, 2013; Peng, et al., 2013; Sun, et al., 2014; Varela, et al., 2014). Rac1 (Rho-related C3 botulinum toxin substrate) is well recognized for its role in actin remodeling and the induction of membrane protrusions, but also has several other downstream effectors, such as activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases or cell surface receptor-mediated signaling resulting in an inflammatory response (Ambriz-Pena, et al., 2014; Cuadrado, et al., 2014; D’Ambrosi, et al., 2014; Marinkovic, et al., 2014b). Numerous stimuli are reported to lead to Rac1 activation, such as vasoactive hormones, cytokines, growth factors, mechanical stress and oxidative stress. Similar to other Rho GTPases, Rac1 is functioning according to the “on and off” switch principal, by cycling between the active GTP-bound and inactive GDP-bound state. The advantage of this switch is to rapidly transmit the extracellular signal, received by the receptor to activate downstream pathways and thereby enabling the cell to respond adequately.

Several classes of proteins are involved in (de)activation of GTPases. The nucleotide exchange from GDP to GTP takes place in the binding pocket of Rac1. This exchange is catalyzed by Guanine nucleotide Exchange Factors (GEFs). GTPase Activating Proteins (GAPs) enable activation of GTPases by facilitating GTP hydrolysis. As inhibitory factors, the Guanine nucleotide Dissociation Inhibitors (GDIs) keep the GTPases in an inactive state in the cytosol and protect them from degradation (Garcia-Mata, et al., 2011).

In recent years, it has become more evident that malfunctioning Rac1 protein, caused by either directly blocking Rac1 activity or indirectly through dysfunctional GEFs, GAPs or GDIs, may underlie several vascular pathologies (Adam, et al., 2007; Kawarazaki, et al., 2012; Kimura, et al., 2014).
In this review, we give an overview of the latest progress in understanding the role of Rac1 in the vasculature, with the emphasis on the dominant cell types of the vessel wall, namely endothelial cells, smooth muscle cells and fibroblasts.

**Endothelial cells**

The endothelium forms a monolayer which covers the inner lining of blood vessels. It functions as a semi-permeable barrier that regulates exchange of nutrients, oxygen and cells between blood and subjacent tissues. Endothelial dysfunction is placed at the basis of many vascular diseases among which atherosclerosis (Libby, 2006), aneurysm formation (Rateri, et al., 2011), diabetes-associated vascular disease (Kim, et al., 2006) and vascularization in cancers (Kowanetz and Ferrara, 2006; Ruegg and Mariotti, 2003). Here, we will focus on the role of Rac1 in endothelial cell function and dysfunction.

**NADPH oxidases**

In endothelial cells, Rac1 has been strongly linked to activation of the NADPH oxidase complex in the cellular membrane. The NADPH-oxidase (NOX) complex is well recognized for its role in killing pathogens in the phagosome of neutrophils, by generating superoxide. Interestingly, the complex is also found in vascular cells, e.g. endothelial cells, smooth muscle cells and adventitial fibroblasts (Konior, et al., 2014). Initially, pioneers in the field of NOX function in endothelial and smooth muscle cells were eager to prove that this cell types have functionally active NADPH oxidases. Being similar to leukocyte enzymes, this oxidases were shown to play outmost important role in maintaining functionality of these cell types and by that, vascular homeostasis (Gorlach, et al., 2000). Since then the in depth functions of the NOX proteins are presented in excellent reviews (Drummond and Sobey, 2014; Lambeth, 2004; Streeter, et al., 2013). The NOX-complex comprises of several subunits, including members of the family of NOX proteins, responsible for the generation of superoxide or other reactive oxygen species (ROS). However, without its critical subunits like p47, p67 or p40 and 22phox, NOX is not able to generate ROS (Hordijk, 2006). For proper
assembly of the full and functional NOX-complex, Rac1 activity is required (Sumimoto, 2008; Bedard and Krause, 2007). In vascular cells, ROS appear to be functionally relevant for the regulation of vascular tone and gene transcription. ROS interact with proteins, lipids and nucleic acids and is able to alter their function. However, chronic or excessive Rac1-mediated ROS production results in oxidative stress, where ROS destroy other molecules, and is considered detrimental (Elnakish, et al., 2013). There are several isoforms of the enzymatic NOX family, of which NOX2 is considered the classical NOX. NOX1, NOX2 and NOX4 are expressed by endothelial cells (Dammanahalli and Sun, 2008; van Buul, et al., 2005). In contrast to NOX1 and NOX2, NOX4 is constitutively active and its activation does not require Rac1 (Martyn, et al., 2006; Meng, et al., 2008).

In endothelial cells, a number of small vasoactive peptide hormones, such as angiotensin-II, endothelin-1 and atrial natriuretic peptide (ANP), are related to blood pressure regulation by influencing Rac1-dependent NOX activation. Angiotensin-II receptor type 1 (AT1R)-mediated signaling is best known to induce NOX activation and generation of oxidative stress. Under physiological conditions, this pathway increases blood pressure, yet when chronically activated, it induces vascular pathogenesis (Gregg, et al., 2003). In mice, chronic infusion of angiotensin-II enhances atherosclerosis and aneurysm formation, which are both pro-inflammatory vascular pathologies with a strong regulatory role for endothelial cells. Decreased endothelial shear stress enhances atherogenesis and aneurysm formation (Higuchi, et al., 2012). In addition, while AT1R deletion in smooth muscle cells has no effect on aneurysm formation in the ascending aorta, endothelial-specific depletion of AT1R attenuated pathology (Rateri, et al., 2011), indicating that endothelial-specific generation of ROS by AT1R may control aneurysm formation. In that light, we recently showed that immunosuppressive drug azathioprine (generic name), is a potent Rac1-inhibitory drug in aortic endothelial cells, and efficiently reduced aneurysm formation in mice after angiotensin-II infusion (Marinkovic, et al., 2013; Marinkovic, et al., 2014b). Endothelial cells treated with
azathioprine metabolites showed reduced c-Jun N-terminal kinase (JNK) phosphorylation in vitro and in vivo (Marinkovic, et al., 2013; Marinkovic, et al., 2014b).

The vasoactive steroid aldosterone also induces ROS generation, via mineralocorticoid receptor-regulated activation of NOX and Rac1, and thereby induced angiotensin converting enzyme (ACE) expression. ACE will generate angiotensin-II and subsequently activate AT1R (Iwashima, et al., 2008). Medication suppressing the Renin-Aldosterone-Angiotensin-II-System (RAAS), such as ACE inhibitors or AT1R blockers, are known to protect against vascular diseases in heart, blood vessels and kidney, probably in part by indirect inhibition of NOX/Rac1 activation.

Next to ligand-receptor induced activation, the NOX complex in endothelial cells can also be activated by fluid shear stress, where a mechanosensitive complex is present and responsible for Rac1 activation upon alterations in shear stress. This complex is composed of VE-cadherin, Par3, p67phox (part of NOX complex), Tiam1 (Rac-specific GEF) and Rac1, of which VE-cadherin is responsible for the mechanosensing, ultimately resulting in MAPkinase activation (Liu, et al., 2013; Yeh, et al., 1999). Many vascular diseases are strongly enhanced by shear stress influencing the endothelium and ligand-receptor mediated signals, especially in atherosclerosis where the site specificity of the lesions is determined by reduced shear stress.

In mice, the role of NOX2 in the pathophysiology of atherosclerosis was studied in NOX2 deficient mice in a pro-atherogenic apolipoprotein E deficient (ApoE-/-) background. These experiments revealed that although NOX2 deficiency showed reduced production of superoxide, lesion formation in the aortic sinus was not affected (Judkins, et al., 2010; Kirk, et al., 2000), while total lesion area of the aorta was reduced by 50% (Judkins, et al., 2010). Endothelial cell specific NOX2 overexpressing mice, also in an ApoE-/- background, showed increased macrophage recruitment, yet no difference in development of atherosclerosis in the sinus or total aorta (Douglas, et al., 2012). Possibly, there is a local difference in NOX
activation of the various NOX isoforms in the different cell types, which contribute to atherosclerosis. In line with this, NOX4 deficiency increases various pathological vascular phenotypes, also in endothelial cells, since loss of NOX4 resulted in a reduction of endothelial nitric oxide synthase expression (eNOS) and nitric oxide (NO) production. This was mediated by reduced transcriptional activity of the anti-oxidant nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor (Schroder, et al., 2012). Thus, the Rac1-independent NOX4 seems to play an important role in the homeostasis of endothelial cells and thereby being protective against vascular disease, whereas other NOX family members that do require Rac1 for their activity, cause a pro-inflammatory phenotype.

In atherosclerotic patients, lipid-lowering statins are used as standard clinical care. Statins reduce low-density lipoprotein (LDL) cholesterol levels by inhibition of the rate limiting enzyme in cholesterol synthesis, which is 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase. Statins have pleiotropic therapeutic effects; for example, they promote the degradation of Rac1 by targeting the anchoring of Rac1 to the membrane via small GTP-binding protein dissociation stimulator (SmgGDS) upregulation (Tanaka, et al., 2013). Once in the cytosol and not protected by Rho-GDIs, Rac1 is rapidly degraded. Statins have been reported to decrease NOX activity and ROS generation via inhibition of Rac1, as summarized by Antonopoulos et al. (Antonopoulos, et al., 2012). However, it has also been shown that simvastatin potently enhances Rac1 activity, as a direct consequence of down-regulating cholesterol synthesis (Kou, et al., 2009). A decrease in the intermediate isoprenoid metabolite geranylgeranyl pyrophosphate was responsible for Rac1 activation, by limiting the substrate for the enzyme geranylgeranyl transferase-1 (GGTase-1) (Kou, et al., 2009). GGTase-1 transfers geranylgeranyl on to specific motifs in many proteins, including RhoA, CDC42 and Rac1, altering protein function. Interestingly, GGTase-1 knockout macrophages have highly increased RhoA, CDC42 and Rac1 activity, and showed a pro-inflammatory phenotype (Khan, et al., 2011). In that light, it is not
surprising that macrophage-specific GGTase-1 knockdown in mice, resulted in increased inflammation in a rheumatoid arthritis model (Khan, et al., 2011). In addition, in proatherogenic LDL-receptor deficient mice, GGTase-1 deficiency in macrophages increased T lymphocyte influx (inflammation) into the vessel wall. Surprisingly, it reduced atherosclerosis by 60%, which was caused by a RhoA-dependent increase in cholesterol efflux from lesion macrophages (Khan, et al., 2013). Inhibition of GGTase-1 in rat arteries induced eNOS and reduced NOX and ROS (Zuckerbraun, et al., 2005), suggesting that statins would improve endothelial cell function, and help reducing cardiovascular disease, yet its influence on Rac1 activity is not resolved completely.

Since Rac1 is the “molecular engine” of most NOX activity, it seems important to control Rac1 activity in order to prevent excessive oxidative stress-induced pathologies (Elnakish, et al., 2013; Konior, et al., 2014). Many (Moldovan, et al., 2006), but not all, downstream effects of Rac1 activation involve NOX activation. In the next paragraphs, cell type specific functions of “vascular” Rac1 are discussed.

**Leukocyte transmigration**

In endothelial cells, Rac1 activation requires a pro-inflammatory stimulus, and thereafter may induce downstream transcription-dependent or -independent actions to facilitate leukocyte attraction and transmigration locally. The most well-known downstream target of Rac1 is serine/threonine p21-activating kinase 1 (PAK1), a critical effector that induces cytoskeleton remodeling and nuclear signaling via activation of the NOX, JNK or NFkB pathways (Kumar, et al., 2006; Taglieri, et al., 2014). For example, cytokine TNFα can mediate chemokine and adhesion molecule VCAM-1 expression in endothelial cells via the pro-inflammatory transcription factor NFkB in a NOX/Rac1-dependent way (Chen, et al., 2004; Marinkovic, et al., 2014b) and AP1/ATF2 via JNK signalling (Marinkovic, et al., 2013; Marinkovic, et al., 2014b). Attracting leukocytes is the first step of an inflammatory response, but leukocyte migration through the endothelial layer requires cell adhesion, which is the second step.
Rac1 is critically involved in the formation of so-called “docking structures” or “transmigratory cups” (Barreiro, et al., 2002; Carman and Springer, 2004; van Buul, et al., 2007), which are membrane protrusions that capture adherent and transmigrating leukocytes and depend on cytoskeletal remodeling (van, et al., 2012b). The main Rho-GEF involved in Rac1-induced docking structures in endothelial cells is TRIO (van, et al., 2012b). While RhoG is a more potent substrate for TRIO-GEF1 than Rac1 (Jai swal, et al., 2013; van, et al., 2012a), RhoG is not activated upon TNFα stimulation whereas Rac1 is (van, et al., 2013). This shows that TRIO can control Rac1 activation upon stimuli that do not necessarily activate RhoG in endothelial cells. Crosslinking or clustering of ICAM-1 recruits TRIO and triggers local activity of Rac1 (van, et al., 2012b). Rac1 can also be activated by VCAM-1 clustering (Marchese, et al., 2012; van, et al., 2002). TNFα-induced VCAM-1 expression involves posttranslational modification by methylation of the C-terminal prenylcysteine in Rac1 to target Rac1 to the cell membrane (Ahmad, et al., 2002; Papaharalambus, et al., 2005). Interestingly, VCAM-1 expression is regulated in a Rac1-dependent fashion, while ICAM-1 is not (Ahmad, et al., 2002; Marinkovic, et al., 2014b; Ng, et al., 2002). This exclusiveness of VCAM-1 regulation has also been observed with transcription factor Ets-2 in endothelial cells (Cheng, et al., 2011), although Rac1 involvement has not been determined here.

Statins, which have been shown to inhibit Rac1-mediated NOX activation, are also reported to be protective against endothelial cell activation (Palinski and Napoli, 2002). In the presence of pro-inflammatory cytokines such as TNFα, statins can suppress Rac1 activation and downstream ROS generation, transcription factor NFκB activity and expression of adhesion molecules such as ICAM-1 and VCAM-1, with ERK5 as a target of statin therapy (Wu, et al., 2013b). The abrogation of Rac1 activation by statins and ERK5 is transcription factor KLF2-dependent, which is the shear stress sensitive factor responsible for healthy endothelium (Komaravolu, et al., 2015).

While ICAM-1 expression is not changed by Rac1 activation, its functionality after clustering is Rac1-dependent and it is severely hampered by Rac1 inhibition (Marinkovic, et al., 2014b). Anti-ICAM-1-antibody-coated beads are shown to efficiently crosslink ICAM-1, thereby
inducing Rac1 activation and transmigratory cup formation in endothelial cells (Marinkovic, et al., 2014b). This can be inhibited by 6-mercaptopurine, a metabolite of azathioprine, which has potent Rac1 inhibitory capacity. 6-Mercaptopurine, which is further converted to 6-thio-GTP, interferes with Rac1 function (Marinkovic, et al., 2014b). Upon angiotensin-II infusion in mice, leukocyte transmigration into the aortic wall was inhibited when mice were treated with azathioprine, with endothelial cells showing decreased p-JNK activation (Marinkovic, et al., 2013;Marinkovic, et al., 2014b).

While Rac1 activation is necessary to form membrane protrusions to facilitate leukocyte transmigration, it is also essential to close the micro-wounds in the endothelial cell layer where leukocytes have passed through, to prevent leakage (Martinelli, et al., 2013). In case of excessive inflammation, Rac1 inhibition can relieve the inflammatory burden by prevention of further leukocyte influx. Yet, micro-wounds created by leukocyte penetration will not be restored when Rac1 is inhibited and could possibly attribute to leaky vessels upon chronic use of Rac1 inhibitors. Thus, tight regulation of endothelial Rac1 is required to respond adequately to changes in the microenvironment.

**Barrier function**

In addition to Rac1-mediated cytoskeletal rearrangements to capture leukocytes, cytoskeletal changes are also involved in endothelial barrier function or cell migration. Maintaining the endothelial barrier is imperative for healthy blood vessels. Vascular leakage leading to edema or tissue inflammation is highly relevant in the microvasculature, and plays a key role in cancer progression (Bid, et al., 2013). Several reports show that Rac1 activation recovers or even stabilizes decreased endothelial barrier function after G-protein coupled receptor activation with vascular endothelial growth factor (VEGF), thrombin, histamine or platelet activating factor (PAF) (Adamson, et al., 2010;Baumer, et al., 2009;Bid, et al., 2013;Hoang, et al., 2011;Knezevic, et al., 2009;Maharjan, et al., 2013;Tan, et al., 2008;Wang, et al., 2011b;Waschke, et al., 2006). Interestingly, apart from proteins, also certain lipids modulate the barrier function. Two endogenous lipid derived molecules, namely the phospholipid...
sphingosine 1-phosphate (S1P), and bile acids (generated from the lipid cholesterol) are known to increase endothelial barrier function. They do so by enhancing Rac1 activity via activation of their respective receptors, S1P-receptor type 1 (van Hooren, et al., 2014) and the G protein-coupled bile acid receptor (Kida, et al., 2014). The sugar hyaluronan, a glucosaminoglycan that is abundantly present in the extracellular matrix, can be generated in a high molecular weight (HMW-HA) or low molecular weight (LMW-HA) form and also modulates the endothelial barrier. HMW-HA and LMW-HA bind to different isoforms of the cell surface receptor CD44, with opposing effects. HMW-HA enhances the barrier function via CD44s association with S1P-receptor type 1 and Rac1 activation, while LMW-HA reduced the barrier function via CD44v10 association with S1P-receptor type 3 and RhoA activation (Singleton, et al., 2006). In light of the concept that sugars and lipids can modulate barrier function, the finding that the pseudo-sugar derivative of cholesterol Sac-1004, blocks stimulus-induced endothelial permeability, is an interesting discovery (Maharjan, et al., 2013). Sac-1004 inhibits endothelial hyperpermeability, which was induced by VEGF, histamine or thrombin, via stabilization of the cortical actin rings and adherens junction proteins at the cell-cell junction by improving Rac1 activity (Maharjan, et al., 2013).

Rac1 is known to regulate cell-to-cell contact by modulation of junctional protein distribution, especially the adherens junctions (Bazzoni and Dejana, 2004). Adherens junctions are predominately composed of transmembrane VE-cadherin and intracellular catenins (Bazzoni and Dejana, 2004). Interference with VE-cadherin causes actin cytoskeleton reorganization (Hordijk, et al., 1999), enhanced permeability (Hordijk, et al., 1999; Timmerman, et al., 2012), cell migration (Breviario, et al., 1995), and angiogenesis (Carmeliet, et al., 1999). While Rac1 activity is essential to maintain junctional integrity, introduction of a constitutively active mutant of Rac1 (Tat-RacV12) into endothelial cells resulted in increased endothelial monolayer permeability by redistributing the VE-cadherin complex (van, et al., 2002). Rac1-dependent ROS production played a key role in this process, leading to phosphorylation of the VE-cadherin complex (Monaghan-Benson and Burridge, 2009; van, et al., 2002). These studies show the importance of balanced Rac1 activity in order to maintain endothelial barrier function.
function. Most likely, local Rac1 activity, induced through specific GEFs may contribute to this delicate balance. However, how exactly this is regulated is intriguing and requires future research.

Within the Rho subfamily, RhoA and Rac1 often have opposing roles and keep each other in balance. Knockdown of focal adhesion kinase (FAK), a mediator of signal transduction by integrins and growth factor receptors in endothelial cells, results in increased RhoA activity and decreased Rac1 activity. This caused a severe dysfunction of adherens junctions and subsequently compromised endothelial barrier function (Schmidt, et al., 2013). However, this effect was rescued by a RhoA inhibitor, that allowed Rac1 to become activated again. Regulation of specific GAPs seemed to contribute in the balancing act between RhoA and Rac1 in fibroblasts. RhoA was shown to inhibit Rac1 activity by antagonism of FILGAP (Ohta, et al., 2006). In addition, Rac1-mediated ROS production resulted in inhibition of the low-molecular-weight protein tyrosine phosphatase (LMW-PTP), followed by increased phosphorylation and activation of its target, p190Rho-GAP, which in turn inhibited RhoA activation (Nimnual, et al., 2003). A similar influence of RhoA or Rac1 on GAPs could be responsible for the opposing effects observed in endothelial cells, which requires future studies.

Migration

Endothelial cell migration is depending on lamellipodia formation. Lamellipodia are membrane protrusions that are induced through local (specific area within the cell) Rac1 activity and downstream activation of the actin-related protein 2/3 (Arp2/3) complexes to induce actin polymerization (Krause and Gautreau, 2014). Several studies have shown that local activation of Rac1 is essential for directional migration (Mayor and Carmona-Fontaine, 2010). Kraynov and colleagues were the first to show local Rac1 activity at the leading edge of a migrating fibroblasts using a FRET-based biosensor for Rac1 activity (Kraynov, et al., 2000). We have confirmed these data in endothelial cells (Figure 1). The actin adapter protein cortactin plays an important role in the re-arrangement of the actin cytoskeleton to
generate and stabilize Rac1-dependent lamellipodia (Krause and Gautreau, 2014). Recently, the involvement of G-protein-coupled receptor-2-interacting protein-1 (GIT1) became apparent in this process, since in GIT1-depleted endothelial cells, cortactin localization and as a consequence lamellipodia formation was strongly reduced. GIT1 promotes the assembly of signaling complexes to the membrane of the leading edge, and thereby activates Rac1 locally, resulting in the formation of lamellipodia (Majumder, et al., 2014).

Rac1-mediated ROS also plays a role in vascular endothelial cell motility (Moldovan, et al., 2006). Generation of ROS has been found at the leading edge of endothelial lamellipodia, exactly at the spots where local Rac1 activity is detected. Scavenging ROS reduced the speed of migration and directionality (Moldovan, et al., 2006). The source of ROS, which regulate endothelial cell migration, may be NOX- or mitochondrial-derived (Wang, et al., 2011b). Rac1 and ROS are involved in phosphorylation of actin filament crosslinking protein MARCKs. This protein is essential for actin assembly and directional cell movement of endothelial cells (Kalwa and Michel, 2011; Kalwa, et al., 2012). siRNA-mediated Rac1 knockdown blocked angiotensin-II-stimulated MARCKS phosphorylation, and siRNA-mediated knockdown of Rac1 or MARCKS disrupted actin polymerization and migration (Kalwa, et al., 2012). In line with these data, we observed similar results using 6-mercaptopurine and 6-thio-GDP as Rac1 inhibitors in a model for endothelial cell migration (Figure 2). Our data indicate that these metabolites of immunosuppressive drug azathioprine inhibit wound-induced Rac1 activity and additionally reduce the length and directionality of endothelial cell migration towards the wound.

VEGF is well known for its capacity to induce angiogenesis through endothelial cell migration to form new, yet leaky, vessels. Interestingly, Rac1 activation reduces VEGF-induced vascular leakage (Hoang, et al., 2011), yet Rac1 is essential for VEGF-induced migration (Wang, et al., 2011a). Again, local activation of Rac1 is very likely to determine these different processes. Recently, Rac1 was also demonstrated to regulate the production of VEGF itself by endothelial cells. An increase in activity of Rac1 and also Cdc42 resulted in
tumor suppressor p53 ubiquitination, reducing p53 protein levels, which in turn induced expression of VEGF in endothelial cells, and thereby promoting angiogenesis as a feed forward mechanism (Ma, et al., 2013). The prominent role of VEGF in angiogenesis makes it a prime target in cancer therapy. Since cell migration is largely Rac1-dependent, a strong focus is now also on development of specific Rac1 inhibitors in the cancer field, to achieve less cancer cell migration and angiogenesis (Bid, et al., 2013; Majumder, et al., 2014). These type of inhibitors could potentially be of importance for other type of angiogenic disorders, such as in diabetic patients, where vascular leakage and angiogenesis in the retina causes injury. Yet in diabetic patients, decreased vascularization of extremities (such as feet) should be monitored with these type of inhibitors. Additionally, the fact that local Rac1 activity determines the final phenotypic outcome in many processes calls for a more specific strategy to target Rac1 activity as a therapeutic reagent. Nevertheless, it is clear that blocking Rac1 activity holds strong potential to fight diseases that show perturbed endothelial monolayer integrity.

**Vascular smooth muscle cells**

The most abundant cell type of a blood vessel is the vascular smooth muscle cell (VSMC). VSMCs are pivotal in vascular contractility and control luminal narrowing to maintain blood pressure. Several pathogenic stimuli can alter VSMC function and contribute to disease. Most VSMC-related diseases concern VSMC proliferation and migration, which are the main topic in this section. Also in VSMC there is a key role for the NOX enzymes and generated ROS in these processes (Konior, et al., 2014).

**Proliferation and migration**

In VSMC, redox-dependent migration and proliferation are central events in physiology and the development of vascular pathology. NOX1 is the prominent Rac1-dependent NOX that contributes to these processes upon activation through a variety of different stimuli (Fernandes, et al., 2009; Janiszewski, et al., 2005; Meng, et al., 2008; Pescatore, et al.,...
2012; Sturrock, et al., 2006). NOX1 deficiency (Nox1(y/-)) inhibited wire injury-induced VSMC hyperplasia in mice, yet VSMC-specific overexpression of NOX1 did not influence hyperplasia (Lee, et al., 2009). NOX1-deficient VSMC derived from the mice exhibited a decreased proliferation and migration response to platelet derived growth factor (PDGF) stimulation. Detailed analysis showed that this phenotype was caused by reduced PAK1 activation and increased cofilin phosphorylation, leading to reduced actin de-polymerization. An essential role for PAK1 in VSMC hyperplasia is shown in a rat restenosis model (Hinoki, et al., 2010; Wang, et al., 2009). Thus NOX1 activation causes Rac1/PAK1-dependent actin polymerization in VSMC (Lee, et al., 2009). Kalirin-9 (Kalrn) is an arterial Rho-GEF with high homology to the Rho-GEF TRIO. Interestingly, Kalrn contains 2 GEF domains for both Rac1 and RhoA. Neointimal hyperplasia, induced by carotid endothelial denudation, was significantly reduced in VSMC-specific Kalrn(-/-) mice compared to control mice. Reduced Kalrn function gave normal RhoA activation but diminished Rac1 activation in serum-, endothelin-1- or PDGF-stimulated VSMC, as assessed by reduced Rac1-GTP levels, PAK1 activation and reduced VSMC proliferation and migration (Wu, et al., 2013a). These data suggest that the Rac-GEF domain is dominant over the Rho-GEF domain in Kalrn under these conditions.

In addition, basic fibroblast growth factor (bFGF) induces NOX1/Rac1-dependent ROS generation and VSMC migration. bFGF-induced migration was attenuated in VSMC derived from NOX1 deficient mice, and reduced VSMC outgrowth from aortic segments of these mice was observed (Schroder, et al., 2007). Transduction of NOX1 restored normal migration. bFGF activated JNK and enhanced phosphorylation of adaptor protein paxillin, which brings together regulators of actin organization. Paxillin is crucial for migration and secretion of matrix-metalloproteinases (MMPs) to facilitate movement (through matrix) (Schroder, et al., 2007). Insulin-like growth factor-1 (IGF-1) could also increase MMP-2 and MMP-9 activity and promoted VSMC migration via Rac1 and (Rac1-independent) NOX4 activation (Meng, et al., 2008). NOX4 knockdown inhibited VSMC migration due to reduced ROS generation (Meng, et al., 2008). These NOX4 data do not overlap entirely with the data that NOX4 is
important for preservation of the VSMC contractile phenotype, thus protecting VSMC function (Clempus, et al., 2007). Here, knockdown of NOX4 reduced expression of VSMC differentiation markers smooth muscle α-actin (SM α-actin), smooth muscle myosin heavy chain, and calponin, as well as SM α-actin stress fibers. In endothelial cells, it has been shown that NOX4 induced activation of redox-sensitive Nrf2 and its target genes (Schroder, et al., 2012). In VSMC, PDGF promotes nuclear translocation of Nrf2 as an anti-oxidant response against excessive oxidative stress (Ashino, et al., 2013). Nrf2 depletion by siRNA, enhanced PDGF-promoted Rac1 activation and ROS production and phosphorylation of downstream extracellular signal-regulated kinase-1/2 (ERK1/2). In line with these data, in vivo, Nrf2-deficient mice showed enhanced neointimal hyperplasia in a wire injury model (Ashino, et al., 2013). Thus the Nrf2-mediated pathway seems a negative feedback mechanism to protect the cell, which may be NOX4-mediated.

Interestingly, 8-Hydroxy-2-deoxyguanosine (8-OHdG), which is a product of DNA oxidation upon excessive oxidative stress, can decrease Rac1 activity. 8-OHdG has been shown to inhibit Rac1 by binding in the Rac1 GTP-active site, preventing Rac1 recycling. 8-OHdG decreased VSMC hyperplasia in a carotid artery ligation model probably by inhibition of Rac1 (Huh, et al., 2012).

The cholesterol lowering statins inhibit geranylgeranyl pyrophosphate generation and thereby limit the substrate for the enzyme GGTase-1 (Kou, et al., 2009). The effect of decreased GGTase-1 function on VSMC reveals that inhibition of GGTase-1 decreased activation of RhoA and Rac1 as well as proliferation, and thereby decreased intimal hyperplasia after balloon injury in rats. TNFα- or angiotensin-II-induced activation of NOX and ROS was decreased by GGTase-1 inhibition. While GGTase-1 inhibition in macrophages activated RhoA and Rac1, it reduced RhoA and Rac1 activity in VSMC, which may be part of the atheroprotective pleiotropic effect of statins (Zuckerbraun, et al., 2005).

In conclusion, proliferation and migration of VSMC is largely regulated by NOX1/Rac1 and ROS, which can be beneficial in vascular repair, but also detrimental in VSMC hyperplasia disorders.
Contraction

NOX1 activation is known to enhance calcium (Ca\(^{2+}\)) mobilization in VSMCs, which does not only affect VSMC migration (Duran-Prado, et al., 2013; Li, et al., 2011; Rossi, et al., 2009; Zimmerman, et al., 2011). VSMC contraction is highly Ca\(^{2+}\)-dependent and the role of Rho GTPases herein is extensively discussed in a recent review by Loirand and Pacaud (Loirand and Pacaud, 2014). While the role of GTPase RhoA in VSMC contraction is clear, inducing vasoconstriction upon activation due to the rise of cytosolic Ca\(^{2+}\), the role of Rac1 is still quite obscure. VSMC contraction requires phosphorylation of myosin light chain (MLC) by the Ca\(^{2+}\)-calmodulin-dependent MLC-kinase (MLCK), which is activated by a rise in cytosolic Ca\(^{2+}\) concentration. MLCK is dephosphorylated again by the Ca\(^{2+}\)-independent MLC phosphatase (MLCP). Upon increased influx of Ca\(^{2+}\), activation of RhoA leads to activation of Rho-kinase (Rock) which phosphorylates MLCP, and thereby inactivating it (Uehata, et al., 1997). This inactivation prolongs the phosphorylated state of MLC and thus increased contractility of VSMC.

Loirand and Pacaud extensively describe that Rac1 activation in VSMC is not that straightforward (Loirand and Pacaud, 2014). Collectively, the context of Rac1 activation seems to determine if Rac1 activation leads to vasoconstriction or vasodilation. Rac1 activation can inactivate RhoA via PAK1-dependent inhibition of cGMP conversion to GMP, thus promoting cGMP-dependent protein kinase (PKG) activity, subsequent RhoA phosphorylation and inactivation. Another Rac1/PAK1-mediated vasodilatory pathway affects endothelial eNOS and thus NO production, and NO increases cGMP generation and PKG activation. Rac1/PAK1 activation also inhibits MLCK, thus inhibits phosphorylation of MLC and downstream contraction. In contrast, Rac1/PAK3 activation enhances phosphorylation of caldesmon, which induces vasoconstriction. In addition, Rac1-mediated NOX activation results in increased ROS generation, which also induces vasoconstriction. Clearly, further research on the functional relevance of the different context of Rac1 activation and its effect on VSMC contraction is necessary.
Fibroblasts

Fibroblasts are the primary cell type responsible for collagen synthesis, composing the structural network of most organs. In blood vessels, the fibroblasts in the adventitia control homeostasis of the collagen matrix surrounding the vessels, protecting the vessel from excessive stretch. Fibroblasts are also known for their role in wound healing, providing repair of damaged tissue by facilitating fibrosis, attracting leukocytes to clear the damaged tissue and resolve fibrosis (Shinde and Frangogiannis, 2014). Persistent fibrosis can on the other hand induce further organ damage. Perivascular fibrosis by myofibroblasts is considered a pathological feature, for instance in cardiac hypertrophy (Dai, et al., 2012). Fibroblasts also have extensive NOX-mediated signaling. They are responsive to endothelial cell-derived cues (Adiarto, et al., 2012), and strongly influence VSMC behavior (Bazzoni, 2006; Duan, et al., 2010). Because of the limited data of Rac1 in adventitial fibroblasts, we extended the search to relevant Rac1 data in different type of fibroblasts.

Fibrosis

The importance of Rac1 in tissue repair is illustrated by the delayed wound healing response in mice with a specific deletion of Rac1 in fibroblasts. These mice revealed reduced collagen production and fibrosis (Liu, et al., 2009). In cultured Rac1-deficient fibroblasts, adhesion, spreading, and migration was significantly inhibited (Liu, et al., 2009). Rac1-deficient fibroblasts showed decreased myofibroblast formation by reduced SM α-actin (stress fiber) expression as well as matrix contraction (pulling force). In addition, in vivo and in vitro, Rac1-deficient fibroblasts showed reduced generation of ROS (Liu, et al., 2009). In a hepatic fibrosis model, Rac1 caused NOX1 activation, NOX4 up-regulation, ROS generation, and subsequent liver fibrosis, showing again the involvement of the NOX (Aoyama, et al., 2012). Myofibroblast conversion is characterized by increased contractility of the cells, which coincides with decreased ciliation of the cell. Cilia are membrane protrusions sensing environmental mechanical cues. Rac1 enhances deciliation via transforming growth factor-beta (TGFβ), NOX4 and ROS, and sustained phosphorylation of MLC, which promoted the
contractile myofibroblast phenotype (Rozycki, et al., 2014). There is an important role for Integrin-linked kinase (ILK) in myofibroblast formation and wound repair, since fibroblast-restricted inactivation of ILK in mice resulted in decreased myofibroblast conversion and decreased repair (Blumbach, et al., 2010). ILK is recruited to integrin β1 in focal adhesions, mediating the communication between the cell and the extracellular matrix. When this communication is disturbed in ILK-deficient fibroblasts, decreased SM α-actin, TGFβ, and Rac1 activity is observed, while having enhanced RhoA activity. Exogenous TGFβ could restore myofibroblast formation, cell shape and directional migration (Blumbach, et al., 2010).

Fibroblasts also display AT1R-mediated signaling, which is one of the main activators of NOX signalling. AT1R activation induces myofibroblast formation and fibrosis, which is strongly dependent on NOX and ROS (Adam, et al., 2010). A prominent downstream gene of AT1R signaling is TGFβ, which induces connective tissue growth factor (CTGF) expression, which in turn induces matrix (such as collagen) production (Che, et al., 2008). Rac1 is involved in the production of CTGF in fibroblasts, which could be completely abolished by AT1R antagonist losartan (generic name) (Cotton, et al., 2007; Loirand and Pacaud, 2010; Tsai, et al., 2008). AT1R-mediated signaling in fibroblasts also induced a pro-inflammatory milieu, by the production of cytokine interleukin-6 and chemokine MCP-1 (Tieu, et al., 2011), which will provoke an immune response contributing to wound repair or when excessive, harming the repair response. Rac1 activation may easily be involved in this pro-inflammatory gene transcription pattern upon AT1R-mediated signal transduction, since this is also described for other cell types (Marinkovic, et al., 2014b; Marinkovic, et al., 2014a). Collectively, it shows that Rac1 is one of the central mediators of wound healing by promoting fibrosis.

Proliferation and migration

Enhanced proliferation of adventitial fibroblasts is detrimental, such as in hypoxia-induced pulmonary hypertension. Excessive fibroblast proliferation under these conditions was caused by a Rac1/p38 signaling cascade (Carlin, et al., 2007). Different statins inhibited
adventitial fibroblast proliferation, and reduced p38 activation. The statin-mediated effect can be mimicked by inhibition of GGTase-1 or Rac1 (Carlin, et al., 2007). Thus, similar as for the VSMC, statins inhibited proliferation via inhibition of GGTase-1 activity, probably preventing Rac1 activation. Rats infused with angiotensin-II (activating AT1R) displayed not only increased levels of active Rac1, collagen and fibrosis, but also presented enhanced proliferation and phosphorylation of signal transducer and activator of transcription 3 (STAT3), which was efficiently dampened by a AT1R-blocker or statin (Tieu, et al., 2011; Tsai, et al., 2008). Activation of AT1R-mediated JAK/STAT signaling was Rac1-dependent, and the statin blocked Rac1 localization to the membrane (Tsai, et al., 2008). A Rac1-dependent STAT-3-mediated proliferative response was also observed in our gut epithelial cell study, where cyclin D1 was ultimately increased (Marinkovic, et al., 2014a).

Active Rac1 is described to bind STAT-3 and seems to target it to become phosphorylated and activated (Simon, et al., 2000). This complex formation between Rac1 and STAT3 is not always found, however, RhoA, Rac1, and Cdc42 can all mediate phosphorylation and nuclear translocation of STAT3, independently of the interleukin-6 pathway (Debidda, et al., 2005). In embryonic fibroblasts, tumor suppressor p53 stimulates actin cytoskeleton remodeling and limits lamellipodia formation, since in the absence of p53 these features were disturbed and migration was induced. P53 depletion induced STAT3, and inhibition of Rac1, Arp2/3 or NFkB suppressed STAT3 and diminished lamellipodia formation (Guo, et al., 2014). In addition, a role for STAT3 independent of its transcriptional activity is found. In rescue experiments with STAT3-deficient fibroblasts, introduction of STAT3 reduced the high activation level of Rac1 in these cells. Cytoplasmic STAT3 could bind to and thereby inhibit Rac1-specific GEF β-PIX, and normalize Rac1 activity (Teng, et al., 2009). GEF β-PIX plays a key role in the negative regulation of focal adhesion maturation and the promotion of lamellipodial protrusion formation, thus driving cell migration (Kuo, et al., 2011). Collectively, it shows that Rac1/STAT3 activation is involved in fibroblast proliferation and migration, with different mechanisms of STAT3 regulation, and a negative feedback loop to inactivate Rac1.
Apart from angiotensin-II, bFGF is also known to induce proliferation and migration. Inhibition of Rac1 blocks bFGF-induced fibroblast migration, whereas inhibition of RhoA does not. Here, Rac1 activation was dependent on PI3-kinase (PI3K) activation and Rac1-induced downstream JNK activation. Inhibition of each step could inhibit fibroblast migration (Kanazawa, et al., 2010). In endothelial cells and fibroblasts, Rac1-mediated JNK activation has been reported and thus also seems a common Rac1 activation pathway. In line with these data, high glucose could inhibit bFGF-induced Rac1/JNK-dependent fibroblast migration, which is presumably the cause of delayed wound repair in diabetic patients (Xuan, et al., 2014). On the other hand, a neurotrophic factor called nerve growth factor (NGF) has been described to accelerate wound healing. NGF promoted the migration, but not proliferation, of dermal fibroblasts, by inducing an increase in the activity of PI3K/Akt/Rac1/JNK/ERK (Chen, et al., 2014). Blockade with their specific inhibitors impaired the NGF-induced migratory response of fibroblasts.

Clearly, based on the above presented data, targeting Rac1 for inhibition or activation in fibroblasts, may depend on the type of pathology to be treated. While Rac1-mediated fibrosis and migration/hyperplasia is necessary for wound healing, it is undesired when there is excessive fibrosis or hyperplasia, which disturbs the normal function of the vasculature and/or organ perfusion.

PERSPECTIVES

Rac1 activation displays unique (e.g. barrier function, contraction, fibrosis) and common (e.g. proliferation and migration) functions in each cell type of the vessel wall, and can be activated by many different factors (Table 1). A cellular response to environmental cues is important to maintain homeostasis of the vasculature, involving kinases for a fast and transcription factors for a slower response (Table 1). Thus the main functions of Rac1 are necessary to restore this balance. This review shows that many of the Rac1 functions can be attributed to NOX activity and ROS production in the vasculature.
TABLE 1.
Summary of the factors leading to Rac1 activation, and downstream signaling, with enhanced transcription factor activity in endothelial cells, smooth muscle cells and fibroblasts, which have been discussed in the current manuscript. In addition, a number of antagonizing factors are stated.

<table>
<thead>
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<th>Agonists Rac1 activation</th>
<th>Kinases</th>
<th>Transcription factors</th>
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<td><strong>Endothelial cell</strong></td>
<td><strong>Vaso active peptides:</strong>&lt;br&gt;Angiotensin-II&lt;br&gt;Endothelin-1&lt;br&gt;ANP&lt;br&gt;<strong>Pro-inflammatory protein:</strong>&lt;br&gt;TNFα&lt;br&gt;<strong>Lipid(-like) molecules:</strong>&lt;br&gt;Aldosterone&lt;br&gt;Sphingosine-1-phosphate&lt;br&gt;Bile acids&lt;br&gt;<strong>Growth factor:</strong>&lt;br&gt;VEGF</td>
<td><strong>Downstream of Rac1 activation:</strong>&lt;br&gt;PAK1&lt;br&gt;JNK&lt;br&gt;FAK&lt;br&gt;MAPK&lt;br&gt;Prevents Rac1 activation:&lt;br&gt;ERK5</td>
<td><strong>Downstream of Rac1 activation:</strong>&lt;br&gt;NFκB&lt;br&gt;AP-1&lt;br&gt;ATF2&lt;br&gt;Prevents Rac1 activation:&lt;br&gt;KLF2&lt;br&gt;Nrf2</td>
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<td><strong>Smooth muscle cell</strong></td>
<td><strong>Vaso active peptides:</strong>&lt;br&gt;Angiotensin-II&lt;br&gt;Endothelin-1&lt;br&gt;<strong>Growth factors:</strong>&lt;br&gt;PDGF&lt;br&gt;bFGF&lt;br&gt;IGF-1</td>
<td><strong>Downstream of Rac1 activation:</strong>&lt;br&gt;PAK1&lt;br&gt;JNK&lt;br&gt;ERK1/2&lt;br&gt;PKG&lt;br&gt;MLCK (inhibition)</td>
<td>Prevents Rac1 activation:&lt;br&gt;Nrf2</td>
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<td><strong>Fibroblast</strong></td>
<td><strong>Vaso active peptide:</strong>&lt;br&gt;Angiotensin-II&lt;br&gt;<strong>Pro-inflammatory protein:</strong>&lt;br&gt;TNFα&lt;br&gt;<strong>Growth factors:</strong>&lt;br&gt;bFGF&lt;br&gt;TGFβ&lt;br&gt;NGF</td>
<td><strong>Downstream of Rac1 activation:</strong>&lt;br&gt;P38&lt;br&gt;JAK&lt;br&gt;PI3K&lt;br&gt;JNK&lt;br&gt;Upstream of Rac1 activation:&lt;br&gt;ILK</td>
<td><strong>Downstream of Rac1 activation:</strong>&lt;br&gt;NFκB&lt;br&gt;STAT3&lt;br&gt;Prevents Rac1 activation:&lt;br&gt;STAT3 (negative feedback)</td>
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In endothelial cells, Rac1 activation promoted leukocyte transmigration, increased barrier function and VEGF-induced migration (Figure 3). Leukocyte influx is important for damage repair of tissue. Yet, excessive leukocyte accumulation will progress organ damage. Likewise, a VEGF-induced angiogenic response is beneficial to restore blood flow in deprived tissues. However, enhanced angiogenesis may also induce diabetic retinopathy, unstable atherosclerotic lesions, or cancer progression. Thus, for the clinical outcome, Rac1 activation and inhibition are in a delicate balance with each other. Therefore, complete Rac1 inhibition does not seem an attractive therapeutic target. In VSMC, Rac1 promotes proliferation, migration and modulates contraction (Figure 3). VSMC proliferation/migration is essential for vascular repair, yet excessive proliferation/migration may lead to restenosis after angioplasty or pulmonary hypertension. But even in the pathological condition of established atherosclerosis, VSMC proliferation may be beneficial, since it stabilizes the atherosclerotic lesion and makes it less prone to rupture. As for blood pressure regulation, long term hypo- or hypertension should be avoided. In fibroblasts, Rac1 is involved in fibrosis, proliferation and migration (Figure 3). While wound healing is dependent on these functions, the fibrotic area with fibroblast hyperplasia should be resolved eventually to restore organ function. Thus, also for these cells, a balancing act of Rac1 inhibition is demanded.

Interestingly, chronic Rac1 modulation as (indirect) therapeutic strategy has been used in the clinic already for decades, by using the cardioprotective statins (Table 2). However, also inflammatory bowel disease (IBD) patients use Rac1-inhibitory medication chronically, since the immunosuppressive drug azathioprine and its derivatives 6-mercaptopurine and 6-thio-GDP (and 6-thio-GTP) are (probably direct) Rac1 inhibitors in different cell types (Marinkovic, et al., 2014a) (Table 2). These drugs have been used in the clinic for already 60 years, thus proven to be effective and safe. Key to their effectiveness is probably that they do not inhibit all Rac1, so the Rac1 balance is restored. Recently, there has been increasing interest in Rac1 inhibitors from the cancer field (Bid, et al., 2013). Apart from the role of angiogenesis in cancer, cancer cells themselves also display high Rac1 activity causing
cancer progression. Thus, Rac1 inhibition can be a potential therapeutic approach for many
diseases, especially since the vasculature plays a key role in most diseases. Nonetheless,
since Rac1 is also essential in activation of immune cells, general Rac1 inhibition may not
always be harmless. So, if it is required to block (or activate) Rac1 in a specific cell type, cell-
specific GEFs, GAPs or GDIs that regulate local Rac1 activity may be targeted. However,
before reaching this point in the clinic, many research still has to be performed on these
upstream modifiers of Rac1 activity. Until then, the Rac1-inhibitory drug azathioprine may be
of potential interest in other diseases than autoimmune disorders, since there is abundant
clinical experience with this drug.

AUTHORSHIP CONTRIBUTIONS:
Performing migration experiment and wrote a manuscript: G. Marinković
Performing FRET biosensor Rac1 activity experiment: N. Heemskerk
Contributed to the writing of the manuscript: J.C. van Buul
Contributed to the writing of the manuscript writing: V. de Waard
TABLE 2.
The two best described drugs with Rac1 inhibitory capacity are the cholesterol lowering statins and the immunosuppressive drugs azathioprine and 6-mercaptopurine. The mechanisms of Rac1 inhibition are described.

<table>
<thead>
<tr>
<th>Mechanism Rac1 inhibition</th>
<th>Statins</th>
<th>Azathioprine or 6-Mercaptopurine</th>
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<tr>
<td><strong>Indirect Rac1 inhibition:</strong></td>
<td>1. Down-regulation of the cholesterol synthesis by statins decreases the intermediate isoprenoid metabolite geranylgeranyl pyrophosphate, which is the substrate for the enzyme geranylgeranyl transferase-1 (GGTase-1). GGTase-1 transfers geranylgeranyl on to specific motifs on RhoA and Rac1, altering GTPase activity. 2. Statins induce small GTP-binding protein dissociation stimulator (SmgGDS) upregulation, which leads to enhanced Rac1 degradation. 3. Statin treatment induces shear stress-responsive transcription factor KLF2 expression and thereby ERK5 activation, which strongly decreases PAK1 mRNA and protein expression and thereby Rac1-mediated signaling.</td>
<td>1. Azathioprine- or 6-mercaptopurine-derived metabolites 6-thio-GDP and 6-thio-GTP are suspected to bind directly to Rac1 and block Rac1 activation. 2. Note: Both azathioprine and 6-mercaptopurine disturb the purine biosynthesis pathway. Thus next to the GMP pathway, also 6-thio-AMP, -ADP and –ATP is generated, which are not involved in Rac1 inhibition.</td>
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REFERENCE LIST


Marinkovic G, Kroon J, Hoogenboezem M, Hoeben KA, Ruiter MS, Kurakula K, Otermin R, I, Vos M, de Vries CJ, van Buul JD and de W, V (2014b) Inhibition of GTPase Rac1 in endothelium by 6-


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FIGURE LEGENDS

Figure 1. *Local Rac1 activity at the leading edge of a migrating endothelial cell.*
An endothelial cell is transfected with the Rac1 DORA-FRET biosensor and recorded in time to study spatio-temporal Rac1 activity during endogenous protrusion formation. Arrowheads show high FRET, i.e. active Rac1 regions, that coincide with protrusive activity. Bar, 20 µm.

Figure 2. *Rac1-mediated migration is inhibition by 6-mercaptopurine and 6-thio-GDP.*
Rac1 activity is induced in HUVEC endothelial cells by TNFα, and inhibited by 6-MP, 6-T-GDP, 6-T-GTP and TRIO inhibitor ITX-3. In a scratch wound assay, HUVEC migration was tracked in 6 cells per condition. Path length and directional migration was inhibited by 6-MP and 6-T-GDP after 32 hours.

Figure 3. *Functions of Rac1 in the different vascular cell types.*
Migration; overexpression of GFP-tagged TRIO-GEF (green), activating endogenous Rac1, results in polarized Rac1 activity at the leading edge in the direction of migration (arrows). Barrier; an endothelial cell monolayer with adherens cell-cell junction protein VE-cadherin (green). Leukocyte transmigration; scanning electron microscopy shows an apical membrane protrusion from the endothelium surrounding an adherent leukocyte. Using fluorescent microscopy, the F-actin (green) endothelial cell protrusion engulfs a purple adherent cell. Proliferation; proliferating (endothelial) cells with VE-cadherin (green), F-actin (red) and nuclear (blue) staining. Contraction/relaxation; schematic representation of contractile and relaxed muscle cell, with sliding actin-myosin filaments. Fibrosis; perivascular fibrosis (blue) observed after pressure overload in the heart (+), compared to control (-).
Figure 1.
Figure 2.

Rac1 activity

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Migration towards scratch

Path length (µm)

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<td>Path length</td>
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P < 0.001
Figure 3.