

RGS5 promotes arterial growth during arteriogenesis

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Abstract

Arteriogenesis—the growth of collateral arterioles—partially compensates for the progressive occlusion of large conductance arteries as it may occur as a consequence of coronary, cerebral or peripheral artery disease. Despite being clinically highly relevant, mechanisms driving this process remain elusive. In this context, our study revealed that abundance of regulator of G-protein signalling 5 (RGS5) is increased in vascular smooth muscle cells (SMCs) of remodelling collateral arterioles. RGS5 terminates G-protein-coupled signalling cascades which control contractile responses of SMCs. Consequently, overexpression of RGS5 blunted $G\alpha_{q/11}$ -mediated mobilization of intracellular calcium, thereby facilitating $G\alpha_{12/13}$ -mediated RhoA signalling which is crucial for arteriogenesis. Knockdown of RGS5 evoked opposite effects and thus strongly impaired collateral growth as evidenced by a blockade of RhoA activation, SMC proliferation and the inability of these cells to acquire an activated phenotype in RGS5-deficient mice after the onset of arteriogenesis. Collectively, these findings establish RGS5 as a novel determinant of arteriogenesis which shifts G-protein signalling from $G\alpha_{q/11}$ -mediated calcium-dependent contraction towards $G\alpha_{12/13}$ -mediated Rho kinase-dependent SMC activation.

Keywords arteriogenesis; G-protein; remodelling; RGS5; vascular smooth muscle cells

Subject Categories Vascular Biology & Angiogenesis

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Introduction

In industrialized countries, cardiovascular diseases by far constitute the leading cause of morbidity and death. Arteriosclerosis often leads to progressive occlusion of large conductance arteries causing severe ischaemia in the affected tissues (Meier *et al*, 2012; Schaper,

2012). Given the clinical relevance of this disease, significant efforts have been made to attenuate consequences of tissue ischaemia by stimulating angiogenesis—the growth of new blood vessels from pre-existing ones. However, local blood supply strictly depends on the diameter of arterioles or arteries to which a capillary network is connected. Consequently, adaptive growth of pre-existing collateral arterioles bypassing an occluded conductance artery appears to be a prerequisite to compensate for the consequences of arteriosclerosis. Recently, a meta-analysis revealed that patients who suffer from coronary artery disease but develop a collateral circulation have a 36% reduced risk of mortality (Meier *et al*, 2012; Schaper, 2012) and thus underlines clinical relevance of this vascular remodelling process. It is referred to as arteriogenesis and basically driven by a change in biomechanical forces to which the vessel wall is exposed. Upon occlusion of a conductance artery, blood flow in collateral arterioles increases and elicits the release of nitric oxide (NO) from endothelial cells and subsequent relaxation of vascular smooth muscle cells (SMCs) (Unthank *et al*, 1996). Through the accompanying dilatation of the collateral arterioles, wall stress is increased which in turn promotes proliferation and pro-inflammatory responses of endothelial cells and SMCs (Heil *et al*, 2006; Demicheva *et al*, 2008). As a consequence, these cells express adhesion molecules (e.g. vascular cell adhesion molecule 1, VCAM-1) and release chemokines such as MCP-1 triggering the recruitment of mononuclear leucocytes—a prerequisite for the subsequent structural changes of the vessel wall (Heil & Schaper, 2004; Demicheva *et al*, 2008). In the long run, these processes lead to the enlargement and corkscrew-like appearance of the collaterals and finally to their transformation into arteries which are capable of bypassing the occluded artery.

A rate limiting and therefore crucial step of this remodelling process is the shift of the SMC phenotype from the resting, differentiated to the activated, proliferative state. Recently, it has been shown that balancing of G-protein signalling is crucial for the control of the SMC phenotype and thus vascular remodelling processes (Althoff *et al*, 2012). Yet, the intracellular effects of G-protein-dependent signalling critically depend on the activity of a group of regulatory proteins known as regulators of G-protein

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signalling (RGS) (Wieland & Mittmann, 2003; Wieland *et al*, 2007). These proteins contain a GTPase-activating protein (GAP) domain which accelerates hydrolysis of GTP upon binding to the corresponding $G\alpha$ subunit (Berman *et al*, 1996; Ross & Wilkie, 2000). As a consequence, GPCR-induced signalling is effectively suppressed at high expression levels of these proteins and disconnected from the downstream signal transduction cascade.

Among the various RGS proteins, expression of RGS5 seems to be primarily restricted to pericytes and vascular SMCs (Adams *et al*, 2000). It is up-regulated in the developing vasculature (Cho *et al*, 2003) and in pericytes during wound healing or tumour angiogenesis (Berger *et al*, 2005) but down-regulated in SMCs present in advanced atherosclerotic lesions (Li *et al*, 2004). RGS5-deficient mice appear to be hypotensive (Cho *et al*, 2008; Nisancioglu *et al*, 2008) as compared to their wild-type littermates although initial findings with mice made RGS5-deficient by non-genetic strategies had suggested the opposite (Gu *et al*, 2009). Thus, the impact of RGS5 on the function of vascular SMCs remains elusive.

As G-protein signalling appears to be pivotal for vascular remodelling processes (Althoff *et al*, 2012), we hypothesized that RGS5 may similarly affect vascular remodelling during arteriogenesis by modulating G-protein activity. Consequently, we (i) analysed the expression of RGS5 during arteriogenesis, (ii) identified cellular mechanisms linked to a change in RGS5 expression and (iii) investigated the consequences on arteriogenesis evoked by the loss of RGS5.

Results

Increased expression of RGS5 in collateral arteriolar SMCs during arteriogenesis

Arteriogenesis was induced by ligation of the femoral artery of C57BL/6 wild-type mice stimulating the growth of collateral arterioles in the hindlimb (Fig 1A). Immunofluorescence analyses of the remodelling collaterals 7 days post ligation revealed a marked rise in RGS5 abundance in medial SMCs (Fig 1B). A moderate but significant increase of RGS5 abundance was also observed 3 days upon induction of arteriogenesis (Supplementary Fig S1). Likewise, exemplary microarray analyses of mRNA isolated from growing collaterals in the brain indicated a twofold increase in RGS5 expression (data not shown). To identify whether the change in smooth muscle cell RGS5 abundance during vascular remodelling is associated with a loss of the differentiated phenotype, we additionally examined the expression of myocardin. This transcriptional co-activator maintains the expression of SMC marker proteins and is degraded during biomechanically induced remodelling processes (Pfisterer *et al*, 2012). In line with this, arteriogenesis led to a notable decline of myocardin abundance in the medial SMCs (Fig 1C).

Nitric oxide and cyclic stretch—critical determinants of arteriogenesis—increase RGS5 protein levels

Shear stress-induced release of nitric oxide (NO) from endothelial cells plays an important role in the onset and progression of arteriogenesis (Yu *et al*, 2005; Troidl *et al*, 2009b). Thus, we assumed that continuous exposure of vascular SMCs to NO stimulates RGS5

expression. In fact, exposure of human-cultured SMCs to the NO donor NONOate resulted in a delayed but prominent rise in RGS5 mRNA levels over 96 h (Fig 2A). The same effect was achieved with the cell-permeable cyclic GMP analog 8pCPT-cGMP (Fig 2B), pointing to a role of the soluble guanylyl cyclase/protein kinase G pathway in RGS5 expression downstream of the exposure to NO. Finally, NO-mediated up-regulation of RGS5 expression in the medial SMCs was also confirmed by immunofluorescence analyses of isolated mouse arteries treated with NONOate for up to 72 h (Fig 2C).

Likewise, exposure of human-cultured SMCs to cyclic stretch—a biomechanical stimulus that mimics an increase in wall stress which is another highly relevant determinant of arteriogenesis (Demicheva *et al*, 2008)—also significantly increased RGS5 abundance (Fig 2D).

RGS5 modulates GPCR agonist-evoked mobilization of intracellular calcium in cultured SMCs

Recently, baseline signalling mediated by the G proteins $G\alpha_{q/11}$ and $G\alpha_{12/13}$ has been shown to control phenotype changes of SMCs during vascular remodelling processes (Althoff *et al*, 2012). To investigate possible functional consequences of the rise in RGS5 protein abundance for SMCs during arteriogenesis, G-protein-dependent signalling cascades which are directly or indirectly affected by RGS5 (e.g. $G\alpha_{q/11}$ - and $G\alpha_{12/13}$ -dependent signalling) were analysed. To this end, human-cultured SMCs were transduced with an adenoviral RGS5 or GFP control construct and treated with sphingosine-1-phosphate (S1P). This stimulus activates both $G\alpha_{q/11}$ (calcium)- and $G\alpha_{12/13}$ (RhoA)-dependent signalling pathways and therefore was utilized to assess the general activity of the corresponding G-proteins in the presence of RGS5. Overexpression of RGS5 (adenoviral transduction efficiency up to 70–90%) in fact blunted the $G\alpha_{q/11}$ -dependent increase in intracellular calcium evoked by S1P (Fig 3A), angiotensin II (Supplementary Fig S2A) or the $G\alpha_{q/11}$ -specific agonist bradykinin (Supplementary Fig S3). In agreement, loss of RGS5 reinforced mobilization of intracellular calcium in response to a stimulation with S1P (Fig 3B) or angiotensin II (Supplementary Fig S2B). Correspondingly, isolated mesenteric artery segments from RGS5-deficient mice revealed an enhanced contractile response (calcium-dependent) to increasing concentrations of the $G\alpha_{q/11}$ -specific agonist norepinephrine (Fig 3C).

Overexpression of RGS5 in cultured SMCs reinforces GPCR agonist-evoked stress fibre formation via the Rho kinase pathway

RGS proteins do not simply abrogate but modulate G-protein signalling in the cell. Turning off $G\alpha_{q/11}$ -mediated mobilization of intracellular calcium by R4 RGS proteins such as RGS5 should not affect the S1P/ $G\alpha_{12/13}$ -dependent activation of the Rho kinase pathway in RGS5-overexpressing SMCs. Subsequent experiments analysing actin remodelling as a readout for Rho kinase activity in human-cultured SMCs revealed that RGS5 overexpression already reinforced stress fibre formation in these cells without further stimulation (Fig 4A, B and G). Stimulation with S1P increased stress fibre formation even further (Fig 4C, D and G). The RGS5-dependent stress fibre formation was sensitive to the Rho kinase inhibitor Y27632 (Fig 4E, F and G). Additionally, direct analyses of RhoA

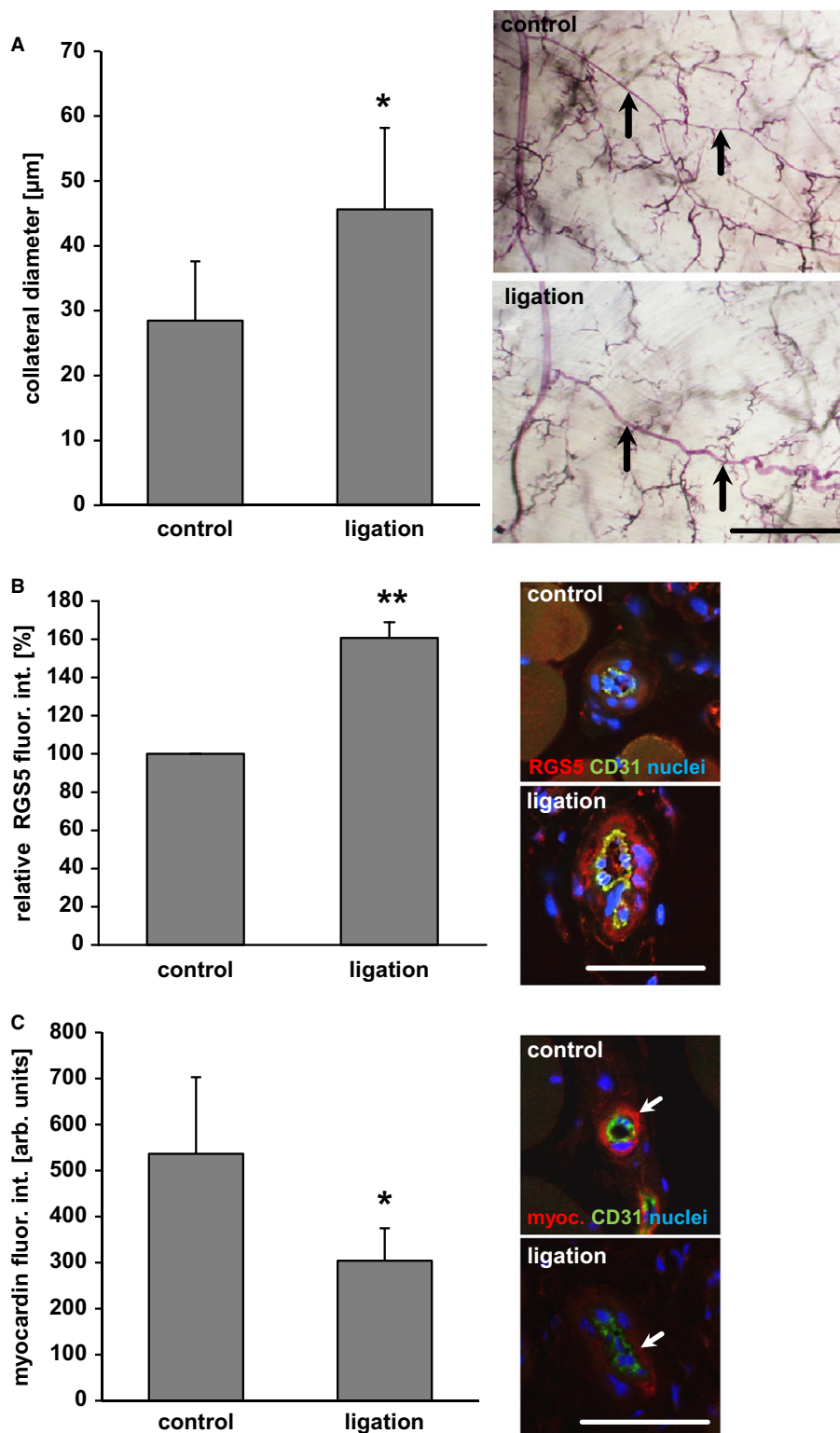


Figure 1. RGS5 protein abundance is increased in remodelling collateral arterioles.

A Arteriogenic remodelling of collateral arterioles in the mouse hindlimb was analysed 7 days post ligation of the femoral artery. Growth of the remodelling arterioles (arrows) is significantly increased during this period (* $P < 0.05$ versus control, $n = 5$; scale bar: 1 mm).

B Quantification of RGS5-specific immunofluorescence intensity (red fluorescence staining) in the SMCs of these arterioles reveals a significant rise in RGS5 abundance over this period (** $P < 0.05$ versus control, $n = 5$, analysing up to 3 collaterals per animal; scale bar: 50 µm).

C Under these conditions, the fluorescence intensity of myocardin (red), detected in the collateral media, was significantly decreased (* $P < 0.05$ versus control, $n = 5$; arrows: endothelial cell; CD31 staining: green; scale bar: 50 µm).

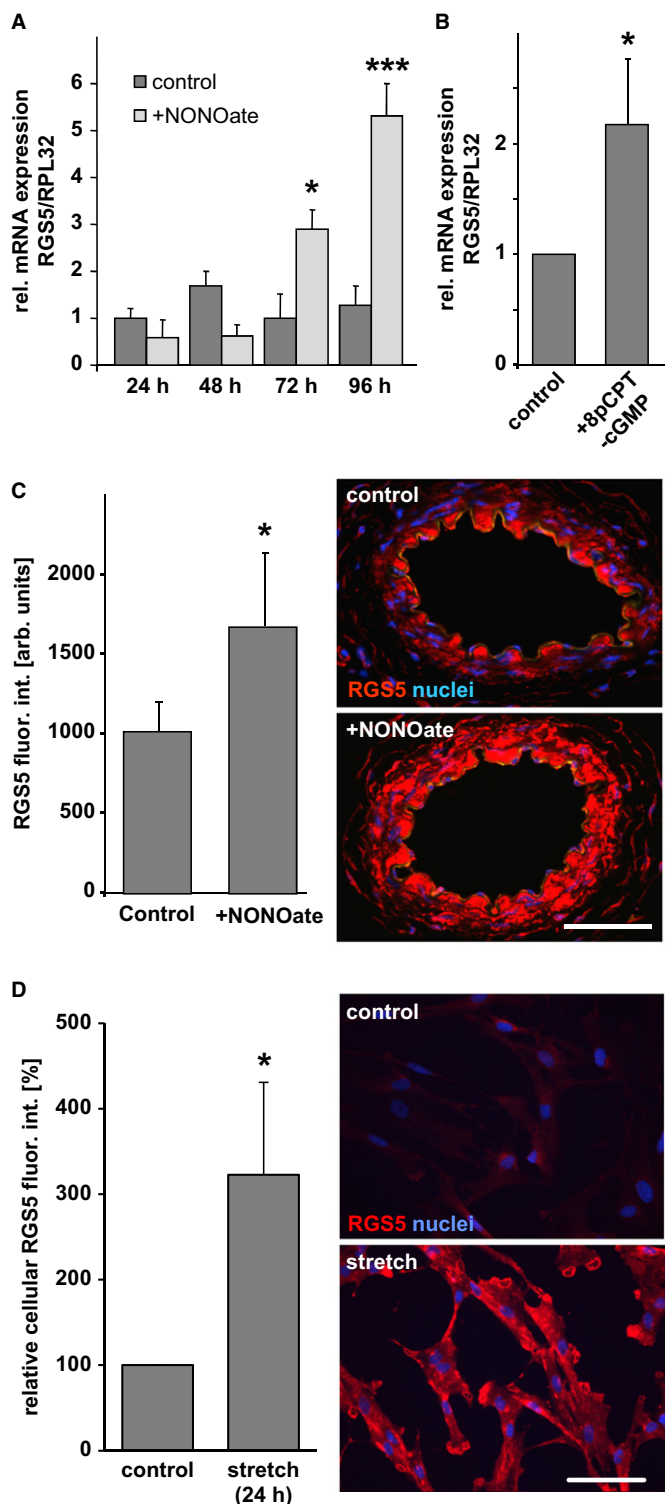


Figure 2. Nitric oxide and cyclic stretch—critical determinants of arteriogenesis—increase RGS5 protein levels.

A, B Cultured human umbilical artery SMCs were exposed to the NO donor NONOate (100 μ M) or the cell-permeable cyclic GMP analog 8pCPT-cGMP (100 μ M) for up to 96 h. Subsequent changes in RGS5 mRNA levels were quantified by real-time PCR analysis (* P < 0.05 versus 0 h; *** P < 0.001 versus 0 h, n = 3; expression of the housekeeping gene RPL32 was utilized as internal standard).

C Treatment (72 h) of isolated femoral artery segments with NONOate also reveals a significant rise in RGS5-specific immunofluorescence (red fluorescence staining) in the medial SMCs (* P < 0.05 versus 0 h, n = 3, nuclei were counterstained with DAPI (blue fluorescence staining), scale bar: 50 μ m).

D Immunofluorescence analyses of control cells cultured under static and stretch-stimulated conditions (0.5 Hz, 0 to 15%) HUASMCs showed an increase in RGS5-specific (red) fluorescence (* P < 0.05 versus control, n = 3; RGS5 abundance in the cells was quantified by determining the fluorescence intensity in at least five microscopic fields of view per condition. Control levels were set to 100%; scale bar: 100 μ m).

accumulation upon S1P stimulation (Fig 4I–M) and diminished the S1P-dependent activation of RhoA (Fig 4N) without affecting baseline proliferation or apoptosis (Supplementary Figs S5 and S6). Furthermore, enhanced stress fibre formation in S1P-stimulated RGS5-overexpressing SMCs was blocked by RGS-Lsc—a specific inhibitor of $G\alpha_{12/13}$ signalling (Wuertz *et al*, 2010)—corroborating that the RGS5-dependent RhoA activation occurred via a $G\alpha_{12/13}$ -regulated pathway (Supplementary Fig S7). Correspondingly, the abundance of globular actin is decreased in RGS5-overexpressing SMCs (Supplementary Fig S8). Collectively, these data imply that RGS5 attenuates $G\alpha_{q/11}$ -dependent signalling while promoting $G\alpha_{12/13}$ -mediated RhoA signalling in vascular SMCs. Exemplary profiling of the activation, that is, phosphorylation of a set of kinases after RGS5 overexpression in cultured human SMCs revealed that the activity of well-known RhoA-regulated protein kinases such as p38a (Zhang *et al*, 1995; Dubroca *et al*, 2005), JNK (Teramoto *et al*, 1996), HSP27 (Dubroca *et al*, 2005) and FAK (Zhang *et al*, 2012) were increased twofold, while RhoA-independent kinases such as ERK1/2 (Zuckerbraun *et al*, 2003) were not affected (data not shown).

RGS5 deficiency impairs the growth of collateral arterioles during arteriogenesis

It is well established that RhoA signalling is crucial for the growth of collateral vessels during arteriogenesis (Eitenmuller *et al*, 2006; Troidl *et al*, 2009a). Based on the aforementioned findings, we therefore hypothesized that the absence of RGS5-dependent amplification of the RhoA pathway will hamper arteriogenesis. To pursue this idea, RGS5-deficient mice were utilized which display no obvious defects in the architecture, morphology and development of the vasculature (Supplementary Fig S9) (Nisancioglu *et al*, 2008) or ischaemia-induced angiogenesis (Supplementary Fig S10). As evidenced by telemetric blood pressure recordings, these mice have a slightly but not significantly increased mean arterial blood pressure (Supplementary Fig S11) as compared to wild-type mice but show normal cardiac functions (data not shown). Analyses of arteriogenic remodelling in RGS5-deficient mice revealed that the blood flow recovery was in fact severely impaired (Fig 5A). As the overall perfusion level of the lower limb does not clearly distinguish between angiogenesis and arteriogenesis, the diminished growth of

activity in SMCs revealed that the S1P-induced formation of RhoA-GTP was much more pronounced in SMCs overexpressing RGS5 (Fig 4H).

In line with this, knockdown of RGS5 expression in human-cultured SMCs through RNA interference (Supplementary Fig S4) diminished the formation of stress fibres at baseline, inhibited their

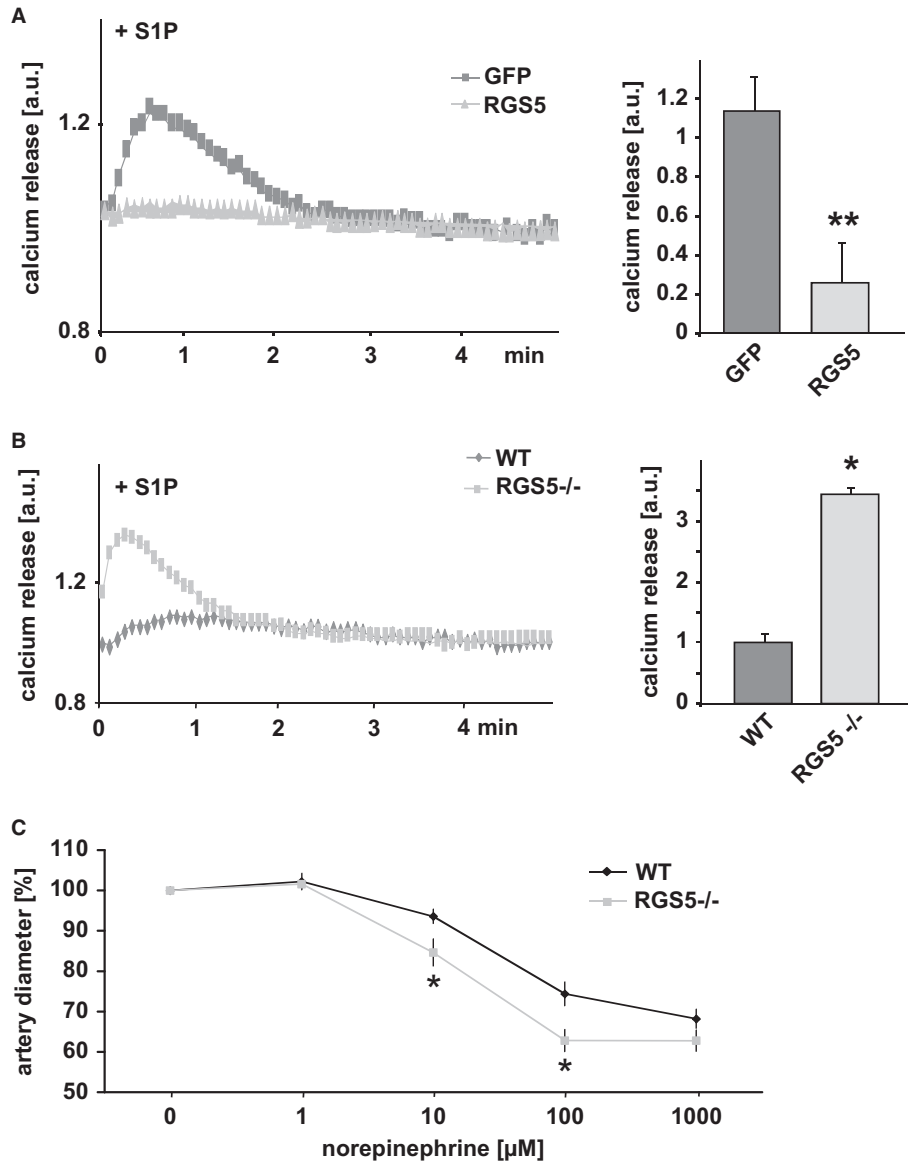
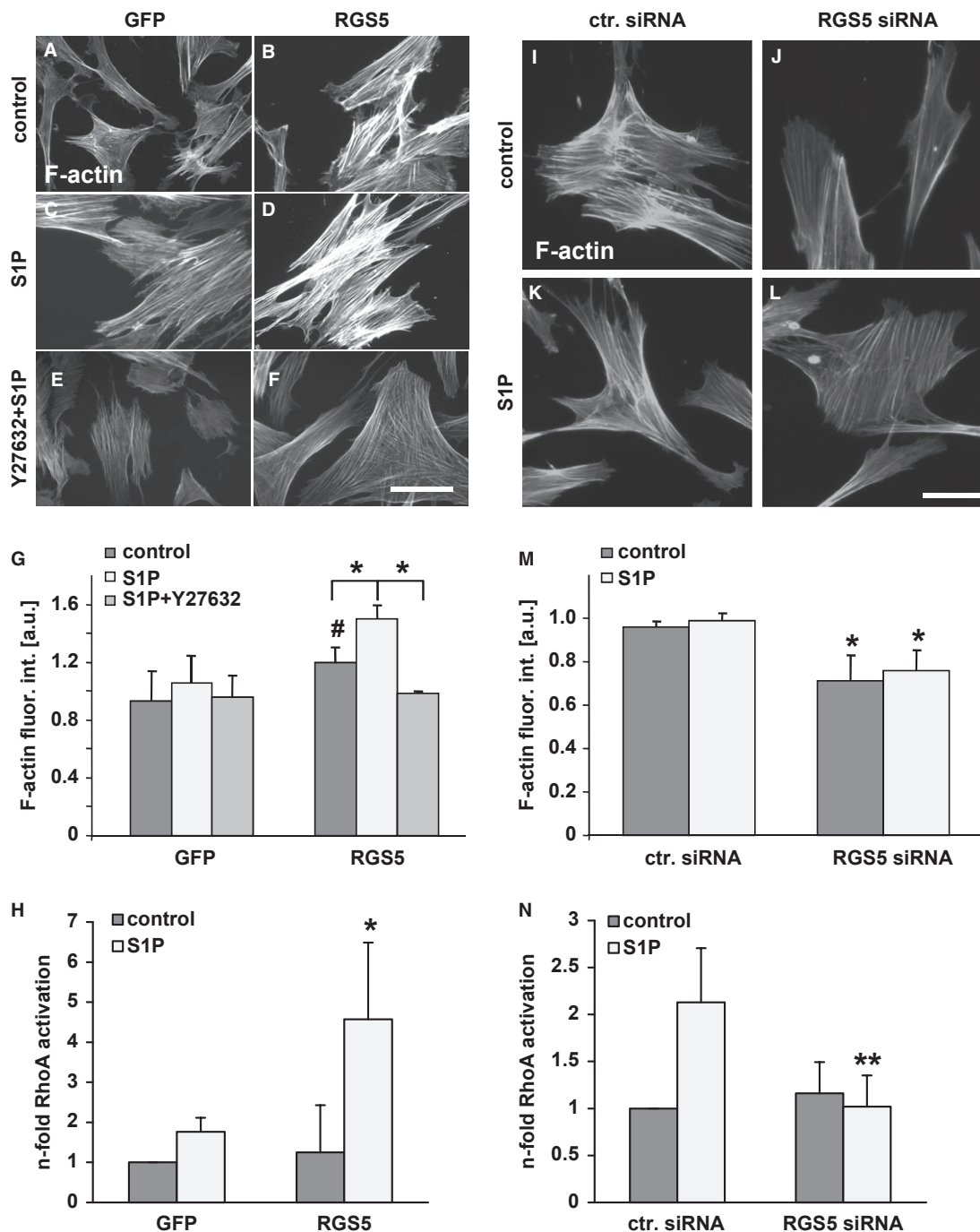


Figure 3. GPCR agonist-induced mobilization of intracellular calcium and arterial constriction is enhanced in RGS5-deficient SMCs.

- A** Cultured human umbilical artery SMCs were transduced with an adenoviral control (GFP) or RGS5 expression vector (RGS5) and then loaded with the calcium-sensing fluorophore Rhod-4 AM. Sphingosine-1-phosphate (S1P, 10 μ M) elicits a rapid but transient rise in intracellular calcium in GFP-expressing cells which is virtually abrogated in cells overexpressing RGS5 (** P < 0.01 versus GFP-expressing cells, n = 4; calcium transients were quantified by determining the area under the curve).
- B** Cultured arterial SMCs derived from wild-type (WT) or RGS5-deficient mice (RGS5^{-/-}) were loaded with the calcium-sensing fluorophore Rhod-4 AM. S1P moderately increased the intracellular calcium concentration in control SMCs. This effect was significantly reinforced in RGS5-deficient SMCs (* P < 0.05 versus control, n = 4).
- C** Similarly, mesenteric artery segments of RGS5-deficient mice responded with a more pronounced constriction to increasing concentrations of norepinephrine as compared to segments of wild-type (WT) animals (* P < 0.05 versus WT, n = 3).

collateral arterioles was additionally verified by morphometrical analyses (Fig 5B and C). Subsequent immunofluorescence analyses in remodelling collaterals by utilizing a specific antibody against (active) RhoA-GTP indicated that RhoA activation was more pronounced in SMCs of wild-type but not RGS5-deficient mice (Fig 5D). Furthermore, the abundance of myocardin and its target gene product smooth muscle actin was decreased in SMCs of wild-type mice but remained unchanged in RGS5-deficient mice (Fig 6A and B). These data indicate a failure of the SMCs in acquiring an

activated phenotype which was also supported by the lack of SMC proliferation in the remodelling collaterals in these animals (Fig 6C) as a prototypic response during arteriogenesis. Moreover, this lack of arteriogenic SMC activation which usually promotes a distinct pro-inflammatory response through release of MCP-1 (Heil *et al*, 2004; Demicheva *et al*, 2008) may be the reason for the diminished recruitment of macrophages in RGS5-deficient mice (Supplementary Fig S12) (Banai *et al*, 1998; Waltenberger *et al*, 1999; Shibata *et al*, 2001; Matsumoto *et al*, 2004). Collectively, our findings suggest that

**Figure 4.**

A–G Cultured human umbilical artery SMCs were transduced with an adenoviral control vector (GFP; A, C, E) or RGS5 expression vector (RGS5; B, D, F). Thereafter, stress fibres (F-actin) were visualized by exposing the cells to TRITC-labelled phalloidin (1:200). RGS5 overexpression alone facilitates stress fibre formation (B and G, $^{\#}P < 0.05$ versus GFP-expressing cells, $n = 3$). Stimulation with sphingosine-1-phosphate (C and D, S1P, 10 μM) increases stress fibre formation in cells overexpressing RGS5. This effect is abolished by pretreatment of control and RGS5-overexpressing cells with 5 μM of the Rho-kinase inhibitor Y27632 and subsequent S1P stimulation (E, F and G, $^*P < 0.05$ versus RGS5-overexpressing cells, $n = 3$; cumulative fluorescence intensity of 3–6 whole cells was measured in at least 6 different fields of view, scale bar 20 μm).

H RGS5 overexpression facilitates S1P-stimulated RhoA activation as evidenced by G-LISA-based analyses ($^*P < 0.05$ versus GFP-expressing/S1P-stimulated cells, $n = 3$).

I–M As compared to SMCs transfected with control siRNA (ctr. siRNA; I and K), knockdown of RGS5 significantly decreases stress fibre formation at baseline (RGS5 siRNA; I, J and M) but stimulation with S1P (K, L and M) does not further affect stress fibre formation upon RGS5 knockdown (M, $^*P < 0.05$ versus ctr. siRNA-transfected cells, $n = 3$; cumulative fluorescence intensity of 3–6 whole cells was measured in at least 6 different fields of view, scale bar: 20 μm).

N Loss of RGS5 in HUASMCs inhibits S1P-stimulated RhoA activation as evidenced by G-LISA-based analyses ($^{**}P < 0.01$ versus ctr. siRNA/S1P-treated cells, $n = 4$).

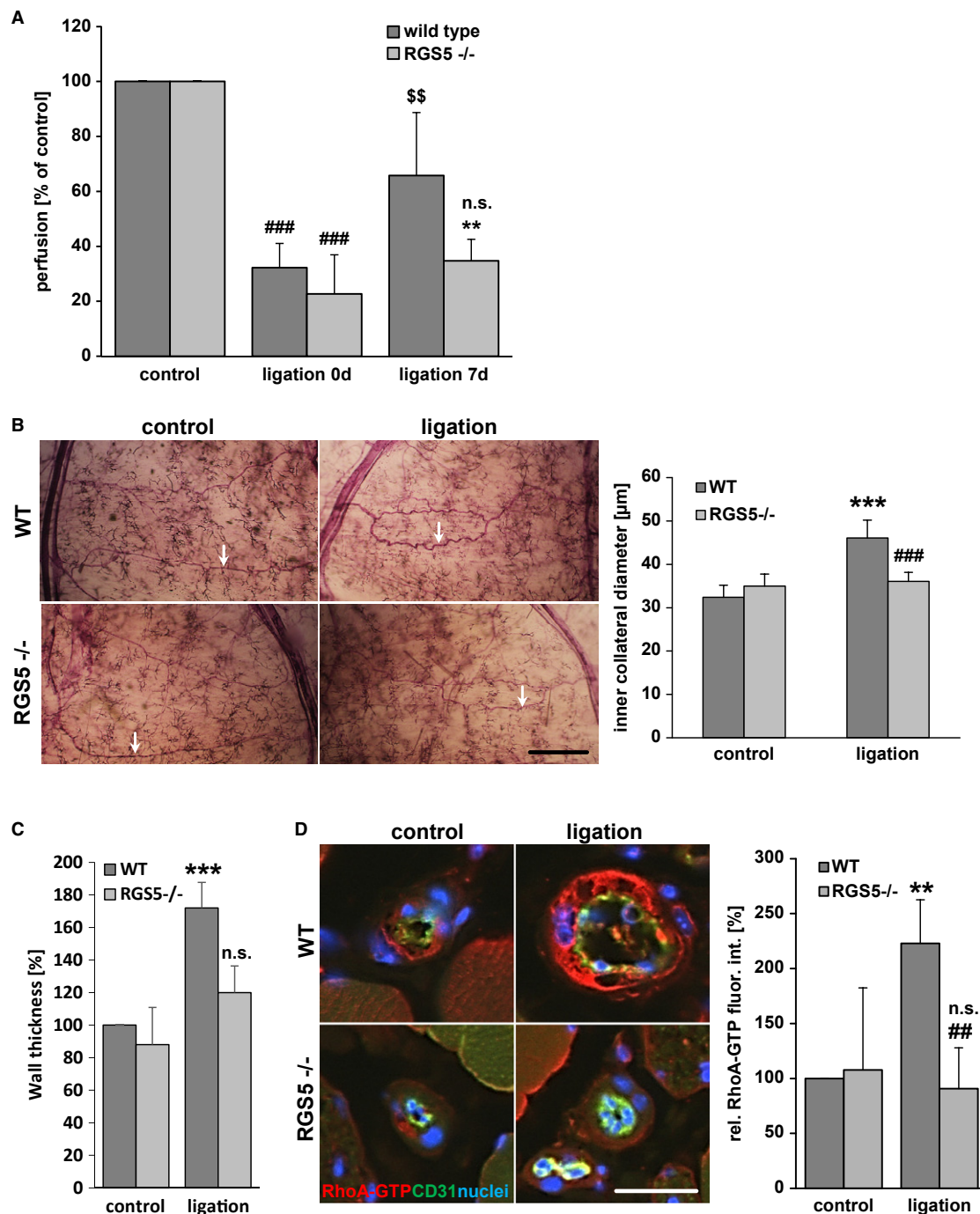


Figure 5. RGS5 deficiency inhibits growth of collateral arterioles.

A To determine collateral-dependent blood flow recovery, hind foot perfusion was measured before, just after and 7 days after ligation. Blood flow recovery (expressed as flow in percent of non-ligated legs) was significantly attenuated in RGS5-deficient mice as compared to wild-type mice (wild type: $###P < 0.001$ versus control, $^{55}P < 0.001$ versus ligation 0 days (wild type); RGS5^{-/-}: $###P < 0.001$ versus control not significant (n.s.) versus ligation 0 days (RGS5^{-/-}), $**P < 0.01$ versus ligation 7 days (wild type), $n = 6-11$; control values were set to 100%).

B Arteriogenic remodelling of collateral arterioles in the mouse hindlimb was analysed 7 days post ligation of the femoral artery. Growth of the remodelling arterioles (arrows) is significantly increased during this period in WT ($***P < 0.001$ versus control, $n = 5$) but not in RGS5-deficient mice (B, $###P < 0.001$ versus WT ligation, $n = 5$; scale bar: 1 mm).

C Likewise, wall thickness is not increased during arteriogenesis in RGS5-deficient mice ($***P < 0.001$ versus control (WT) and not significant (n.s.) versus control (RGS5^{-/-}), $n = 5$).

D Immunofluorescence detection of active RhoA (Rho-GTP) in cross sections of collateral arterioles revealed a significant increase in RhoA activity during arteriogenesis in WT but not in RGS5-deficient mice ($**P < 0.01$ versus control (WT), $###P < 0.01$ versus ligation (WT) and not significant (n.s.) versus control (RGS5^{-/-}), $n = 5$, analysing up to three collaterals per animal; scale bar: 25 μm).

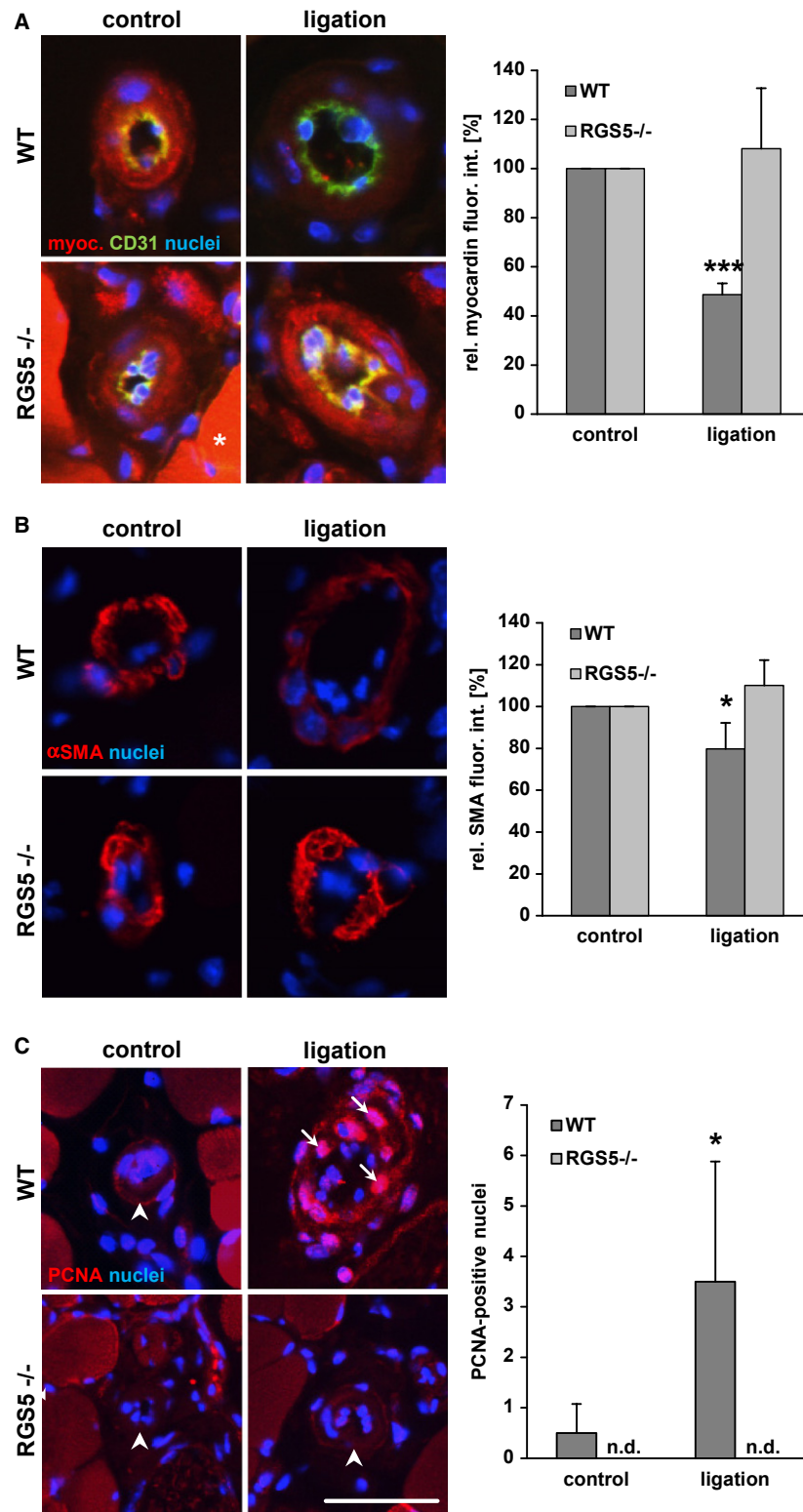


Figure 6. RGS5 deficiency preserves the differentiated SMC phenotype.

A, B Immunofluorescence analyses of cross sections of arterioles undergoing arteriogenesis revealed a significant decline in myocardin (A) and alpha-smooth muscle actin (B) abundance in WT but not in RGS5-deficient mice over this period (* $P < 0.05$, *** $P < 0.001$ versus control, $n = 5$, analysing up to 3 collaterals per animal; asterisk: myoglobin-related red background fluorescence).

C Likewise, PCNA-positive nuclei (C, red fluorescence, arrows) indicating proliferating cells were detected in the SMCs of remodelling collaterals of WT mice but not in RGS5-deficient mice (* $P < 0.05$ versus control, $n = 5$; n.d.: none detected; arrowheads indicate collateral arterioles; scale bar: 50 μ m).

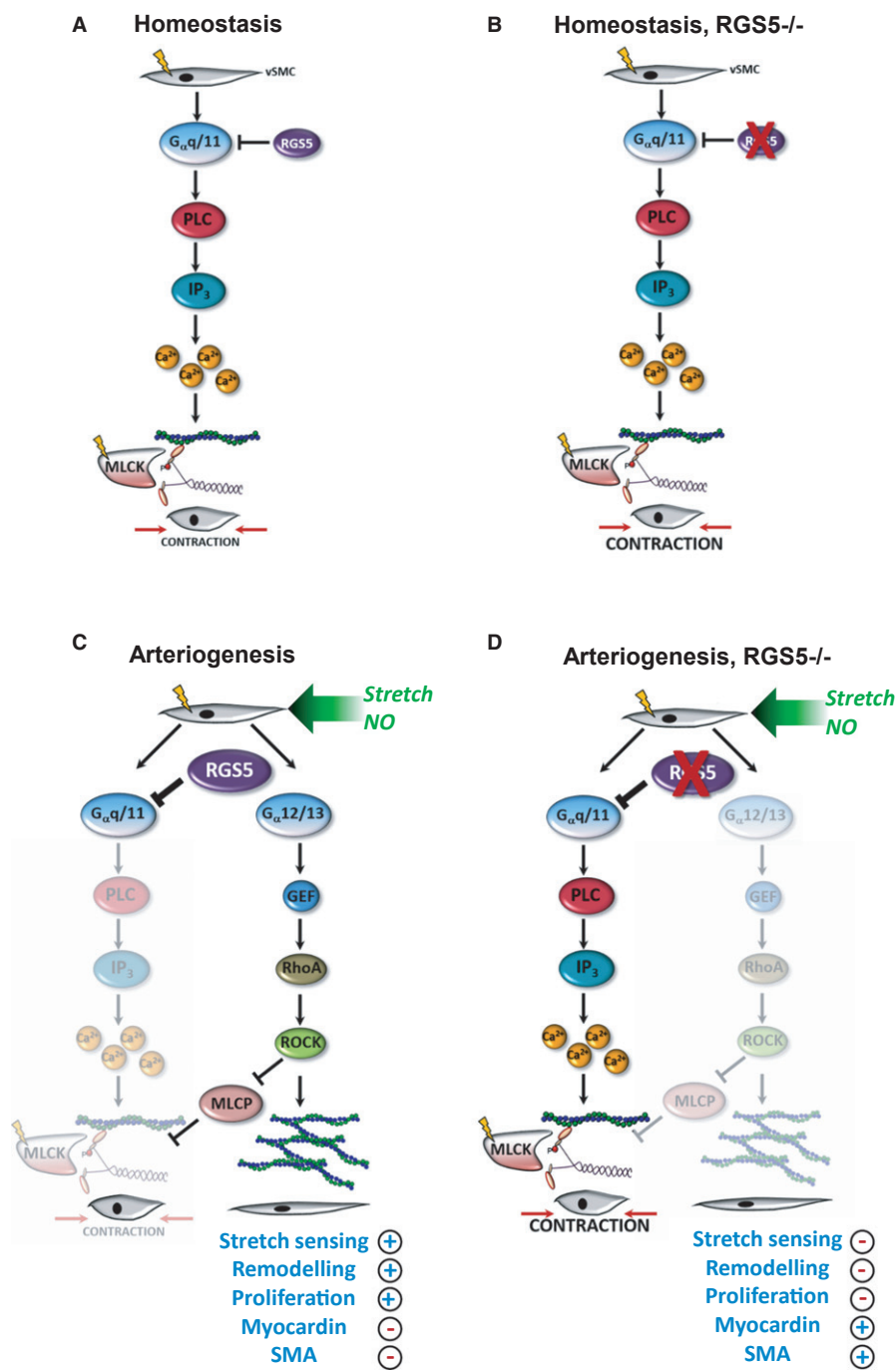


Figure 7. Impact of RGS5 on SMC function.

A Contractile responses of vascular smooth muscle cells (vSMC) were evoked by $G_{\alpha q/11}$ signalling which is sparsely inhibited under baseline conditions.
 B Loss of RGS5 enhances these responses to some extent and leads to a slight increase in blood pressure.
 C The increase in biomechanical stretch and release of nitric oxide (NO) triggers the expression of RGS5 during the onset of arteriogenesis, thereby favouring RhoA-mediated signal responses which promote biomechanical sensitivity of SMCs and the activated SMC phenotype.
 D In RGS5-deficient mice, RhoA signalling is not amplified impairing the SMC phenotype switch under these conditions.

RGS5 expression during arteriogenesis reinforces RhoA-mediated signal responses and promotes an activated SMC phenotype. In RGS5-deficient mice, $G_{\alpha q/11}$ signalling prevails and maintains a quiescent and differentiated SMC phenotype under these conditions (Fig 7).

Discussion

Downstream signalling of G_{α} subunits into the cell and the resulting alterations in SMC tone and phenotype are subject to regulation by different RGS proteins (Hepler *et al*, 1997). Intriguingly, the genes

encoding RGS proteins 2, 4 and 5 are clustered on chromosome 1 within a locus that has been associated with the development of hypertension (Gu *et al*, 2009). RGS2-deficient mice for instance, are hypertensive (Heximer *et al*, 2003; Tang *et al*, 2003; Gross *et al*, 2005), show enhanced signalling through $G\alpha_{q/11}$ and respond differently to treatment with Ang II or the α -adrenoceptor agonist norepinephrine (Heximer *et al*, 2003). RGS4 has been implicated in left ventricular hypertrophy in mice (Tokudome *et al*, 2008; Wang *et al*, 2008) as well as in inhibiting $G\alpha_{q/11}$ signalling in cardiac myocytes (Tamirisa *et al*, 1999; Mittmann *et al*, 2002). Simultaneously, these proteins may affect SMC differentiation as baseline activity of G-protein signalling is essential for the phenotypic switch of SMCs and therefore for proliferation during vascular remodelling processes (Althoff *et al*, 2012). This study focused on the impact of RGS5—an inhibitor of $G\alpha_{q/11}$ signalling—on arteriogenesis as a prototypic biomechanically induced adaptive arterial remodelling process. As compared to its closely related R4 family members RGS2, RGS4 and RGS16, RGS5 is only sparsely expressed in arterial SMCs (Supplementary Fig S13) and can hardly be detected *in vivo* in SMCs of collateral arterioles during vascular homeostasis. In addition, RGS5 deficiency does not induce any compensatory increase in the expression of other R4 RGS homologous (Supplementary Fig S13).

We demonstrated that arteriogenesis stimulates RGS5 expression in SMCs of collateral arterioles, presumably due to their prolonged exposure to increased wall stress or stretch which has been shown to be pivotal for these remodelling processes (Heerkens *et al*, 2007; Demicheva *et al*, 2008). In fact, prolonged stretching of cultured SMCs—mimicking an increase in wall stress *in vivo*—robustly increased RGS5 expression (Fig 2D). While medial SMCs can reduce wall stress temporarily through active constriction, long-term exposure requires remodelling of the vessel wall, typically resulting in an increase in SMC mass. This SMC hyperplasia (arterioles) or hypertrophy (conduit arteries) requires a shift from the resting to the activated phenotype (Scholz *et al*, 2000; Davis-Dusenbery *et al*, 2011) which is characterized, for example, by a decline in myocardin, a pivotal determinant of the expression of SMC marker proteins (Pipes *et al*, 2006; Parmacek, 2007; Pfisterer *et al*, 2012). Thus, RGS5 expression seems to correlate temporarily and spatially with adaptive remodelling processes in the arterial vessel wall.

Flow-induced vasodilation through enhanced shear stress-dependent NO release (Busse *et al*, 1985) from the collateral arteriolar endothelial cells figures prominently in the onset of arteriogenesis (Yu *et al*, 2005; Demicheva *et al*, 2008; Troidl *et al*, 2009b; Dai & Faber, 2010), presumably due to the concomitant flow-dependent increase in wall stress (Demicheva *et al*, 2008). Moreover, our results suggest that continuous exposure of arterial SMCs to NO in fact up-regulates RGS5 expression via the soluble guanylyl cyclase/protein kinase G pathway. Together with the increase in wall stress, this may cause the pronounced increase in RGS5 expression occurring in the SMCs of the remodelling arterioles during arteriogenesis. However, mouse models triggering the formation of collaterals are limited because they are usually based on a sudden occlusion of a conduit artery to alter biomechanical load within the collateral circulation. In contrast, arteriogenesis in human patients occurs slowly by the progressive occlusion of conduit arteries due to arteriosclerosis.

Experimental evidence delineating the functional consequences of an altered expression of RGS5 in vascular SMCs is rather scarce.

Therefore, we employed both a loss-of-function and a gain-of-function approach via siRNA-based knockdown and adenoviral overexpression of the protein, respectively. Taken together, both approaches establish RGS5 as an important regulator of the SMC phenotype that attenuates $G\alpha_{q/11}$ signalling by accelerating the GTP hydrolysis rate of this G-protein subunit (Gu *et al*, 2009). In agreement with our hypothesis that increased RGS5 expression blocks $G\alpha_{q/11}$ -phospholipase C β -inositol-1,4,5-trisphosphate-mediated calcium mobilization, overexpression of RGS5 in airway SMCs negatively impacts GPCR-mediated mobilization of intracellular calcium and subsequent constriction (Yang *et al*, 2012). Moreover, RGS5 allows for an enhanced $G\alpha_{12/13}$ -mediated activation of RhoA in SMCs not only by suppressing $G\alpha_{q/11}$ activity but also by sequestration of the active protein. This would allow a predominant coupling of agonist-activated receptors to $G\alpha_{12/13}$ subunits and therefore increase the activity of RhoA- and Rho-dependent signalling pathways.

RhoA is in fact involved in SMC contraction as it inhibits MLCP activity through ROCK. Nevertheless, RhoA activity may not directly affect the SMC phenotype, but it may act context-dependently to control chronic adaptive changes in the cytoskeleton (e.g. stress fibre formation) which is a prerequisite for SMCs to adequately respond to changes in biomechanical forces especially biomechanical stretch (Zhao *et al*, 1995; Numaguchi *et al*, 1999; Liu *et al*, 2007; Wojtowicz *et al*, 2010). Consequently, promoting a sustained RhoA activity by shifting $G\alpha_{q/11}$ to $G\alpha_{12/13}$ signalling appears to be rate limiting for arteriogenesis as this remodelling process is strictly dependent on Rho-dependent signalling (Eitenmuller *et al*, 2006) and adequate SMC responses to biomechanical stress (Demicheva *et al*, 2008). Likewise, the Rho signalling pathway also controls SMC proliferation during arteriogenesis (Troidl *et al*, 2009a), which was absent in RGS5-deficient mice. Despite solid evidence indicating that RhoA signalling promotes the expression of SMC differentiation markers (Wamhoff *et al*, 2004; Althoff *et al*, 2012; Pagiatakis *et al*, 2012), activation of this pathway in biomechanically stimulated SMCs may have a different outcome. In this context, Rho-dependent regulation of subcellular ERK1/2 localization (Zuckerbraun *et al*, 2003) may control the activity of myocardin, which is dependent on this kinase in SMCs exposed to biomechanical stress (Pfisterer *et al*, 2012). In fact, inhibition of Rho kinase has been shown to suppress stretch-induced ERK activation (Numaguchi *et al*, 1999) which underlines the relevance of the Rho signalling pathway for regulating adequate SMC responses to biomechanical stimuli. Moreover, Rho-mediated signalling in biomechanically stimulated SMCs controls the activity of the pro-inflammatory transcription factor activator protein 1 (AP-1) (Cattaruzza *et al*, 2001; Mohamed & Boriek, 2010), and blockade of Rho activity prevents vascular inflammation (e.g. diminished MCP-1 expression and macrophage infiltration) during stent-induced neointima formation (Matsumoto *et al*, 2004). Considering that AP-1-mediated pro-inflammatory responses of SMCs are rate-limiting for arteriogenesis (Demicheva *et al*, 2008), inadequate RhoA activity is likely to be incompatible with collateral growth and may contribute to the lack of macrophage infiltration during arteriogenesis in RGS5-deficient mice (Supplementary Fig S12). In conclusion, sustained repression of Rho signalling severely hampers the phenotypic switch in SMCs during arteriogenesis which is consistent with our observation that activation of RhoA was blunted in RGS5-deficient mice.

Besides arteriogenesis, Rho signalling controls SMC proliferation, apoptosis and migration and thus plays an important role in neointima formation (Shibata *et al*, 2001; Matsumoto *et al*, 2004). Considering our findings, RGS5 may amplify this process by promoting RhoA activity. In fact, neointima formation induced by ligating the carotid artery (Kumar & Lindner, 1997; Kumar *et al*, 1997) was attenuated in RGS5-deficient mice (Supplementary Fig S14). Although this type of vascular remodelling is triggered by different biomechanical and molecular stimuli than arteriogenesis, these findings underline the importance of RGS5 in controlling the activity of SMCs during adaptive changes of the arterial vessel wall. Consequently, pharmaceutical modulation of RGS5 activity by small molecule approaches, as has been shown for RGS4 (Roman *et al*, 2007), may have great clinical relevance with respect to stimulating arteriogenesis or counteracting neointima formation during arteriosclerosis or in-stent restenosis. On the one hand, inhibition of RGS5 activity may block vascular remodelling by abolishing the amplification of the Rho signalling pathway. On the other hand, depending on the local activity of RGS5 in distinct vascular entities, RGS5 inhibition may have complex consequences on the regulation of the local myogenic tone. For instance, PPAR γ - and PPAR δ -controlled expression of RGS5 has been reported to blunt angiotensin II-mediated activation of protein kinase C, controlling the myogenic tone of mesenteric arteries (Ketsawatsomkron *et al*, 2012).

In line with the inhibitory effect of an increased level of RGS5 on GPCR agonist-evoked calcium mobilization in arterial SMCs, RGS5-deficient arteries showed an improved vasoconstrictor response. Likewise, arterial SMCs derived from RGS5-deficient mice not only revealed an enhanced sensitivity to GPCR agonist-induced signalling via G $\alpha_{q/11}$, but also a distinct deficit in stress fibre formation already at baseline. In contrast, RGS5-deficient mice have repeatedly been reported to be hypotensive (Cho *et al*, 2008; Nisancioglu *et al*, 2008) or hypertensive (Holobotovskyy *et al*, 2013). The discrepancies in these findings are likely to be based on the use of different techniques (i.e. tail cuff versus telemetric measurement), genetic backgrounds and stress levels (increased cardiac output/systolic blood pressure). Our own telemetric blood pressure recordings show instead that mice utilized in our study have a modestly but not significantly increased systolic and diastolic blood pressure.

Collectively, our findings reveal that (i) vascular remodelling and the switch in SMC phenotype during arteriogenesis is accompanied by and dependent on up-regulation of RGS5 in arteriolar SMCs and (ii) RGS5 shifts G $\alpha_{q/11}$ -PLC β -MLCK-mediated constriction to G $\alpha_{12/13}$ -mediated RhoA activation and subsequent stress fibre formation in arterial SMCs—a prerequisite for arteriogenesis to take place (summarized in Fig 7). Therefore, stimulation of RGS5 expression in these cells may favour such an adaptive vascular remodelling process while diminishing RGS5 expression likely impairs activation of vascular SMCs and thus attenuates remodelling of the vessel wall.

Materials and Methods

Materials

The anti-human/mouse RGS5 antibody was purchased from Sigma-Aldrich (GW22900, Schnellendorf, Germany). Its specificity on tissue samples was confirmed by comparing the RGS5 staining of capillary

pericytes in muscle tissue of wild-type and RGS5-deficient mice (Supplementary Fig S15). The monoclonal anti-mouse CD31 antibody (clone: MEC 13.3) was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The anti-mouse α SMA antibody was purchased from Dianova (Hamburg, Germany), the anti-mouse PCNA antibody was ordered from Abcam (Cambridge, UK), the anti-mouse myocardin antibody was purchased from Santa Cruz (Heidelberg, Germany), and the anti-active-RhoA (Rho-GTP) antibody (#26904) was ordered from New East Biosciences (Pennsylvania, USA). TRITC-labelled phalloidin was obtained from Invitrogen. Bradykinin, angiotensin II, norepinephrine and NONOate were purchased from Sigma-Aldrich, sphingosine-1-phosphate was obtained from Cayman Chemical (New Orleans, USA) and 8pCPT-cGMP from BioLog (Bremen, Germany). The adenoviral RGS5, GFP and Lsc vectors were kindly provided by Prof. Wieland (Department of Experimental Pharmacology, Heidelberg University).

Arteriogenesis mouse model

All animal studies were performed with permission of the Regional Council Karlsruhe and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). RGS5-deficient mice were based on a C57BL/6 background. In these mice, the RGS domain coding sequence of RGS5 was replaced by GFP (Nisancioglu *et al*, 2008). At least 10- to 12-week-old male C57BL/6 (wild type, WT) or RGS5-deficient (RGS5 $^{-/-}$) mice were anesthetized with isoflurane, and the femoral artery was ligated just distal to the origin of the deep femoral artery. Each experimental group was comprised of 5–6 mice. To assess collateral-dependent blood flow recovery, perfusion of the mouse foot was determined before, just after and 7 days after ligation by laser Doppler analysis (PeriFlux 4001 Master, Perimed, Germany). On day 7 after surgery, mice were euthanized and the left ventricle of the heart was cannulated and perfused for 2 min at 100 mmHg with Ringer solution containing 100 μ M adenosine and 10 μ M sodium nitroprussid at 37°C followed by zinc fixative for the purpose of immunohistological stainings. A 4% paraformaldehyde containing a coloured pigment (HKS Gouache 318; Schmincke, Germany) that cannot pass the capillary system was used to visualize the arterial system. Thereafter, hindlimbs were dissected and processed for histological analysis.

Visualization of the arterial system

Mouse hindlimb specimens perfused with pigment-containing paraformaldehyde were postfixed in 4% formaldehyde (18 h) and dehydrated using a series of ethanol and isopropanol following standard protocols. The tissue was then incubated in a mixture of benzyl alcohol and benzyl benzoate (1:1, v/v) having the same refractive index of the tissue for at least 18 h. This procedure induces transparency of the tissue and allows detailed analysis of the pigment-loaded arterial system. Growing collaterals in the hindlimb were identified by their constant course on the surface of the adductor muscles facilitating their identification in transparent tissue and histological preparations (Heil & Schaper, 2004). The luminal diameter of the collateral arterioles was measured using the morphological analysis software Cell \wedge R from Olympus (Hamburg, Germany) in at least three different sites of an individual arteriole.

Telemetric blood pressure measurement

For blood pressure measurements in conscious, naïve male wild-type (WT) or RGS5-deficient (RGS5^{-/-}) mice, telemetric devices were implanted (PA-C10, Data Sciences International) as described earlier (Mills *et al*, 2000). In brief, catheter tips were advanced into the aortic arch through the left common carotid artery. The signal transducer unit was placed into a subcutaneous pocket on the right ventrolateral side of the animal. Mice were allowed to recover after surgery for 1 week before blood pressure recording was started for five consecutive days using the Dataquest A.R.T. software 4.0. The data were acquired every 30 min for 30 s, and the means were calculated from the values obtained during 6 h either at night-time or daytime.

Perfusion of isolated mouse arteries

C57BL/6 wild-type and RGS5-deficient mice (Nisancioglu *et al*, 2008) were sacrificed; third- or fourth-order mesenteric arteries were extracted and inserted into the chamber of a myograph (Culture Myograph, DMT, Copenhagen, Denmark) containing Tyrode's buffer. Arteries were perfused with Tyrode's buffer at a longitudinal pressure gradient of 20 mmHg (70 mmHg at the inflow and 50 mmHg at the outflow) with a resulting flow of approximately 0.07 ml/min. Constriction was induced with different concentrations of norepinephrine, and vessel diameter was measured using the VediView Software (DMT, Copenhagen, Denmark).

Cell culture and adenoviral transduction

Human arterial smooth muscle cells (HUASMCs) were isolated from human umbilical cord arteries, and mouse smooth muscle cells were isolated from third- or fourth-order mesenteric arteries of wild-type and RGS5-deficient mice (Nisancioglu *et al*, 2008) and cultured in DMEM (Invitrogen) supplemented with 15% FCS and 50 U/ml penicillin, 50 µg/ml streptomycin and fungizone (Invitrogen, Darmstadt, Germany). To isolate umbilical cord arterial smooth muscle cells, the umbilical artery was cannulated and flushed with HBSS to remove blood and endothelial cells. The artery media were cut into pieces which were placed in a Petri dish and covered with DMEM medium containing 15% FCS and antibiotics. After 10–14 days, the smooth muscle cells which grew out of the artery were removed by trypsin treatment and transferred into a T75 cell culture flask. Mouse vascular smooth muscle cells were isolated from third- or fourth-order mesenteric arteries. After separating the vessel from surrounding fat and connective tissue, the artery was cut into pieces and incubated in 1% collagenase (Sigma-Aldrich, C0130) in DMEM with 15% FCS and antibiotics overnight. Upon centrifugation, the artery pieces were resuspended in fresh medium. Outgrowing smooth muscle cells were removed by trypsin treatment and transferred into a T75 cell culture flask. Cell type specificity was routinely checked by performing smooth muscle actin immunofluorescence stainings. Only cells cultured up to passage 3 were used throughout. The isolation of HUASMCs was approved by the local ethics committee (Heidelberg, Germany) and conformed to the principles outlined in the Declaration of Helsinki (1997). In order to expose HUASMCs to cyclic stretch, they were cultured on plastic dishes or BioFlex™ 6-well plates (Flexcell, Hillsborough, NC). Stretching was performed using a Flexcell FX-5000™ Tension System with 13%

cyclic elongation at 0.5 Hz. Cyclic elongation is needed to prevent that the cells evade the biomechanical stimulus through rearranging their focal contacts. HUASMCs were transduced (200 MOI) in 2 ml DMEM w/o supplements using recombinant adenoviruses encoding either GFP or GFP plus RGS5 or GFP plus RGS-Lsc under the control of independent CMV promoters (He *et al*, 1998). Viruses were removed after 18 h, and the experiments performed 48 h later.

siRNA-based gene silencing

For functional studies of RGS5, a siRNA-based silencing approach was used. Control siRNA (siGENOME Non-Targeting siRNA Pool #2, Thermo Scientific, Germany) and customized RGS5-targeting siRNA (sense: CCUGAAGUCUGAAUUCAGU, antisense: CCAUGAAUGUGGACUGGCA) was purchased from Sigma-Aldrich and used at a concentration of 100 nM. HUASMCs were transfected by utilizing the MATRAsi technique (IBA bioTAGnology, Göttingen, Germany) according to the manufacturer's instructions. Transfected cells were incubated for 48 h, and the siRNA-based knockdown efficiency was verified before further usage (Supplementary Fig S4).

Measurement of intracellular calcium mobilization

Cells were seeded in black, clear bottom 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and used for adenoviral transduction as described before. Thereafter, medium was removed, and cells were loaded with 2.5 µM Rhod-4 AM (AAT Bioquest, Sunnyvale, USA) in HBSS with Ca²⁺/Mg²⁺ (PAA, Pasching, Austria) containing 20 mM Hepes and 0.001% detergent (Pluronic F12, AAT Bioquest) for 30 min at 37°C and another 30 min at room temperature in the dark. GPCR agonist-evoked mobilization of intracellular calcium was measured using the f_{max} Fluorimeter (Molecular Devices, Biberach, Germany). Relative fluorescence units were measured every 5 s for a period of 5 min. Fluorescence intensities were normalized against non-stimulated controls.

PCR analysis

Total RNA was isolated from cultured cells or isolated mouse arteries by solid-phase extraction with the RNeasy MiniKit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. Subsequently, reverse transcription (RT, SensiScript Reverse Transcription kit, according to manufacturer's instructions) and either conventional PCR or quantitative real-time RT-PCR (qRT-PCR) was performed for the target sequences; the latter was performed in a LightCycler (LC, Roche Diagnostics, Germany). Primers based on the following sequences were used for amplification (annealing temperature 60°C): huRGS5, forward: GGTGGAACCTTCCTGAGCAGC, reverse: AGAGCGCACAAAGCGAGGCA; huRPL32, forward: AGGCATTGACAACAGGGTTC, reverse: GTTGACATCAG-CAGCAC TT; LC mRPL32, forward: GGGAGCAACAAGAAAACCAA, reverse: ATGTGTGGACCAGGAACCTTGC (annealing: 60°C); LC mRGS5, forward: GCGGAGAAGGCAAAGCAA, reverse: GTGGTCAATGTTCA CCTCTTTAGG; LC mRGS2 forward: ATCAAGCCTTCTCCTGAGGAA, reverse: GCCAGCAGTTCATCAATGC; LC mRGS4, forward: GGG CTGAATCGTTGGAAAAC, reverse: ATTCGACTTCAGGAAAGCTT T; LC mRGS16, forward: CCTGGTACTTGCTACTCGCTTTT, reverse: AGCACGTCGTGGAGAGGAT. Fluorescence was monitored (excitation

at 470 nm and emission at 530 nm) at the end of the annealing phase. Threshold cycle (C_t) was set within the exponential phase of the PCR. Quantification of the PCR product was performed using the $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001). Amplification of the 60S ribosomal protein L32 (RPL32) cDNA served as an internal standard.

Immunofluorescence analyses

Deparaffinized vessel sections and methanol-fixed HUASMCs were blocked in Tris buffer (pH 7.6) containing 1% (w/v) BSA. Primary antibodies were diluted in blocking solution as follows: RGS5 1:200, CD31 1:50, PCNA and SMA 1:500, myocardin 1:100. Samples were incubated with the antibodies for 18 h at 4°C. After rinsing, sections or cells were incubated with secondary antibodies for 2 h at room temperature, additional 10 min with 4',6-Diamidin-2-phenylindol (DAPI, Invitrogen) in PBS and mounted in Mowiol (Calbiochem). Quantification of fluorescence intensity in cultured cells or medial SMCs in arteries was performed by utilizing the morphological analysis software Cell[^]R from Olympus (Hamburg, Germany). Cytoplasmic fluorescence intensities were analysed by comparing size-matched areas in three to five randomly selected microscopic fields of view. When analysing the fluorescence intensity of medial SMCs in arteries, three to five size-matched areas per section and artery were quantified. Nuclei containing PCNA were detected by a purple colour upon merging the blue DAPI-fluorescence with the corresponding red fluorescence.

RhoA activation assay

To measure the amount of activated, that is, GTP-bound, RhoA, the RhoA G-LISA Kit (colorimetric format) from Cytoskeleton (TebuBio, Offenbach, Germany) was used. HUASMCs were cultured in 6-well plates and transfected with either control/RGS5 siRNA or GFP/RGS5 construct-containing adenoviral vectors as described above. Thirty hours after transfection, the culture medium was changed to 5% FCS-containing DMEM for another 18 h to obtain more responsive cells prior to S1P stimulation. Afterwards, cells were stimulated with 10 μ M S1P for 2 min and then lysed for protein extraction and further assay procedure according to manufacturer's instructions.

Statistical analysis

All results are expressed as mean \pm SD. Differences between two experimental groups were analysed by unpaired Student's *t*-test or one sample *t*-test if applicable, with a probability value of $P < 0.05$ considered statistically significant. Differences among three or more experimental groups were analysed by one-way ANOVA followed by a Bonferroni post hoc test for selected pairs of groups, with a probability value of $P < 0.05$ considered statistically significant.

Supplementary information for this article is available online: <http://embomolmed.embopress.org>

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The paper explained

Problem

Recent reports underlined that the development of a collateral circulation through arteriogenesis reduces the risk of mortality due to coronary artery disease by 36%. This natural compensatory vascular remodelling process is initiated when arteriosclerotic lesions occlude conduit arteries which subsequently lead to an increase in the pre- to poststenotic pressure difference. As a consequence and depending on the (progressive) stenosis level, the local perfusion of collateral arterioles and small arteries gradually increases and promotes an adaptive growth to enhance their perfusion capacity. However, therapies promoting collateral growth are not available as the mechanisms controlling this complex arteriolar remodelling process remain elusive. To define suitable targets for corresponding pharmacologic therapies, the delineation of key molecules orchestrating the activity of vascular smooth muscle cells as basis for the reorganization of the vessel wall architecture is indispensable.

Results

Against this background, our study indicated for the first time that balancing of G-protein signalling in arteriolar smooth muscle cells by RGS5 is a prerequisite for arteriogenesis to occur. While RGS5 appears to be abundantly expressed in smooth muscle cells of remodelling collaterals, thereby promoting a $G\alpha_{12/13}$ -mediated RhoA-dependent activation of these cells, genetic ablation of RGS5 in mice impairs collateral growth by inhibiting activation of these cells as evidenced by a blockade of their proliferation and RhoA activity.

Impact

From a clinical point of view, enhancing the abundance and/or stability of RGS5 appears to support the activated vascular smooth muscle cell phenotype and thus may be a promising therapeutic strategy to locally promote arteriogenesis and favouring the development of natural bypasses.

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Author contributions

Each author contributed to the submitted work as follows: CA and AF acquired, analysed and interpreted the data. LP, MHö and KT acquired and analysed the data. GG and TW drafted the manuscript and made critical revisions for important intellectual content. TK and MHe designed the research, analysed and interpreted the data and drafted the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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