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Different Migration of Vascular Smooth Muscle Cells from Human Coronary Artery Bypass Vessels

Role of Rho/ROCK Pathway

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Key Words
Cell migration · Signal transduction · Atherosclerosis · Bypass graft disease

Abstract
Background: We examined whether vascular smooth muscle (VSMC) or endothelial cell (EC) migration from internal mammary artery (MA) differed from VSMC or EC migration from saphenous vein (SV). ***Methods and Results:*** Migration to PDGF-BB (1–10 ng/ml) was lower in VSMC from MA than SV; however, attachment, movement without chemokine, and chemokinesis were identical. Unlike VSMC, migration of EC was similar in response to several mediators. Expression of PDGF receptor-β was lower in VSMC from MA than SV, while α-receptor expression was higher. PDGF-BB-induced RhoA activity was lower in MA than SV, while basal activity was identical. Rosuvastatin and hydroxyfasudil impaired PDGF-BB-induced migration of VSMC from MA and SV. Mevalonate and geranylgeranylpyrophosphate rescued inhibition by rosuvastatin. PDGF-BB induced less stress fiber formation in VSMC from MA than SV. A dominant negative RhoA mutant inhibited stress fiber formation to PDGF-BB, while a constitutively active mutant resulted in maximal stress fiber formation in MA and SV. Rosuvastatin and hydroxyfasudil impaired PDGF-BB-induced stress fiber formation in MA and SV. ***Conclusions:*** VSMC migration to PDGF-BB is lower in MA than SV, which is at least in part related to lower activity of the Rho/ROCK pathway.

Introduction

Internal mammary artery (MA) and saphenous vein (SV) are used as conduits for coronary artery bypass grafting. Long-term patency rates of MA grafts, however, are higher than those of SV, with more than 85% of MA grafts patent after 10 years as compared to less than 50% of SV grafts [1–3]. As a consequence, morbidity and mortality are increased in patients receiving only venous grafts [1, 2, 4]. Graft occlusion occurs due to thrombosis, neointima formation, and accelerated atherosclerosis [2, 5]. Although the frequency of acute thrombotic bypass graft occlusion has declined since the introduction of platelet inhibitory drugs, bypass graft disease remains a major problem [4].

Vascular smooth muscle cell (VSMC) migration plays an important role in the development of neointima formation and atherosclerotic lesions [6, 7]; hence, it is also

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involved in the pathogenesis of bypass graft disease [8, 9]. The remarkable patency of MA grafts appears to be related to intrinsic functional properties [10, 11]. Indeed, proliferation rates are lower in VSMC from MA than SV [12]. However, migration rates of VSMC from these vessels have not yet been compared.

Endothelial cells (EC) are important regulators of vascular homeostasis. Endothelial erosions are a frequent event in atherogenesis, as the absence of a complete endothelial lining favors thrombus formation [13]. Migration of EC is required for repair of eroded areas and as such is an important protective mechanism. Since migration rates of EC from MA versus SV are not known, the relevance of endothelial migration for patency of these grafts remains unclear.

Therefore, the present study was designed to compare for the first time migration of VSMC as well as EC from MA versus SV.

Methods

Cell Culture

VSMC of MA and SV were isolated from patients undergoing coronary artery bypass grafting, which was approved by the Ethics Committee of the University Hospital. Isolation was performed by the explantation method; each isolate was identified as VSMC by immunofluorescent staining for smooth muscle α-actin (No. 1148818, Roche Diagnostics, Mannheim, Germany). Cells were grown in Dulbecco's MEM supplemented with 10% fetal calf serum (FCS) as described and used up to passage 12 [14]. EC isolation was performed enzymatically; each isolate was identified as EC by immunofluorescent analysis of acLDL uptake. Cells were grown in EGM-2 supplemented with 10% FCS (all from Cambrex Corporation, East Rutherford, N.J., USA) and used up to passage 5 [14]. Cells from MA and SV were only compared when they originated from the same patient and reached the same passage. Lactate dehydrogenase release was analyzed by a Cytotoxicity Detection Kit (Roche Diagnostic GmbH, Mannheim, Germany) as described in a previous study [15].

Cell Migration and Attachment

Migration was examined in a modified Boyden chamber (Neuroprobe, Gaithersburg, Md., USA). For chemotaxis, the directional movement of cells towards a chemokine concentration gradient, different concentrations of PDGF-BB (R&D Systems, Minneapolis, Minn., USA), FCS, VEGF, or bFGF were diluted in PBS + 0.1% BSA and added to the lower compartment [16]. For chemokinesis, the random movement of cells in the absence of any concentration gradient, PDGF-BB was added to both compartments. For movement without chemokine, 0.1% BSA was added to both compartments [16]. Fifty microliters of the cell suspension (5×10^5 cells/ml) were placed in the upper compartment. The chambers were incubated at 37° for 5 h; then the upper surface of the filter was scraped off. The cells were fixed in 100% methanol for 10 min and stained with Diff-Quick solution (Dade Diagnostics, Auckland, New Zealand). The number of migrated cells was counted at 400× magnification. To assess migration in the presence of hydroxyfasudil or rosuvastatin, VSMC were pretreated for 30 min with the respective inhibitor. For examining attachment, subconfluent VSMC were seeded on 6-well tissue culture dishes and incubated with PDGF-BB for 5 h; afterwards, cells were washed with PBS and the number of attached cells determined by a hemacytometer.

RhoA Activity and Expression

VSMC were stimulated with PDGF-BB for 30 min and then lysed in buffer as described previously [17]. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Munich, Germany). Western blotting and RhoA pulldown were performed as described [18]. The antibodies against RhoA, PDGF receptor-α (PDGFR-α), PDGFR-β were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA) and that against α-tubulin from Sigma Chemical Company.

Adenoviral Transduction and Immunofluorescence

Adenoviral vectors for overexpression of constitutively active RhoA (Rho 63) and dominant negative RhoA (Rho 19) were kindly provided by Z. Yang (University of Fribourg, Switzerland) [19]. Cells were transduced with the respective adenoviral vector at moi 800. After a 24-hour growth period, the cells were rendered quiescent for 48 h before stimulation with PDGF-BB (10 ng/ml) for 5 h. Hydroxyfasudil or rosuvastatin were added 30 min before stimulation with PDGF-BB.

After stimulation, cells were washed with ice-cold PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and washed 3× with PBS. Stress fiber formation was visualized by staining for F-actin bundling [20]. Cells were blocked with 1% BSA in PBS for 20 min followed by incubation with 3.3 μg/ml TRITC-phalloidin (Sigma, St. Louis, Mo., USA) for 30 min. Afterwards, the cells were again washed 3× with PBS, and DAPI staining was performed as described previously [21].

Statistics

Cells from MA and SV were only compared when they originated from the same patient. For all experiments, 'n' designates the number of patients. Comparison were performed with unpaired Student's t test.

Results

Migration of VSMC and EC

Migration of MA VSMC was 7.0 ± 2.4% in response to 1 ng/ml, 20.1 ± 4.4% to 3 ng/ml, and 32.1 ± 8.4% to 10 ng/ml PDGF-BB (fig. 1a; $p < 0.05$ for 1 vs. 10 ng/ml). In contrast, migration of SV VSMC reached 44.7 ± 8.5% in response to 1 ng/ml, 85.4 ± 6.9% to 3 ng/ml, and 100% to 10 ng/ml PDGF-BB (fig. 1a; $p < 0.0005$ for 1 vs. 10 ng/ml). Thus, migration to PDGF-BB was lower in VSMC from MA than SV and resulted in a significant difference between the two vessels at all PDGF-BB concentrations

examined (1 ng/ml: $p < 0.005$; 3 ng/ml: $p < 0.0001$; 10 ng/ml: $p < 0.0001$; fig. 1a). No difference in attachment, movement without chemokine, or chemokinesis of VSMC from MA as compared to SV was observed (p = n.s.; fig. 1b).

Migration of EC from MA and SV was identical in response to 10% FCS (MA: 43.8 ± 5.7, SV: 50.4 ± 7.2 cells/hpf; n = 5; p = n.s.; table 1). A similar pattern was observed after stimulation with 10 ng/ml bFGF (MA: 13.2 ± 2.2, SV: 14.1 ± 1.8 cells/hpf; n = 5; p = n.s.; table 1) or 10 ng/ml VEGF (MA: 23.4 ± 4.7, SV: 23.2 ± 2.9 cells/hpf; n = 5; p = n.s.; table 1).

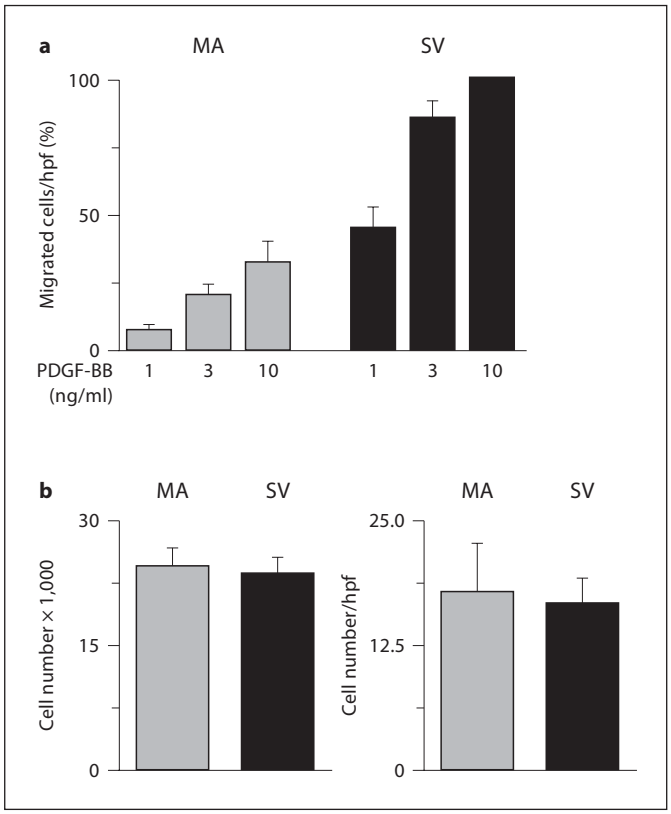


Fig. 1. Migration of VSMC from MA and SV. **a** PDGF-BB induces lower migration rates of VSMC from MA as compared to SV. **b** No significant difference in attachment (left panel) or movement without chemokine (right panel) is observed between VSMC from MA and SV.

PDGFR Expression

While VSMC express PDGFR-α and PDGFR-β, VSMC migration is known to be mediated by PDGFR-β activation [22]. Expression of these receptors was examined by Western blot analysis. PDGFR-α expression was higher in VSMC from MA than SV ($p < 0.0001$), while PDGFR-β expression was lower in VSMC from MA than SV ($p < 0.005$; fig. 2a).

Rho/ROCK Pathway and Migration

In addition to the different PDGFR expression, signal transduction of VSMC from MA and SV may differ per se. The Rho/ROCK pathway is involved in VSMC migration and lies downstream of PDGFR-β [9, 23]. Therefore, RhoA activity was determined in VSMC from MA and SV. Basal RhoA activity did not differ in VSMC from MA and SV (p = n.s.; n = 4; fig. 2b). Stimulation with PDGF-BB only weakly increased RhoA activity over control conditions in VSMC from MA ($p = 0.08$), while a pronounced activation occurred in cells from SV ($p < 0.05$). Consistent with this observation, PDGF-BB-induced RhoA activity was lower in VSMC from MA as compared to SV ($p < 0.005$, fig. 2b).

Rosuvastatin (10^{-7} to 10^{-5} M), an inhibitor of RhoA activation, impaired PDGF-BB-induced migration of VSMC from MA and SV in a concentration-dependent manner (MA: 80.6 ± 37.3% for 10^{-7} M, 33.8 ± 11.7% for 10^{-6} M, 29.6 ± 11.9% for 10^{-5} M; n = 5; $p < 0.01$ for 10^{-6} and 10^{-5} M; SV: 67.3 ± 5.2% for 10^{-7} M, 46.5 ± 4.6% for 10^{-6} M, 29.3 ± 2.9% for 10^{-5} M; n = 5; $p < 0.01$ for 10^{-7} to 10^{-5} M; fig. 3). The inhibitory effect of the statin was rescued by treatment of VSMC with 10^{-4} M mevalonate (MA: 72.0 ± 9.0% for rosuvastatin + mevalonate vs. 27.8 ±

Table 1. Migration of EC from MA and SV

Chemotaxin	MA EC	SV EC	p value
10% FCS, cell number/hpf	43.39 ± 5.73	50.42 ± 7.25	n.s.
10 ng/ml bFGF, cell number/hpf	13.20 ± 2.20	14.14 ± 1.75	n.s.
10 ng/ml VEGF, cell number/hpf	23.40 ± 4.65	23.17 ± 2.93	n.s.

EC migration is similar in response to 10% FCS, 10 ng/ml bFGF, and 10 ng/ml VEGF.

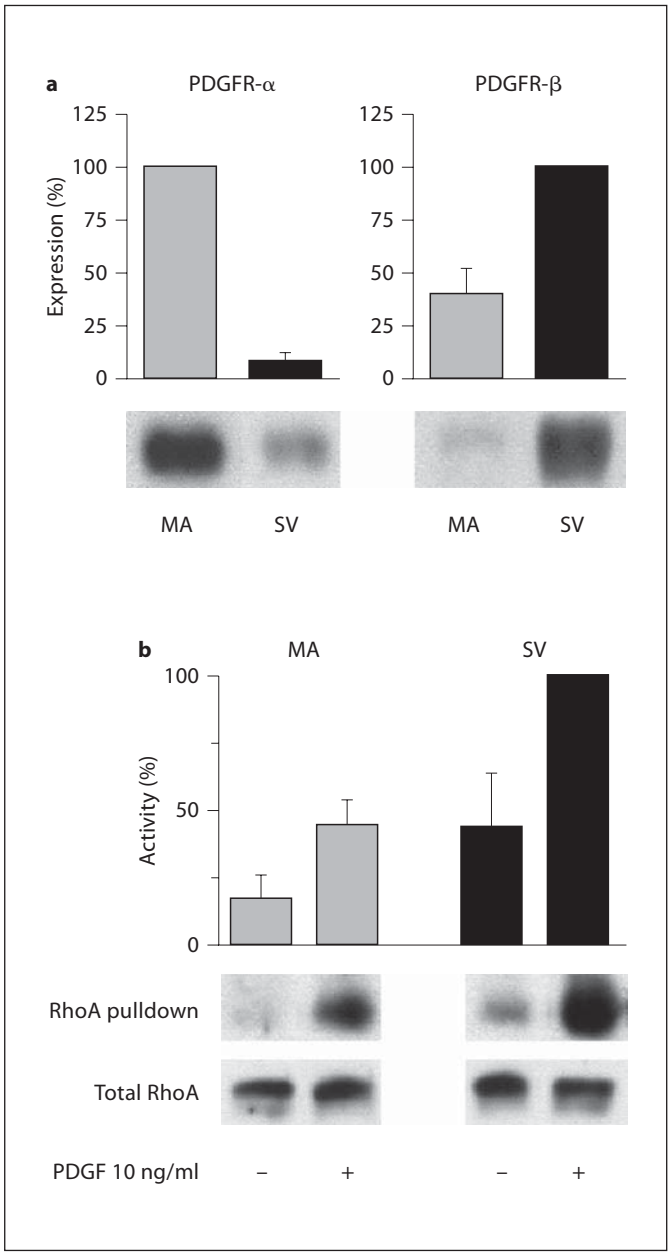


Fig. 2. RhoA activation in VSMC from MA and SV. **a** Western blotting analysis demonstrates that PDGFR-α expression is higher in VSMC from MA than SV, while expression of PDGFR-β is lower in VSMC from MA than SV. **b** Basal RhoA activity is similar in VSMC from MA and SV. PDGF-BB (10 ng/ml) significantly increases RhoA activity in SV VSMC, but not in MA VSMC. RhoA activity after stimulation with PDGF-BB is more pronounced in SV VSMC than MA VSMC. No change in total RhoA expression occurs. RhoA activity is normalized to total RhoA and α-tubulin expression.

9.3% for rosuvastatin alone, n = 4, $p < 0.05$; SV: 95.7 ± 4.6% for rosuvastatin + mevalonate vs. 42.3 ± 4.0% for rosuvastatin alone, n = 4, $p < 0.001$; fig. 3). A similar rescue was achieved with 10^{-4} M geranylgeranylpyrophosphate (GGPP; n = 4; fig. 3). Hydroxyfasudil (10^{-5} M), an inhibitor of ROCK activation, also inhibited PDGF-BB-induced migration of VSMC from MA and SV (MA: 87.3 ± 8.3% inhibition; SV: 87.4 ± 5.9% inhibition; n = 4; $p < 0.0001$ vs. control for both vessels). Movement without chemokine of VSMC from MA and SV was inhibited by both 10^{-5} M rosuvastatin (MA: 38.0 ± 8.8% inhibition, SV: 54.0 ± 13.7% inhibition, $p < 0.01$ vs. control for both vessels) and 10^{-5} M hydroxyfasudil (MA: 66.0 ± 2.0% inhibition, SV 63.0 ± 1.4% inhibition; $p < 0.0001$ vs. control for both vessels). No cytotoxic effect of any of these drugs was observed (n = 4; p = n.s.; data not shown).

Rho/ROCK Pathway and Stress Fiber Formation

Stress fiber formation in VSMC is regulated by the Rho/ROCK pathway [24]. In order to confirm the differential RhoA activation in VSMC from MA and SV, stress fiber formation was investigated in these cells. Consistent with the pattern of RhoA activation, stress fiber formation after stimulation with PDGF-BB was less pronounced in VSMC from MA than SV (fig. 4). Adenoviral transduction of a dominant negative RhoA mutant (Rho19) inhibited stress fiber formation in response to PDGF-BB in VSMC from MA and SV (fig. 4a). A constitutively active RhoA mutant (Rho63) induced pronounced stress fiber formation in VSMC from MA and SV both in the presence or absence of PDGF-BB (fig. 4a). Transduction with a control vector (ΔE1) did not affect stress fiber formation in VSMC from either vessel (fig. 4a). Similar to the dominant negative RhoA mutant, pharmacologic inhibition of RhoA with rosuvastatin and ROCK with hydroxyfasudil impaired PDGF-induced stress fiber formation in VSMC from both MA and SV (fig. 4b).

Discussion

VSMC migration is a key event in the pathogenesis of atherosclerosis and bypass graft disease [6, 8, 9]. The present study demonstrates for the first time that migration is lower in VSMC from MA than SV, while it is similar in EC from the two vessels. Since no difference in attachment, movement without chemokine, or chemokinesis of VSMC is observed, the difference in motility occurs only in the presence of a chemotactic gradient and hence is consistent with a true difference in migration. As

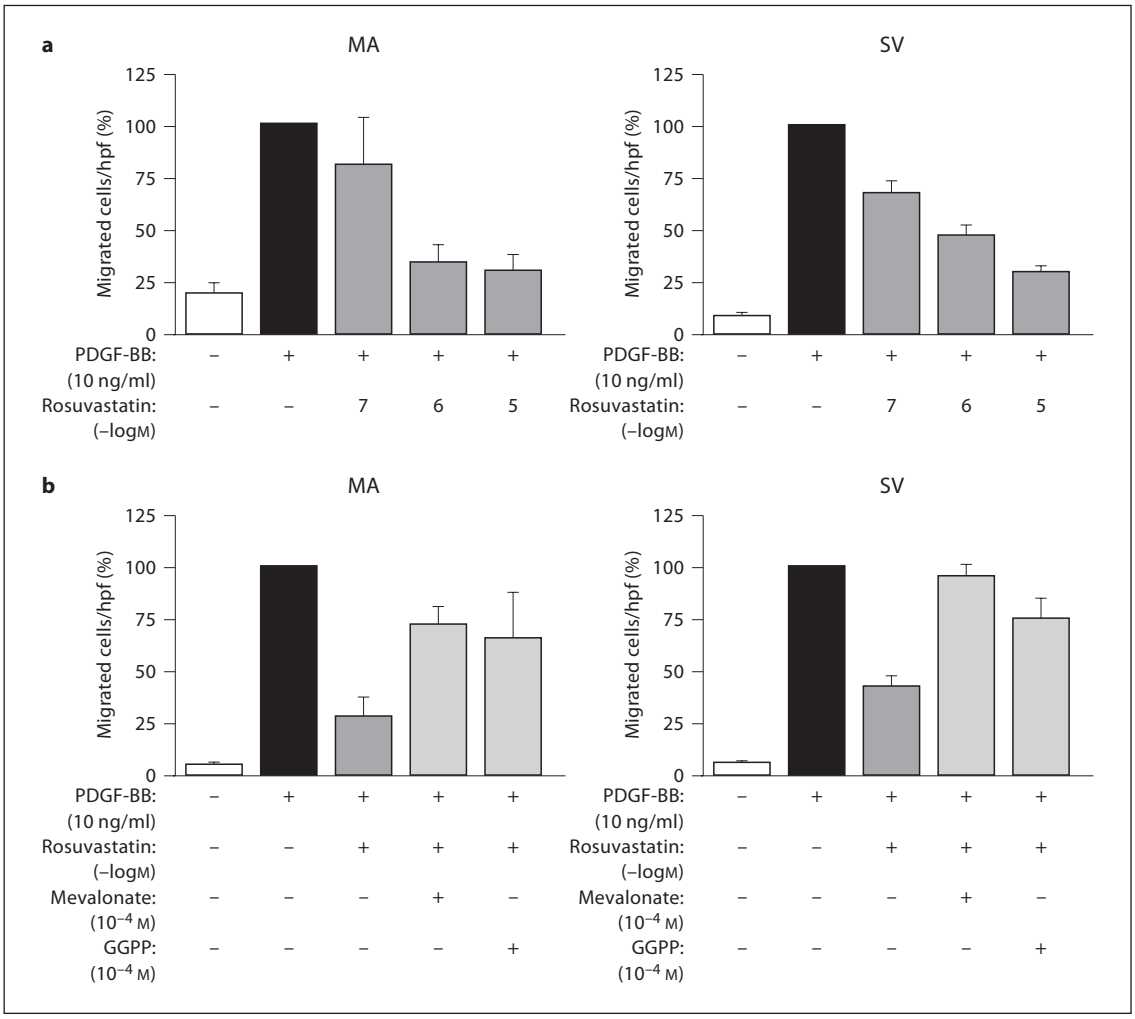
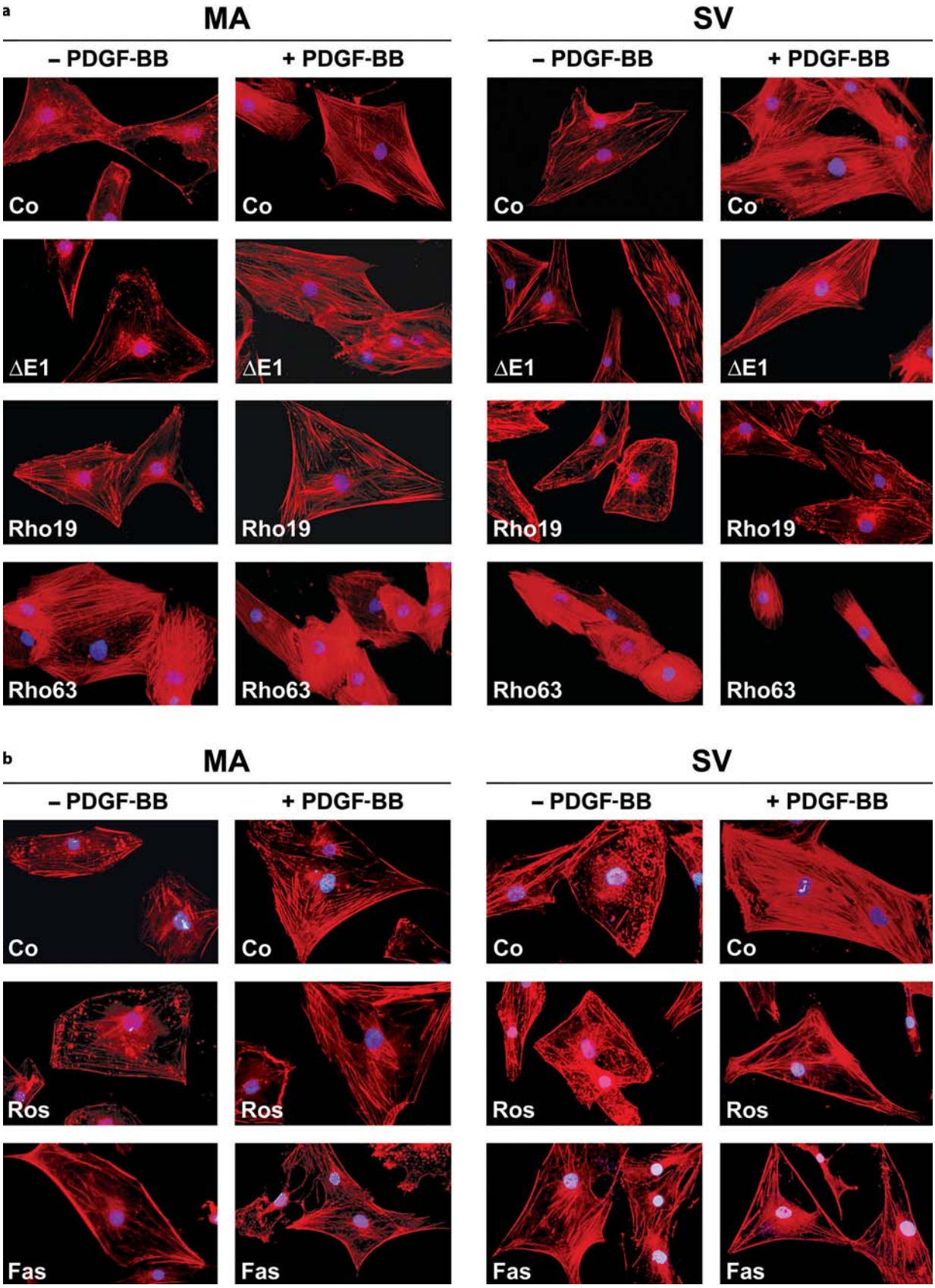


Fig. 3. Statin inhibits migration of VSMC from MA and SV. **a** Rosuvastatin induces a concentration-dependent inhibition of VSMC migration from MA and from SV. **b** Mevalonate and GGPP rescue inhibition of migration by rosuvastatin in VSMC from MA and from SV.

PDGF-BB is known to play a major role in atherogenesis and bypass graft disease, the lower migration rates of VSMC from MA are consistent with the atherosclerosis resistance of this vessel [7, 25].

The vascular system is heterogenous not only in morphological, but also in functional terms; indeed, VSMC from different vascular beds vary with respect to both aspects [12, 26], and the distribution of receptors along the vascular tree is particularly heterogenous [27]. Hence, it is not surprising that PDGFR expression differs in VSMC from MA as compared to SV. Although PDGF-BB activates two receptors on VSMC, only PDGFR-β mediates migration [22]. Our observations demonstrate that PDGFR-β expression is lower in VSMC from MA than SV, which is indeed consistent with the lower migration rates of the arterial VSMC.

Heterogeneity of VSMC at the receptor level does not exclude additional differences in signal transduction. Small GTPases of the Rho family play a crucial role in VSMC migration [28], and RhoA is a well-described intracellular target of PDGF-BB located downstream of PDGFR-β [23]. Therefore, the lower RhoA activity observed in VSMC from MA as compared to those from SV is consistent with both the lower migration rates and the lower PDGFR-β expression of MA. This is in contrast to ERK activation; a previous study, which did, however, not examine migration in VSMC from MA and SV, observed that PDGF-induced ERK activation was similar in these



cells [12]. Hence, these observations support the interpretation that there are additional levels of heterogeneity between VSMC from MA and SV apart from that occurring at the receptor level.

RhoA activation is also important for the assembly of actin stress fibers [29–31]. A role for RhoA in stress fiber formation of VSMC from MA and SV was confirmed using overexpression of mutant forms of RhoA. Indeed, PDGF-BB-induced stress fiber formation was impaired when cells were transduced with a dominant negative RhoA mutant; conversely, abundant stress fiber assembly was detected after overexpression of a constitutively active RhoA mutant. In line with this observation, stress fiber formation was also impaired by inhibition of RhoA or ROCK with rosuvastatin or hydroxyfasudil, respectively. Hence, RhoA activation induces stress fiber formation in VSMC from human bypass vessels. Stimulation with PDGF-BB elicited only a weak increase in stress fiber formation in VSMC from MA, while this effect was pronounced in VSMC from SV, demonstrating that the lower Rho A activity in VSMC from MA as compared to SV is reflected in a less pronounced stress fiber formation. This finding is consistent with the lower expression of PDGFR-β in VSMC from MA as compared to SV. The specific role of stress fiber formation in VSMC migration, however, remains somewhat controversial, and further studies are needed to clarify this issue [30–33].

Inhibitors of RhoA such as HMG-CoA reductase inhibitors (statins) impair migration of SV VSMC as well as neointima formation in human SV [9]. Similarly, the ROCK inhibitor hydroxyfasudil inhibits VSMC migration as well as intimal hyperplasia [20]. As VSMC from SV exhibit higher migration rates and higher RhoA activity than those from MA, the present study implies that inhibition of VSMC migration by inhibitors of the Rho/ROCK pathway may be beneficial for the treatment of bypass graft disease. Indeed, for the first time, statin treatment was shown to render VSMC from SV similar to those from MA in terms of both migration capacity and RhoA activity. A beneficial effect of statins in the setting of SV graft disease is underscored by the observation that such inhibitors can also impair vasospasm, a frequent problem with SV grafts [31, 34].

Taken together, the present data indicate for the first time that migration in response to PDGF-BB is lower in VSMC from MA than SV, and that this difference occurs due to a lower PDGFR-β expression resulting in a lower RhoA activation. The latter is reflected in a lower stress fiber formation in VSMC from MA than SV. Given the importance of VSMC migration for the pathogenesis of bypass graft disease, these findings reveal an important and novel mechanism for the superior patency rates of MA grafts. Conversely, inhibition of the Rho/ROCK pathway by rosuvastatin or hydroxyfasudil may prove beneficial for the treatment and/or prevention of bypass graft disease.

Acknowledgement

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Fig. 4. Stress fiber formation in VSMC from MA and SV. **a** PDGF-BB-induced stress fiber formation is less pronounced in VSMC from MA than SV (upper panel). Transduction with a constitutively active RhoA mutant induces stress fiber formation in both vessels (middle panel), while a dominant negative mutant prevents stress fiber formation (lower panel). **b** Preincubation with rosuvastatin (Ros, middle panel) or hydroxyfasudil (Fas, lower panel) prevents stress fiber assembly in VSMC from both MA and SV. Figures show representative sections; all pictures were taken at 200× magnification.

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