Biology of Vascular Smooth Muscle Cells from Coronary Artery Bypass Vessels

Inauguraldissertation

der Philosophisch-naturwissenschaftlichen Fakultät

der Universität Bern

vorgelegt von

Karin Frischknecht

von Herisau (AR)

Leiter der Arbeit:

Prof. Dr. F.C. Tanner

Kardiovaskuläre Forschung

Universität Zürich

2004

Biology of Vascular Smooth Muscle Cells from Coronary Artery Bypass Vessels

Inauguraldissertation

der Philosophisch-naturwissenschaftlichen Fakultät

der Universität Bern

vorgelegt von

Karin Frischknecht

von Herisau (AR)

Leiter der Arbeit:

Prof. Dr. F.C. Tanner

Kardiovaskuläre Forschung

Universität Zürich

Von der Philosophisch-naturwissenschaftlichen Fakultät angenommen.

Bern, 16. Dezember 2004 **Der Dekan: Prof. Dr. P. Messerli**

Acknowledgements

The present work was performed at the Department of Cardiovascular Research at the University Zürich and the University Hospital Bern.

It was financially supported by the Swiss National Foundation (grant number 3200B0-102232/1), the Swiss Heart Foundation, the Novartis Foundation, and the Schweizerische Rentenanstalt.

In the first instance I would like to express my gratitude to my supervisor, Prof. Felix C. Tanner, for giving me the opportunity to do my thesis under his supervision. I thank him for all his advice, help and teaching over the course of the past three years.

I am very grateful to Professor Thomas F. Lüscher as the head of our institute for his support. I also want to thank Professor Beat Suter and Professor Daniel Schümperli from the Institute of Cell Biology at the University Bern for supervising my work and Professor Zhihong Yang from the Department of Medicine at the University Fribourg to act as second reviewer of my thesis.

A very big thank-you goes to Helen Greutert for her help and advice, but also for sharing some of her experimental set-ups and “know-how” and taking over the cell culturing every time I was absent from the lab.

My thoughts also go to the numerous members of the institute, who helped me in doing experimental work and by setting up the necessary facilities.

Above all, I would like to thank my mum, my brother Beat, Thomas and Silvan, to whom all I dedicate this thesis. Thank you for your support and your love.

Summary

Long-term patency rates of coronary artery bypass vessels are determined by atherosclerosis. Proliferation and migration of vascular smooth muscle cells (VSMC) play a key role in neointima formation and generation of atherosclerosis. Patients who only receive a saphenous vein (SV) bypass graft have a significantly worse long-time survival as compared to those patients, who together with the SV also receive an internal mammary artery (MA) bypass graft. More recently, the radial artery (RA) has also been used as a bypass graft. RA grafts show an excellent patency, which is not significantly lower than that of MA. The present study was undertaken in order to analyze the proliferation and migration of VSMC of coronary artery bypass vessels from the different bypass grafts. Both proliferation and migration was examined in response to platelet-derived growth factor (PDGF-BB), which is an important mitogen and chemotaxin in atherosclerosis. Moreover thrombus formation is a major source of PDGF-BB and a key event in the pathogenesis of bypass graft disease.

In the first part of this study I tried to find out, whether, and if so, why proliferation of VSMC from RA, MA, and SV differs according to patency rates of the three vessels. RA VSMC exhibited only minimal growth to PDGF-BB, which was comparable to MA and differed from SV. PDGF receptor α expression was similar, while receptor β expression was higher in RA as compared to MA or SV. Cell cycle distribution and cell cycle regulation of the three vessels were identical. In contrast, cell death was higher in VSMC from MA than from RA and even more so from SV. The differences in proliferation of the three vessels was abrogated by specific caspase inhibitors. Akt activation was lower in VSMC from MA than from RA, and even more so than from SV. The differences in proliferation of the three vessels was abrogated by constitutively activated Akt. Taken together, these findings indicate that different apoptosis levels due to different Akt activation rather than different cell cycle regulation account for the different increase in cell number of VSMC from RA, MA, and SV. Thus, VSMC apoptosis may protect RA and MA from bypass graft disease.

In the second part of my work, I compared the migration of VSMC from RA, MA and SV. VSMC from RA and MA migrated slower than that from SV in response to PDGF-BB. Migration of all three vessels was concentration-dependent. Rho activity of VSMC from RA and MA was lower as compared to SV, suggesting that different Rho activity of the three vessels determines the different migration rates. Addition of the Rho inhibitor rosuvastatin or the ROCK inhibitor hydroxyfasudil blunted migration of VSMC from all three vessels, demonstrating that the Rho / ROCK pathway is necessary for migration in these cells.

Thus, the third part of the study focused on the interaction of mediators from platelets, such as PDGF, and the coagulation cascade, such as thrombin. The interplay of PDGF-BB and thrombin at the level of cell cycle regulation and signal transduction was assessed in SV VSMC. Thrombin potentiated threshold concentrations of PDGF-BB. This effect was concentration-dependent and inhibited by the direct thrombin inhibitor hirudin. Thrombin did not alter expression of PDGF receptors. Cell cycle protein expression of G1 phase was neither affected by thrombin nor PDGF-BB alone. In contrast, thrombin plus PDGF-BB caused p27 downregulation, without effecting expression of other cell cycle regulatory proteins in G1 phase. Inhibition of PI3 kinase completely abolished both VSMC proliferation and p27 downregulation in response to thrombin plus PDGF-BB. In contrast, inhibition of mammalian target of rapamycin (mTOR) and the MAP kinase cascade only slightly reduced proliferation and did not affect p27 expression. Thus, thrombin potentiates proliferation to threshold concentrations of PDGF-BB in a concentration-dependent manner. This potentiation is mediated by activation of PI3 kinase leading to downregulation of p27, while neither mTOR nor the MAP kinase cascade are significantly involved.

In summary, proliferation and migration of VSMC from coronary artery bypass vessels differ according to long-term patency rates. This indicates that intrinsic differences in the biological properties of this cell type may influence the propensity to atherosclerosis in these vessels. Such differences could be identified with respect to regulation of apoptosis and migration, but not cell

cycle progression. The improved understanding of the biology of these vessels may influence the current treatment of patients and thus translate into clinical applications.

Table of Contents

1. General Introduction 1

1.1. Coronary artery bypass grafting 1

1.2. The role of vascular smooth muscle cells 4

1.3. The potential role of platelet-derived growth factor 5

1.4. The potential role of thrombin 5

1.5. References 8

2. Methods 10

2.1. Cell culture and proliferation 10

2.2. Cell attachment and migration 11

2.3. Flow cytometry and death assays 11

2.4. Caspase inhibition 12

2.5. Western blot analysis and kinase assays 13

2.6. Rho A activity and expression 13

2.7. Adenoviral transduction 14

2.8. Statistics 14

2.9. References 14

3. Hypothesis 16

4. Part One 18

4.1. Introduction 18

4.2. Results 20

4.2.1. Increase in cell number 20

4.2.2. Receptor expression 23

4.2.3. Cell cycle 23

4.2.4. Cell death 27

4.2.5. Caspase Inhibition 29

4.2.6. Akt Activity 31

4.2.7. Celecoxib treatment 31

4.3. Discussion 36

4.4. References 39

5. Part Two 42

5.1. Introduction 42

5.2. Results 43

5.2.1. Migration 43

5.2.2. PDGF-receptor expression 43

5.2.3. Signal transduction 46

5.3. Discussion 49

5.4. References 50

6. Part Three 52

6.1. Introduction 52

6.2. Results 56

6.2.1. Proliferation rates 56

6.2.2. Receptor expression 60

6.2.3. Cell cycle 60

6.2.4. Signal transduction 63

6.2.5. Potency of potentiation 67

6.3. Discussion 71

6.4. References 75

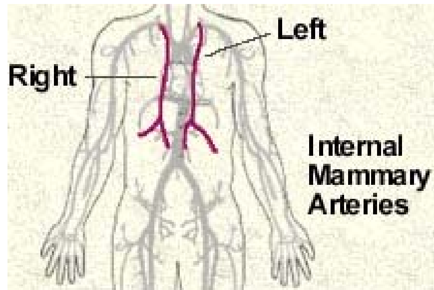
7. Conclusions and Outlook 79

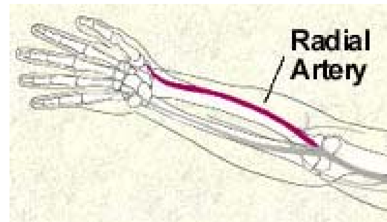
1. General Introduction

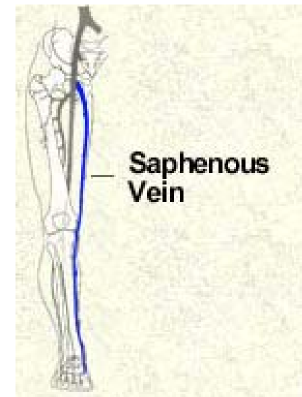
1.1. Coronary artery bypass grafting

Atherosclerosis is the most common cause of artery diseases, such as coronary artery disease, peripheral artery disease and cerebral artery disease. It can become manifest in myocardial infarction and stroke. Not surprisingly, therefore, atherosclerosis accounts for about half of both morbidity and mortality in Western countries.[1] Patients with coronary artery disease involving two or three coronary arteries and persisting symptoms despite pharmacological therapy can be treated by a coronary artery bypass grafting (CABG). The choice of conduits in coronary artery surgery is restricted to lower extremity superficial veins and a variety of small arteries. The two classic bypass grafts are the saphenous vein (SV) and the internal mammary artery (MA) (Figure 1). In the 1960s both the first successful human SV coronary artery bypass was performed by Garrett and colleagues and the first direct MA artery to coronary artery anastomosis in a human was done by Kolessov and colleagues.[2,3,4] For a long time the SV was the graft most often used in coronary artery surgery.[5] Reasons for that were the following: the SV is quick and easy to harvest, it is always long enough, it consists of stronger tissue, therefore it is not as delicate as the MA, and it is larger in caliber than the MA, rendering surgery technically easier. Nevertheless, with time and notable exceptions it can be assumed that all vein grafts used in the aortocoronary circuit will fail and few will exceed 20 years. Long-term patency rates of MA, however, are much better as compared to that of SV reaching 97.5% for the MA as compared to 67% for the SV after 9 years.[6] Similarly, mortality rates of patients with 2-vessel and 3-vessel disease are improved when the MA is included in the revascularisation (Figure 2A).[7] Today, these facts make the MA the graft of choice to revascularize the coronary artery.

A







B

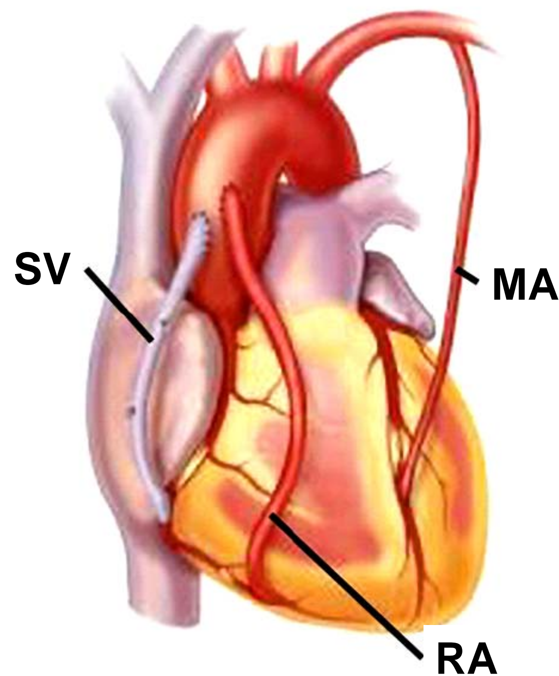
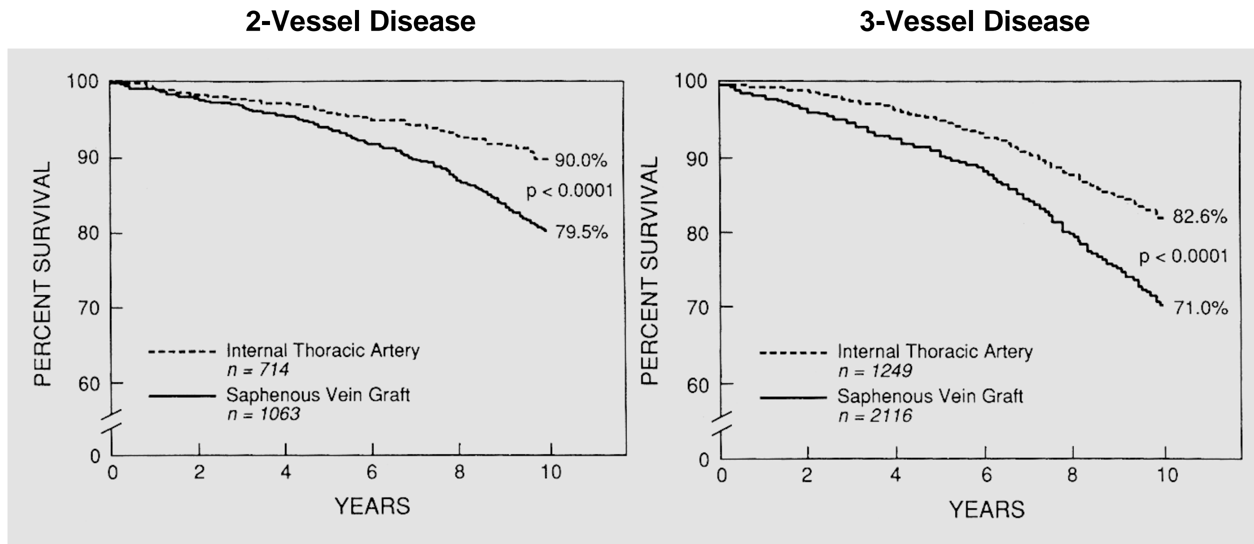


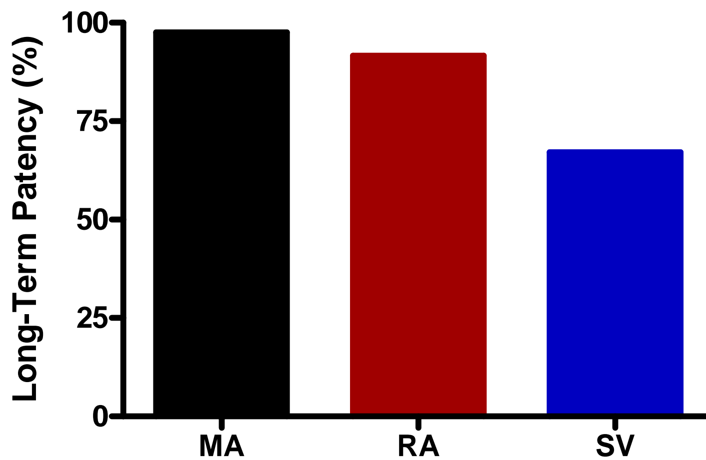
Figure 1. Blood vessels used for different bypass grafts **A.** The internal mammary artery (MA) is located on the inside of the chest cavity, the radial artery (RA) in the forearm, and the saphenous vein (SV) in the leg. **B.** The MA is detached from the chest wall and the open end attached to the coronary artery below the blocked area. A section of RA and SV is harvested. One end is inserted into the aorta and the other end is attached to the coronary artery below the blocked area.

A



Loop F.D. et al. N Engl J Med. 1986;314:1-6

B



Possati G. et al. Circulation. 2003;108:1350-1354

Figure 2. Patency rates of coronary artery bypass vessels **A.** Survival rate after coronary artery bypass operation. Mortality of patients with 2- or 3-vessel disease is reduced when the MA is included. **B.** Long-term patency rates of RA (91.6%) bypass grafts look quite promising and are in between those of MA (97.5%) and SV (67.1%).

Unfortunately, the MA does not provide enough material to do a complete revascularization; therefore and because of the advantageous properties of the MA as compared to the SV, other types of arteries were considered as coronary artery bypass grafts. As an alternative arterial graft, the radial artery (RA) is used increasingly.[7,8,9] The RA gained wide popularity because of its favourable anatomic position, calibre, length, and ease to harvest. Excellent early, mid-term, and long-term patency rates were reported (Figure 2B).[6,9] Nevertheless, native RA can become atherosclerotic and, consistent with this observation, grafted RA can develop neointimal changes favoring graft occlusion.[10,11]

Compared to the MA, the RA has a thicker wall and a higher density of vascular smooth muscle cells (VSMC) indicating a tendency of this vessel towards vasospasm. Further, the RA shows a more pronounced neointima formation rendering the graft susceptible to the development of atherosclerosis. Indeed, in up to 2% of patients the RA can not be harvested for bypass grafting because of severe atherosclerotic calcification. The functional properties of RA VSMC are not well characterized yet. Such information, however, may be useful for inhibiting neointima formation and thereby preventing atherosclerotic changes leading to improved graft function.

1.2. The role of vascular smooth muscle cells

Cardiovascular risk factors induce progressive vascular injury leading to vascular dysfunction. Features of dysfunctional arteries include platelet aggregation, monocyte invasion, VSMC proliferation and migration (Figure 3). Moreover, endothelium, platelets, and monocytes release mitogens as well as chemokines for VSMC; indeed, high concentrations of these substances are found in atherosclerotic lesions.[1] Therefore, proliferation and migration of VSMC importantly contribute to the generation of atherosclerotic lesions.

Coronary artery bypass graft disease occurs faster than atherogenesis; such lesions develop within a few weeks to months. The pathogenesis, however, is similar; indeed, a grafted vessel is subjected to endothelial injury and bypass graft disease should be seen as the response of the vessel to this injury. Early after implantation, thrombosis plays a critical role both by predisposing to

acute occlusion and by serving as a stimulus for neointima formation. Within several weeks after implantation, virtually all vein grafts develop intimal hyperplasia, which renders the graft susceptible to atherosclerosis although only modestly compromising the graft lumen. Thus, bypass graft disease is a repair process involving changes similar to those occurring in atherogenesis, and proliferation and migration of VSMC are important for the development of these changes. As VSMC accumulate in the subendothelial space of grafted vessels and are a major mediator of neointima formation they play an important role in long-term patency rates.[12]

1.3. The potential role of platelet-derived growth factor

Growth factors such as platelet-derived growth factor (PDGF) are released from aggregating platelets, monocytes, and VSMC. As platelet adhesion to bypass grafts begins soon after surgery, VSMC are exposed to PDGF from this early time point on.[13] Moreover, PDGF is a potent mitogen in proliferation and migration. Therefore, PDGF may be an important determinant of neointima formation in bypass grafts.[13] However, the presence of this mitogen does not explain why different grafts develop a different degree of neointima formation. Indeed, such differences may rather be related to different intrinsic properties of VSMC from coronary artery bypass vessels.

1.4. The potential role of thrombin

Generally injuries damage the endothelial layer covering the intimal surface of blood vessels. One of the first events after such endothelial cell denudation is the generation of thrombin at the site of injury. Thrombin is a serine protease of the coagulation cascade that specifically cleaves fibrinogen to fibrin, thereby stabilizing newly formed clots (Figure 4). Thrombin also activates platelets to adhere, aggregate, and release the contents of their α granules and is thereby a mediator of the intravascular coagulation seen in acute myocardial infarction and unstable angina pectoris. The thrombin receptor, which mediates these cellular effects, is highly expressed in atherosclerotic lesions and in the neointima produced by vascular injury.[14,15] Thus, a growing body of evidence

implicates thrombin in vascular disease. Therefore thrombin may play an important role in VSMC regulation.

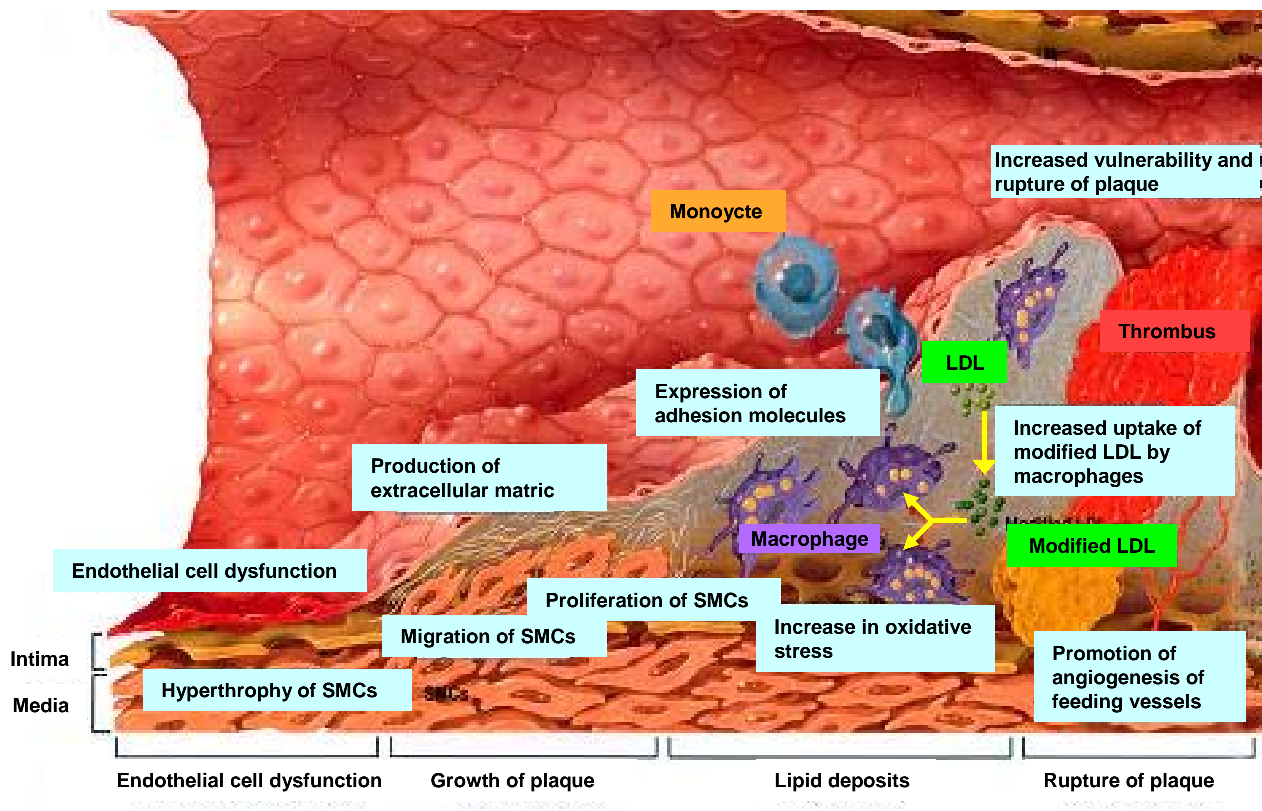


Figure 3. Progression of atherosclerosis. Migration, hypertrophy, and proliferation of smooth muscle cells, as well as an increased production of fibronectin and collagen which form the extracellular matrix, are the first steps in atherogenesis. By the expression of adhesion factors in the endothelial cells, the penetration of monocytes, which differentiate into macrophages, into the intima is promoted. An increase in the oxidation function of macrophages accelerates modification of LDL and promotes ingestion of the modified LDL by macrophages. These actions result in aggravation of atherosclerosis by effects at each stage of the disease.

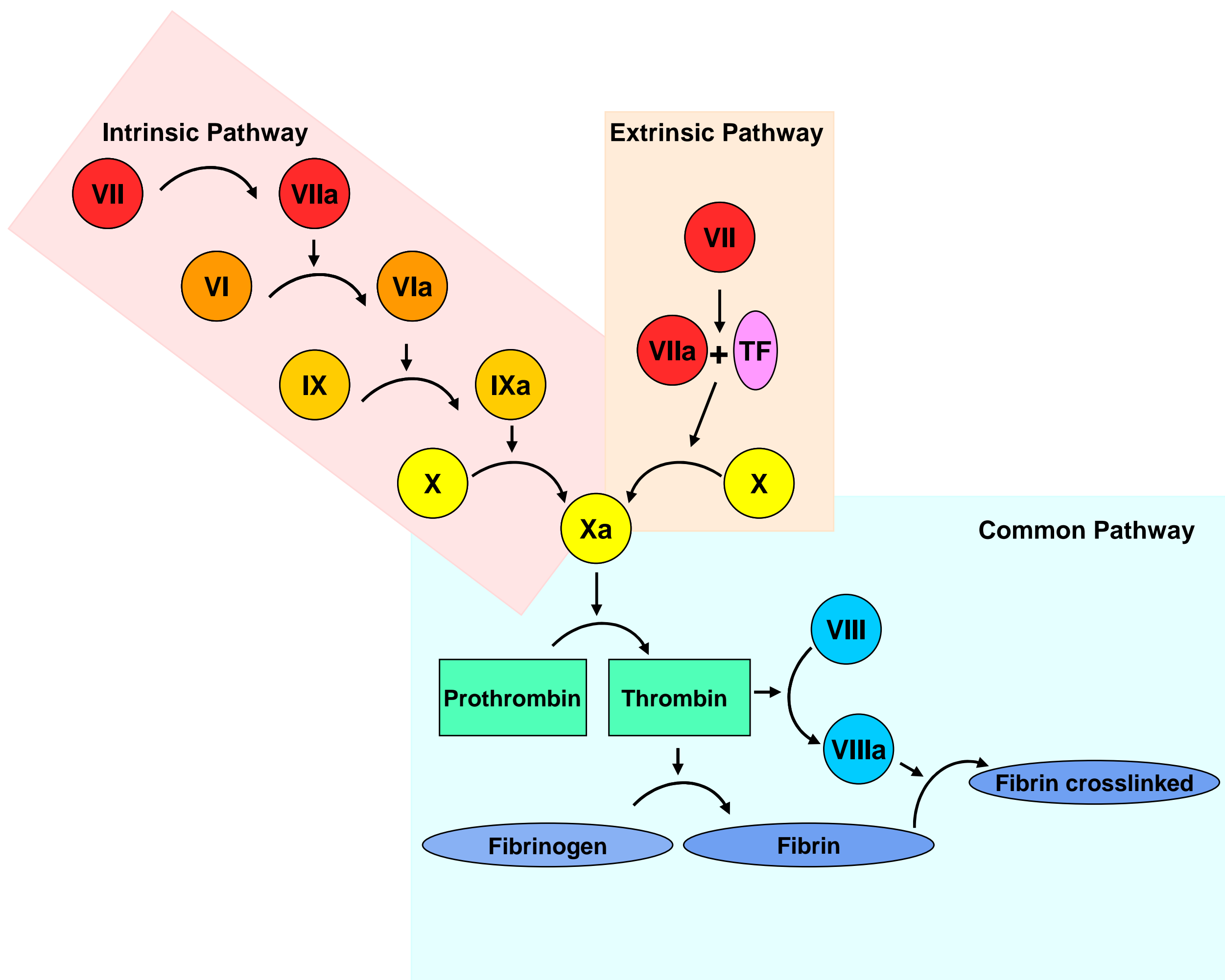


Figure 4. The coagulation cascades: The intrinsic cascade is initiated when contact is made between blood and exposed endothelial cell surfaces. The extrinsic pathway is initiated upon vascular injury which leads to exposure of tissue factor (TF), a subendothelial cell-surface glycoprotein that binds phospholipid. The two pathways converge at the activation of factor X to Xa. Factor Xa has a role in the further activation of factor VII to VIIa. Active factor Xa hydrolyzes and activates prothrombin to thrombin. Thrombin can then activate factors XI and VIII furthering the cascade. Ultimately the role of thrombin is to convert fribrinogen to fibrin and to activate factor XIII to XIIIa. Factor XIIIa cross-links fibrin polymers solidifying the clot.

1.5. References

1. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993; 362:801-809
2. Garrett HE, Dennis EW, DeBakey ME. Aortocoronary bypass with saphenous vein graft. Seven-year follow-up. *JAMA*. 1973;223:792-794.
3. Kolessov VI. Mammary artery - coronary artery anastomosis as a method for treatment of angina pectoris. *J Thorac Cardiovasc Surg*. 1967;54:535-544
4. Olearchyk AS. Vasilii I. Kolesov. A pioneer of coronary revascularization by internal mammary-coronary artery grafting. *J Thorac Cardiovasc Surg*. 1988;96:13-18.
5. Grondin CM, Campeau L, Thornton JC, Engle JC, Cross FS, Schreiber H. Coronary artery bypass grafting with saphenous vein. *Circulation*. 1989;79:I24-I29
6. Possati G, Gaudino M, Prati F, Alessandrini F, Trani C, Glieca F, Mazzari MA, Luciani N, Schiavoni G. Long-term results of the radial artery used for myocardial revascularization. *Circulation*. 2003;108:1350-1354
7. Loop FD, Lytle BW, Cosgrove DM, Stewart RW, Goormastic M, Williams GW, Golding LA, Gill CC, Taylor PC, Sheldon WC. Influence of the internal-mammary artery graft on 10-year survival and other cardiac events. *N Engl J Med*. 1986;314:1-6
8. Grondin CM. Late results of coronary artery grafting: is there a flag on the field? *J Thorac Cardiovasc Surg*. 1984;87:161-166
9. Acar C, Jebara VA, Portoghese M, Beyssen B, Pagny JY, Grare P, Chachques JC, Fabiani JN, Deloche A, Guermonprez JL. Revival of the radial artery for coronary artery bypass grafting. *Ann Thorac Surg*. 1992;54:652-659
10. Acar C, Ramsheyi A, Pagny JY, Jebara V, Barrier P, Fabiani JN, Deloche A, Guermonprez JL, Carpentier A. The radial artery for coronary artery bypass grafting: clinical and angiographic results at five years. *J Thorac Cardiovasc Surg*. 1998;116:981-989

11. Ruengsakulrach P, Sinclair R, Komeda M, Raman J, Gordon I, Buxton, B. Comparative histopathology of radial artery and risk factors for development of intimal hyperplasia and atherosclerosis. *Circulation*. 1999;100(19Suppl):II139-144
12. Loscalzo J. Vascular matrix and vein graft failure. Circulation. 2000;101:221-223
13. Ross R. Platelets, platelet-derived growth factor, growth control, and their interactions with the vascular wall. *J Cardiovasc Pharmacol*.1985;7:186-190
14. Nelken NA, Soifer SJ, O'Keefe J, Vu TK, Charo IF, Coughlin SR. Thrombin receptor expression in normal and atherosclerotic human arteries. *J Clin Invest*. 1992;90:1614-1621
15. Wilcox JN, Rodriguez J, Subramanian R, Ollerenshaw J, Zhong C, Hayzer DJ, Horaist C, Hanson SR, Lumsden A, Salam TA, et al. Characterization of thrombin receptor expression during vascular lesion formation. *Circ Res*. 1994;75:1029-1038

2. Methods

2.1. Cell culture and proliferation

VSMC of RA, MA, and SV were isolated from patients undergoing CABG, which was approved by the local Committee on Ethics of Human Research. 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), 2 mM L-glutamine and 100 U/ml streptomycin (all from Invitrogen Life Technologies, Groningen, The Netherlands). Cells from passage 3 to 12 were used for experiments. For G0/G1 arrest, cells were kept in serum-free medium containing 0.1% BSA Fraction V (Sigma, St.Louis, MO). Cells were maintained in this medium for 48 hours prior to stimulation with 10% FCS, 1 to 10 ng/ml human platelet-derived growth factor (PDGF) BB (No. 220-BB, R&D Systems, Minneapolis, MN), 0.1 to 9.0 U/mL thrombin (No. T 7009, Sigma, St.Louis, MO, USA), 100 µM thrombin receptor activator peptide (TRAP, No. S 7152, Sigma, St.Louis, MO, USA). In some experiments, cells were incubated with 3 U/mL hirudin (No. 377853, Calbiochem, Darmstadt, Germany), 1 µM wortmannin (No. W1628), 100 nM rapamycin (No. R0395 ; both Sigma, St.Louis, MO, USA), 50 µM LY294002 (No. 9901), 30 µM PD98059 (No. 9900; both Cell Signaling Technology, Beverly, MA), 10 µM celecoxib (Pfizer, Zürich, Switzerland), 10 µM hydroxyfasudil (No. H4413, Sigma, St.Louis, MO, USA), 10 µM rosuvastatin (generous gift from Astra Zeneca, Switzeland). Cell number was determined after 0, 2, 4, 6 days by an Improved Neubauer hemacytometer. G1/S-progression was evaluated by ^{3}H-thymidine incorporation; 20 hours after mitogenic stimulation, VSMC were pulsed with ^{3}H-thymidine for 4 hours, and incorporated ^{3}H-thymidine was detected by a β-counter (Betamatic V, Kontron Instruments, Milan, Italy).[1]

Toxicity of the inhibitors LY294002, PD98059, rapamycin, rosuvastatin, hydroxyfasudil on VSMC was quantified by a colorimetric assay based on lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells (Roche Diagnostics, Mannheim, Germany). VSMC were

stimulated with mitogens in the presence or absence of the respective inhibitor for 30 hours. VSMC culture supernatant was collected and 100 µl was incubated for 30 minutes with an equal amount of reaction mixture. Absorbance was measured at 490 nm using an ELISA reader (MR 5000, Dynatech Laboratories, Alexandria, VA).

2.2. Cell attachment and migration

For assessing attachment, subconfluent VSMC were seeded on 6-well tissue culture dishes and incubated with 10 ng/ml PDGF-BB (No. 220-BB, R&D Systems, Minneapolis, MN) for 5 hours. Cells were then washed, attached cells were trypsinized and cell number was determined by an Improved Neubauer hemacytometer. VSMC migration was examined in a Boyden chamber (Neuroprobe, Gaithersburg, MD). Polycarbonate filters (5 µm pore) were coated with type I collagen (Vitrogen®, Nutacon, Leimuiden, The Netherlands) for 1 hour at room temperature and air-dried for 10 minutes immediately before use. To assess migration in the presence of the Rho A inhibitors hydroxyfasudil (10 µM) or rosuvastatin (10 µM). VSMC were pretreated for 30 minutes with the respective inhibitor. Different concentrations of the chemotactic substances (PDGF-BB 1-10 ng/ml) were added to the lower compartment. 50 µl of the cell suspension ($5x10^5$ cells/ml) were placed in the upper compartment. The chambers were incubated at 37° for 5 hours; then the upper surface of the filter was scraped off. The filters were fixed in 100% methanol for 10 minutes, stained with Diff-Quick (Dade Diagnostics, Auckland, New Zealand). The number of migrated cells was counted at 400 x magnification.

2.3. Flow cytometry and death assays

For analysis of PDGF receptor expression, VSMC were detached with 2 mM EDTA pH 8.0 in PBS, collected, and washed with PBS containing 20% FCS in part one, whereas in part three of this work VSMC were kept in DMEM + 0.1% BSA for 48 hours, then stimulated with 3 U/ml thrombin for 30 hours before detachment of cells. Cells were stained with anti-PDGF receptor α (No. 556001, Pharmingen, San Diego, CA) or β antibody (No. MAB1263, R&D systems, Minneapolis, MN) for 1

hour on ice, and then incubated with rabbit anti-mouse IgG phycoerythrin conjugated antibody (No. P9287, Sigma, St.Louis, MO) for 1 hour on ice. Cells were washed, fixed with 1% paraformaldehyde, and analyzed by FACScalibur cytometer using CELLQuest software (Becton Dickinson, Franklin Lakes, NJ).

For analysis of cell cycle distribution, cells were harvested, washed twice in PBS, fixed in 2% paraformaldehyde for 60 minutes, and permeabilized in 0.2% Tween 20. The cells were then treated with 1 U RNase in 1 ml PBS for 30 minutes at 37°C, resuspended in 0.03 mg/ml propidium iodide, and analyzed by flow cytometry.[2] For quantification of fragmented DNA, VSMC were harvested, washed twice in PBS, fixed with 2% p-formaldehyd for 1 hour, permeabilized in 0.2% Tween 20 for 15 minutes at 37°C, stained with 0.03 mg/ml propidiumiodide for 1 hour at 37° and analyzed by flow cytometry. Cell death was also examined using a colorimetric assay for lactate dehydrogenase (LDH) activity (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Apoptosis was determined by staining trypsinized VSMC with 10 μg/ml Hoechst 33258 (No. B1155, Sigma, St.Louis, MO) for 5 minutes, which were then washed twice with PBS + 10% FCS, resuspended in 25 μl PBS and put onto a microscope slide. 300 cells were analyzed by fluorescent microscopy and the number of Hoechst-positive cells showing condensed chromatin was determined.[3]

2.4. Caspase inhibition

Cells were growth arrested and then stimulated with 10 ng/ml of PDGF-BB plus 50 μM of the specific caspase inhibitor Z-VAD-fmk (Bachem, Heidelberg, Germany) or 30 μM of another specific caspase inhibitor Boc-D-fmk (No. 218745, Calbiochem, Darmstadt, Germany). Cell number was determined after 4 days of mitogenic stimulation.

2.5. Western blot analysis and kinase assays

Western blot analysis of cell cycle proteins was performed on whole-cell lysates. VSMC were lysed in buffer as described.[4] Protein concentration was determined using the Bio-Rad assay (Bio-Rad Laboratories GmbH, Munich, Germany). 100 μg of protein were loaded per lane, resolved by SDS-PAGE under reducing conditions, and blotted on Immobilon-P transfer membranes (Millipore, Bedford, MA).[4] Membranes were incubated with antibodies against Cyclin E (No. sc-198), cdk2 (No. sc-163), p27Kip1 (No. sc-528; all from Santa Cruz Biotechnology Inc., Santa Cruz, CA), p21Cip1 (No. 65961A), p57Kip2 (No. 556346; both from Pharmingen, San Diego, CA), Akt (No. 9272), p-Akt (No. 9271; both from Cell Signaling Technology, Beverly, MA), acetylated tubulin (No. T-6793, Sigma, St.Louis, MO), Rho A No. (sc-179, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitations for cdk2 kinase assays were performed as described.[4] The kinase reaction was performed for 30 minutes at 37°C in the presence of 10 µCi [32-P]ATP (Amersham Pharmacia Biotech, Buckinghamshire, UK) and 1 µg histone H1 (Boehringer, Mannheim, Germany).[4] Labeled proteins were resolved on 12% SDS-PAGE and subjected to autoradiography. Immunoprecipitations for Akt kinase assays were carried out according to manufacturer's instructions by a Akt kinase assay kit (No. 9840, Signaling Technology, Beverly, MA). The kinase reaction was performed for 30 minutes at 30°C in the presence of 200 µM ATP and 1 µg GSK-3 fusion protein. Samples were centrifuged, supernatant was loaded on a 12% SDS-PAGE gel and Western Blotting for phosphorylated GSK3-β was performed as described.[4]

2.6. Rho A activity and expression

VSMC were stimulated with PDGF-BB (10 ng/ml) for 30 minutes and then lysed in buffer as described.[4] Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany). For Rho A activity, 100 µg of protein were incubated with Rhotekin Rho bound to glutathion-agarose beads (No.14-383, Upstate, Lake Placid, NY) at 4°C for 45 minutes. The beads were washed four times with lysis buffer; afterwards, bound proteins were

eluted in sample buffer and analyzed by Western blotting. All values for active Rho A were normalized to total Rho A expression.

2.7. Adenoviral transduction

Cells were transduced with 800 pfu of an adenoviral vector expressing a constitutivly active Akt mutant (Ad-HA-m/p-PKB), kindly provided by Brian A. Hemmings, Ph.D, Friedrich Miescher Institute Basel, Switzerland and Zhihong Yang, MD, University of Fribourg, Switzerland, or a control vector with GFP insert in DMEM + 2% FCS for 1 hour at 37°C.[5,6] Medium was then removed and cells were stimulated with proliferation medium. Cell number was determined after 4 days of mitogenic stimulation.

2.8. Statistics

Values represent mean±SEM from 4 to 6 independent experiments. For each of these experiments, VSMC from a different patient were used. Statistical evaluation was performed by analysis of variance (ANOVA). A p-value of <0.05 was considered to indicate a significant difference.

2.9. References

1. Rechler MM, Podskalny JM, Goldfine ID, Wells CA. DNA synthesis in human fibroblasts: stimulation by insulin and by nonsuppressible insulin-like activity. *J Clin Endocrinol Metab*. 1974;39:512-521
2. Tanner FC, Meier P, Greutert H, Champion C, Nabel EG, Lüscher TF. Nitric oxide modulates expression of cell cycle regulatory proteins. *Circulation*. 2000;101:1982-1989
3. Tokunou T, Shibata R, Kai H, Ichiki T, Morisaki T, Fukuyama K, Ono H, Iino N, Masuda S, Shimokawa H, Egashira K, Imaizumi T, Takeshita A. Apoptosis induced by inhibition of

cyclic AMP response element-binding protein in vascular smooth muscle cells. *Circulation*. 2003;108:1246-52

4. Tanner FC, Yang ZY, Duckers E, Gordon D, Nabel GJ, Nabel EG. Expression of cyclin-dependent kinase inhibitors in vascular disease. *Circ Res*. 1998;82:396-403

5. Ming XF, Viswambharan H, Barandier C, Ruffieux J, Kaibuchi K, Rusconi S, Yang Z. Rho GTPase/Rho kinase negatively regulates endothelial nictric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. *Mol Cell Biol*. 2002;22:8467-77

6. Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM, Hemmings BA. Role of translocation in the activation and function of protein kinase B. *J Biol Chem*. 1997;272:31515-24

3. Hypothesis

- **Part One:**

  Proliferation of Vascular Smooth Muscle Cells (VSMC) from Coronary Artery Bypass Vessels

Hypothesis 1

Patency rates of RA bypass grafts (91.6%) after 9 years are in between those of MA (97.5%) and SV (67.1%). Patency loss is related to neointima formation. Permissive for such changes is VSMC proliferation. Thus, VSMC proliferation will differ in RA as compared to MA and SV.

Hypothesis 2

VSMC from RA as compared to MA and SV have different proliferation rates in response to serum and PDGF-BB, which are proportional to the degree of in vivo neointima formation. These different proliferation rates will be due to intrinsic differences in cellular function either related to differential receptor expression and/or signal transduction and/or cell cycle regulation.

- **Part Two:**

  Migration of VSMC from Coronary Artery Bypass Vessels

Hypothesis 3

Not only proliferation, but also migration is important in the development of bypass graft disease. Migration of VSMC from RA will therefore be different to that of MA and SV.

Hypothesis 4

VSMC from RA, MA and SV have different migration rates. This different migration rates will be due to differences in cellular function, related to different signal transduction.

- **Part Three:**

  Interaction of Mediators Derived from Coagulation Cascade and Platelets on VSMC Proliferation

Hypothesis 5

Fibrin formation and platelet aggregation occur at the same sites of vascular injury and promote each other. As mediators derived from coagulation cascade and platelets are present at the same sites, there will be interactions at the level of VSMC proliferation.

Hypothesis 6

The different proliferation rates of VSMC stimulated with PDGF only, thrombin only, or PDGF plus thrombin will be due to differences in PDGF receptor expression and/or signal transduction and/or cell cycle regulation.

4. Part One

Proliferation of Vascular Smooth Muscle Cells from Radial Artery - A Comparison with Mammary Artery and Saphenous Vein

4.1. Introduction

Coronary artery disease can be treated by bypass grafting (CABG). Internal mammary artery (MA) and saphenous vein (SV) are routinely used bypass grafts. However, patency rate of SV grafts is lower than that of MA grafts.[1] Therefore, alternative arterial grafts such as the radial artery (RA) are used increasingly.[2] Patency rate of RA seems to be nearly as good as that of MA, and much better than that of SV. [3,4]

Vascular smooth muscle cells (VSMC) accumulate in the subendothelium of grafted vessels mediate neointima formation. Hence, VSMC proliferation plays an important role in long-term patency of bypass grafts.[5] VSMC are a heterogenous population, and intrinsic differences of VSMC from different vascular beds have been observed. Therefore, VSMC proliferation may differ between RA, MA, and SV. Growth factors such as platelet-derived growth factor (PDGF) are released from aggregating platelets and produce extracellular matrix. As platelet adhesion to bypass grafts is initiated within minutes after grafting, VSMC of grafted vessels are exposed to PDGF from this early time point on; therefore, growth factors such as PDGF may regulate neointima formation in bypass grafts.[6] Moreover, VSMC from different bypass grafts may respond to growth factors in a different manner, leading to a higher degree of neointima formation in SV as compared to RA and MA.

Not only proliferation, but also apoptosis of VSMC plays an important role in the pathogenesis of human vascular diseases such as atherosclerosis and bypass graft disease.[7,8,9] Indeed, apoptosis of VSMC may reduce neointima formation, and the balance of VSMC proliferation versus apoptosis may be a more important determinant of long-term patency following bypass graft operation than

either parameter alone. Therefore, in the present study, we investigated how VSMC proliferation and apoptosis in presence of PDGF-BB is regulated in RA as compared to MA, and SV. I observed that proliferation rates of RA, MA, and SV correspond to patency rates. However, neither PDGF receptor expression nor VSMC cycle regulation could account for this difference; instead, different apoptosis rates due to different Akt activation were identified.

4.2. Results

4.2.1. Increase in cell number

VSMC from RA, MA, and SV were isolated by the explant method (Figure 1). Isolation was successful in 78% of RA, 56% of MA, and 100% of SV explants (Table 1). This was due to both a different number of explants exhibiting outgrowth and a different cellular outgrowth of the cells suggesting that VSMC proliferation may differ between RA, MA, and SV. Therefore, proliferation pattern of VSMC from random isolates of RA, MA, and SV was examined. Cell number was determined every other day for up to 6 days. In response to 10% FCS increase in cell number of VSMC from RA was higher than that of MA, but lower than that of SV (cell number on day 6: RA: 51‘520±7‘538, MA: 28‘365±6‘324, SV: 69‘750±11‘588; MA vs. SV: p<0.01; MA or SV vs. RA: p=n.s.; n=13 to 15; Table 1). The same pattern was observed when VSMC from all three vessels were derived from the same patient (cell number on day 6: RA: 55’281±8’755, MA: 27’938±6’733, SV: 92’031±22’432; MA vs. SV: p<0.02, MA or SV vs. RA: p=n.s.; n=4; Figure 2A). Thus, increase in cell number of VSMC from RA was in between that of MA and SV. To study the effect of a defined mitogen, VSMC were stimulated with 10 ng/ml of PDGF-BB. In contrast to the observation with FCS, RA VSMC exhibited only minimal increase in cell number to PDGF-BB, which was comparable to MA and differed from SV (RA: 15‘493±2‘116, MA: 15‘446±2‘768, SV: 33‘094±3‘028; RA vs. MA: p=n.s.; RA or MA vs. SV: p<0.01; n=4; Figure 2B). PDGF-BB induced exit from G0/G1 phase was determined by ^{3}H-thymidine incorporation 30 hours after stimulation of arrested cells. Consistent with increase in cell number, ^{3}H-thymidine incorporation was lower in RA and MA as compared to SV (DPM/1000 cells: RA: 150±21, MA: 138±20, SV: 269±14; RA vs. MA: p=n.s., RA or MA vs. SV: p<0.001; n=4; Figure 2C). Thus, RA VSMC growth is in between that of MA and SV after stimulation with 10% FCS, while it is as low as that of MA with PDGF-BB.

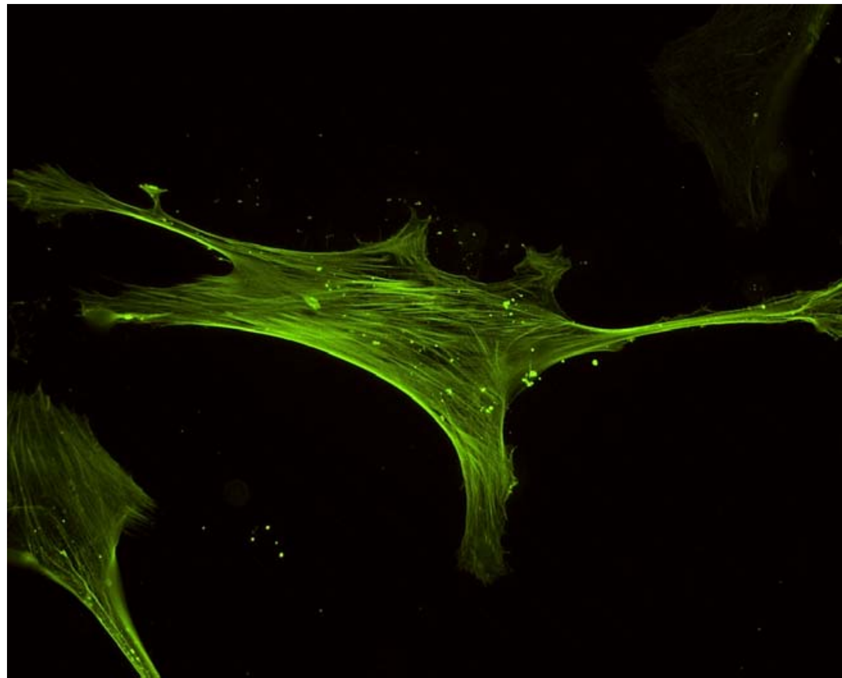


Figure 1. Vascular smooth muscle cells were identified by immunofluorescent staining for smooth muscle α actin.

	Vessel	MA	RA	SV
A.	**Explant outgrowth rates**	**55.6%**	**77.8%**	**100%**
B.	**VSM cell number (x 1'000)**			
	day 0	**13.2 ± 1.4**	**14.6 ± 1.0**	**15.6 ± 1.5**
	day 2	**17.0 ± 1.9**	**23.4 ± 2.0**	**29.1 ± 3.4**
	day 4	**21.7 ± 3.4**	**36.2 ± 4.6**	**45.6 ± 4.8**
	day 6	**28.4 ± 6.3**	**51.2 ± 7.5**	**69.8 ± 11.6**

Table 1. Different proliferation of vascular smooth muscle cells (VSMC) from human coronary artery bypass vessels. **A.** Explant outgrowth resulting in successful VSMC isolation was lower in internal mammary artery (MA) than radial artery (RA), and saphenous vein (SV) (n=9). **B.** Increase in VSMC number to serum was lower in random isolated from MA than RA, and SV (n=13 to 15).

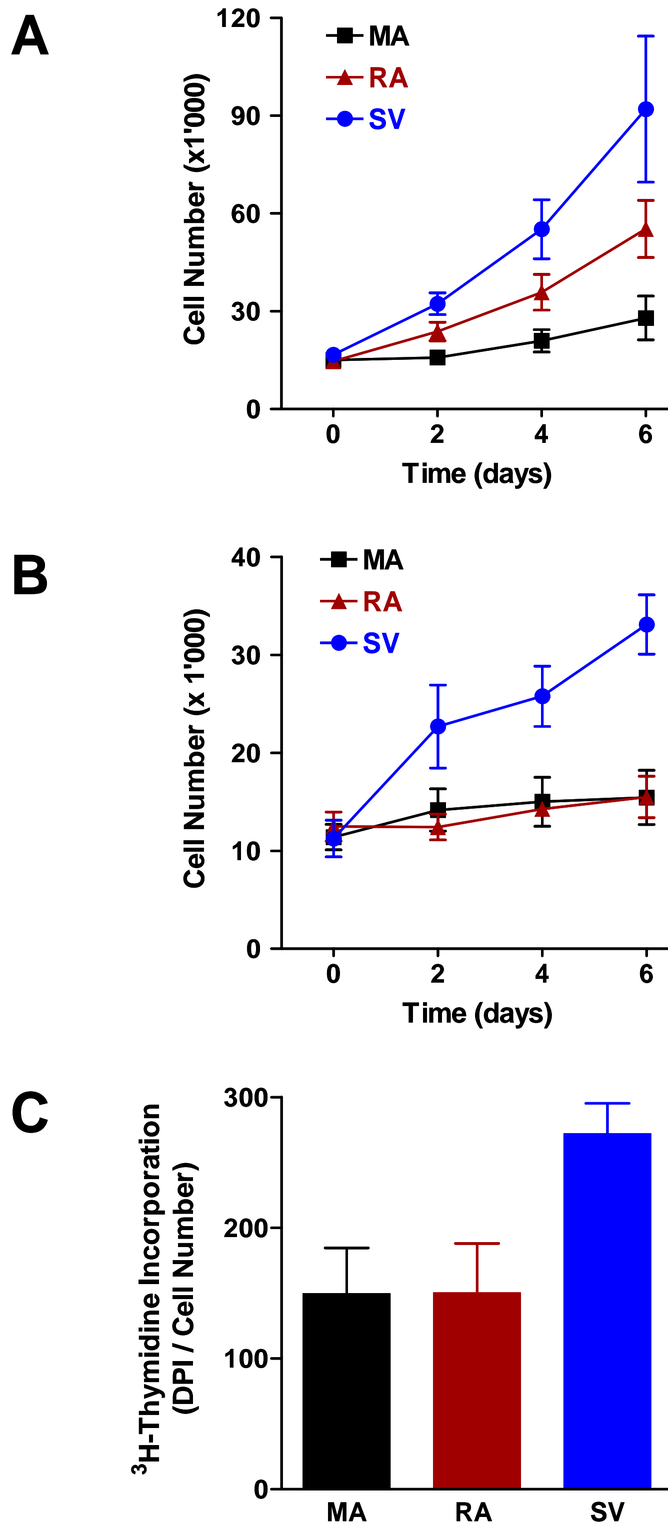


Figure 2. Different increase in cell number of vascular smooth muscle cells (VSMC) from human coronary artery bypass vessels. **A.** VSMC of radial artery (RA), internal mammary artery (MA) and saphenous vein (SV) were serum starved for 48 hours and then stimulated with 10% FCS. Cell number was determined on day 0, 2, 4, 6 after stimulation (n=4). **B.** VSMC of RA, MA, and SV were serum starved for 48 hours and then stimulated with 10 ng/ml PDGF-BB. Cell number was determined on day 0, 2, 4, 6 after stimulation (n=4). **C.** VSMC of RA, MA, and SV were serum starved for 48 hours and then stimulated with 10 ng/ml PDGF-BB. Exit from G0/G1 phase was measured 30 hours after PDGF stimulation by quantification of 3H-thymidine incorporation (n=4).

4.2.2. Receptor expression

The different increase in cell number of VSMC from RA, MA, and SV in response to PDGF-BB may be related to different expression of PDGF-receptors. Therefore, surface expression of PDGF-receptor α and β was determined by FACS analysis. Expression of PDGF-receptor α was similar in VSMC from all three vessels (fluorescence intensity shift: RA: 1.48±0.18, MA: 1.72±0.23, SV: 1.22±0.04; p=n.s.; n=5; Figure 3A), whereas PDGF-receptor β expression was higher in RA as compared to MA or SV (RA: 2.83±0.35, MA: 1.59±0.14, SV: 2.33±0.40; RA vs. MA: $p<0.02$; RA or MA vs. SV: p=n.s.; n=5; Figure 3B). Western blot analysis of PDGF-receptors confirmed these findings (Figure 3). Thus, the pattern of PDGF-receptor expression did not account for the different increase in cell number of VSMC from RA, MA, and SV.

4.2.3. Cell cycle

The differences in increase in cell number of VSMC from RA, MA, and SV may be due to differences in signal transduction or cell cycle regulation or both. In each case, differences in cell cycle progression would be present. As mitogenic signals are integrated in G1 phase, differences in G1 progression would be expected. Therefore, we compared cell cycle distribution and G1 progression of VSMC from RA, MA, and SV. Preliminary experiments examining cell cycle protein expression at 0, 6, 12, 18, 24, 30 hours of mitogenic stimulation revealed that differences in expression of these proteins progressively develop over the 30 hours of observation (n=5; data not shown). Hence, the analysis was performed at 0 and 30 hours of mitogenic stimulation. Propidium iodide incorporation after 30 hours of PDGF-BB (10 ng/ml) stimulation demonstrated similar cell cycle distribution in VSMC from all three vessels (% of cells in G0/G1-phase: RA: 70.25±9.93, MA: 79.5±5.78, SV: 74.25±5.00; S-phase: RA: 7.5±2.10, MA: 5.25±0.86, SV: 9.5±3.20; G2/M-phase: RA: 16.75±4.90, MA: 11±2.89, SV:14±2.04; n=4; p=n.s.; Figure 4). Western blot analysis for cell cycle proteins was performed and expression was evaluated by densitometry. The blot best representing the mean of the densitometric values is shown as an original recording (Figure 5).

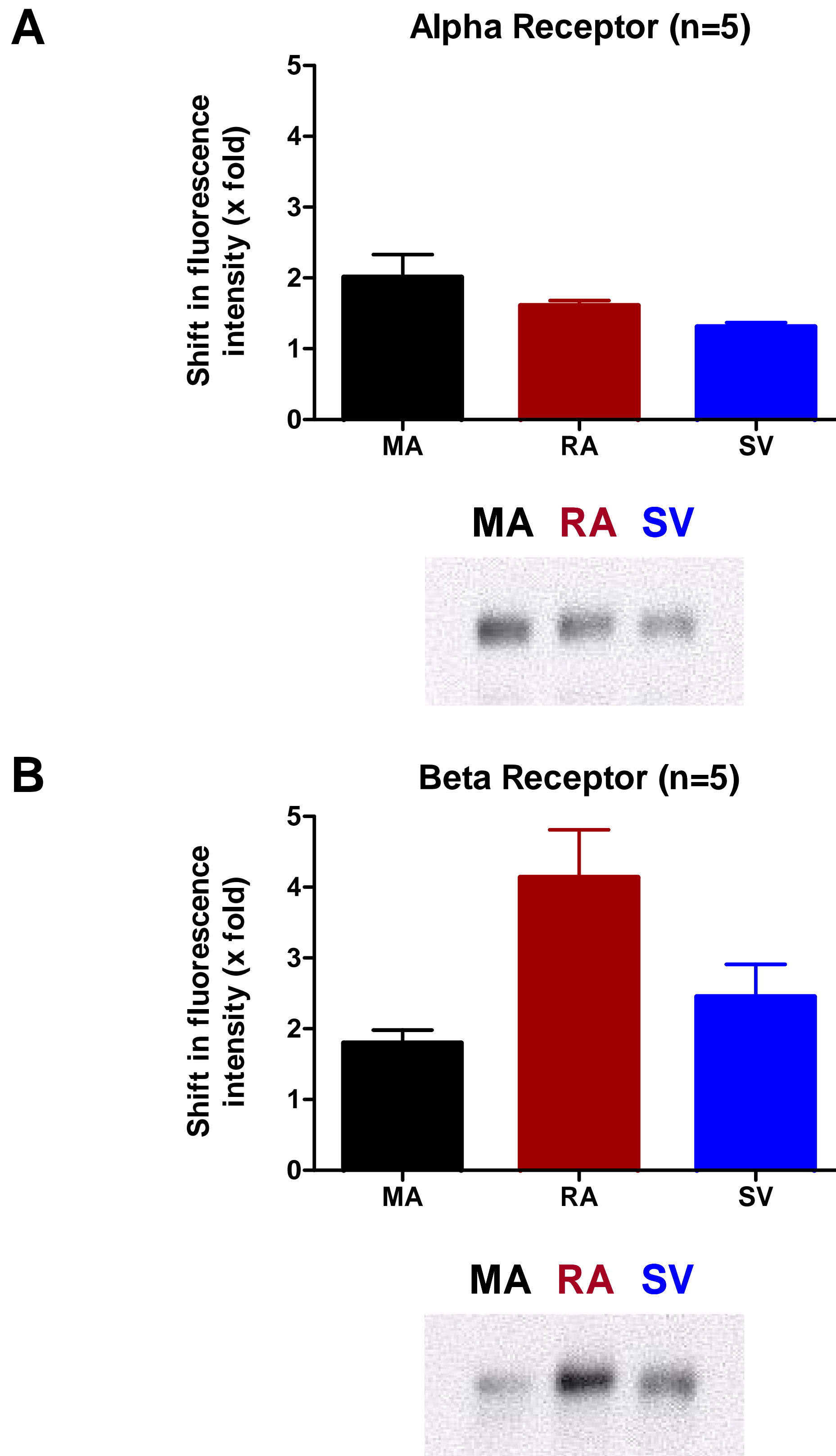


Figure 3. Different surface expression of PDGF receptors on vascular smooth muscle cells (VSMC) from human coronary artery bypass vessels. **A.** PDGF receptor α surface expression of randomly proliferating radial artery (RA), internal mammary artery (MA) and saphenous vein (SV) VSMC was analyzed by FACS and quantified by determining the fluorescence intensity shift relative to isotype signal (n=5). **B.** Analysis of PDGF receptor β by FACS (n=5).

Consistent with cell cycle distribution, VSMC from RA, MA, and SV exhibited an identical pattern of cell cycle protein expression (Figure 5A). Indeed, in VSMC from all three vessels, cyclin E expression remained unaltered. Further, the lower part of the cdk2 double band representing threonine-phosphorylated and hence activated cdk2 was induced. The cyclin-dependent kinase inhibitor (CKI) p21 was slightly upregulated, p27 was downregulated, and p57 remained unchanged. Consistent with these observations, cdk2 kinase activity was identical in VSMC from all three vessels (Figure 5B). Thus, both cell cycle distribution and G1 progression were identical in VSMC from RA, MA, and SV. Therefore, cell cycle regulation did not account for the different increase in cell number of VSMC from these vessels.

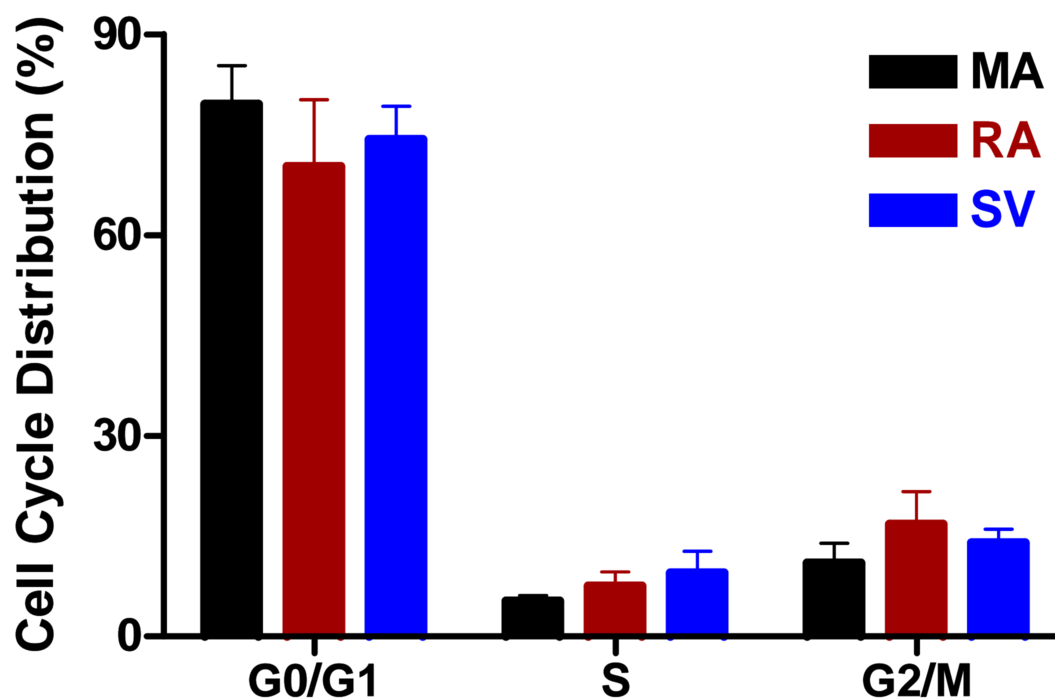


Figure 4. Identical cell cycle distribution of vascular smooth muscle cells (VSMC) from human coronary artery bypass vessels. VSMC isolated of radial artery (RA) internal mammary artery (MA) and saphenous vein (SV) were serum starved for 48 hours and then stimulated with 10 ng/ml PDGF-BB for 30 hours. Cell cycle distribution was determined by propidium iodide incorporation and analyzed by FACS (n=4).

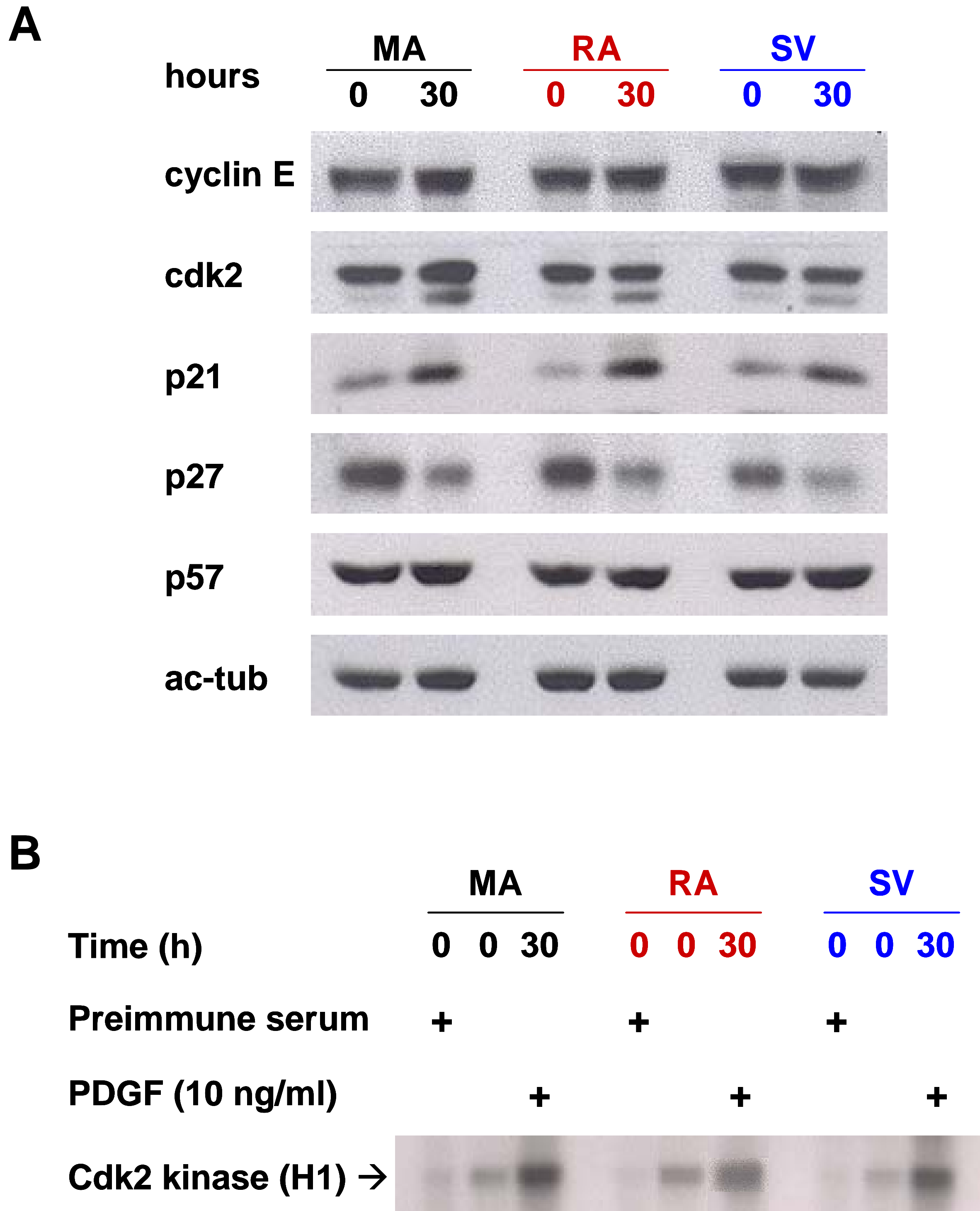


Figure 5. Identical protein expression of vascular smooth muscle cells (VSMC) from human coronary artery bypass vessels. **A.** VSMC of radial artery (RA), internal mammary artery (MA), and saphenous vein (SV) were serum starved for 48 hours and then stimulated with 10 ng/ml PDGF-BB for 30 hours. Cells were lysed and cell cycle regulatory protein levels were determined by Western blotting (n=4). **B.** VSMC of RA, MA and SV were serum starved for 48 hours and then stimulated with 10 ng/ml PDGF-BB for 30 hours. Cells were lysed and cdk2 kinase activity was determined by H1 kinase assay (n=2).

4.2.4. Cell death

As neither PDGF receptor expression nor cell cycle regulation account for the differences in cell number increase of VSMC from RA, MA, and SV, cell viability 30 hours after PDGF-stimulation was examined. FACS analysis of DNA fragmentation revealed that the number of dead VSMC from RA was in between that of MA and SV (percentage of dead cells: RA: 1.46±0.36, MA: 1.89±0.56, SV: 1.00±0.27; p=n.s.; n=5; Figure 6A), suggesting that cell death rates of RA are in between that of MA and SV. Quantification of cell death by lactate dehydrogenase (LDH) activity confirmed this observation. Indeed, cell death of VSMC from RA was in between that of MA and SV (dead cells in % of maximal release: RA: 14.28±4.54, MA: 31.12±6.43, SV: 8.04±3.15; MA vs. SV: $p<0.05$; RA vs. MA or SV: p=n.s.; n=5; Figure 6B). To investigate if cell death was related to apoptosis, VSMC were stained with Hoechst 33258 and the percentage of Hoechst-positive cells showing condensed chromatin was determined. Apoptosis rate of RA VSMC was in between that of MA and SV (% of apoptotic cells: MA: 3.04±0.49, RA: 2.75±0.86, SV: 1.46±0.25; n=4; p=n.s.; Figure 6C). This pattern of cell death was consistent with the differences in increase of cell number of VSMC from RA, MA, and SV.

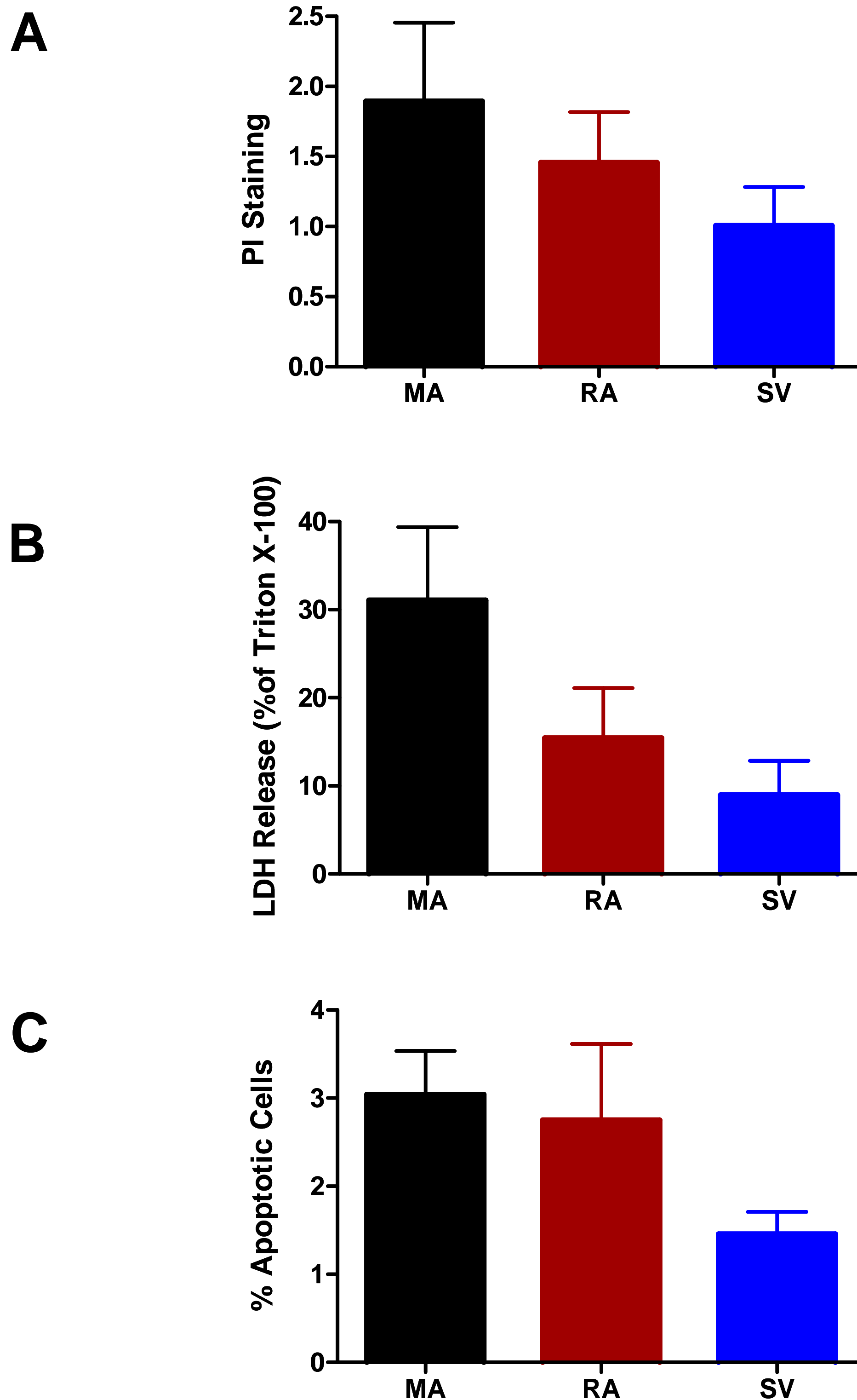


Figure 6. Different cell death rates of vascular smooth muscle cells (VSMC) from human coronary artery bypass vessels. VSMC of radial artery (RA), internal mammary artery (MA), and saphenous vein (SV) were serum starved for 48 hours and then stimulated with 10 ng/ml PDGF-BB. **A.** Fragmented DNA 30 hours after stimulation was analyzed by FACS (n=5). **B.** LDH release was determined 30 hours after stimulation (n=5). **C.** Hoechst-positive cells were identified 30 hours after stimulation by fluorescence microscopy (n=4).

4.2.5. Caspase Inhibition

As activation of caspases is a key event in apoptosis, VSMC were treated with specific caspase inhibitors to prevent apoptosis. VSMC were stimulated with PDGF-BB over 4 days (increase in cell number: RA: 12’448±2’853, MA: 6’510±2’328, SV: 29’688±6’285; RA vs. SV: $p<0.05$; MA vs. SV: $p<0.01$; MA vs. RA: p=n.s; n=6). Addition of the specific caspase inhibitor Z-VAD-fmk abolished differences in cell number increase of RA versus MA and SV, confirming that the different cell death rates are related to apoptosis (increase in cell number: RA+VAD: 29’948±2’560, MA+VAD: 32’292±10’128, SV+VAD: 38’854±9’736; p=n.s.; RA vs. RA+VAD: $p<0.01$; MA vs. MA+VAD: $p<0.05$; SV vs. SV+VAD: p=n.s.; n=6). These data could be confirmed by addition of another specific caspase inhibitor Boc-D-fmk (increase in cell number: RA+Boc: 27’031±2’666, MA+Boc: 29’375±8’657, SV+Boc: 29’531±6’341; p=n.s.; RA vs. RA+Boc: $p<0.01$; MA vs. MA+Boc: $p<0.05$; SV vs. SV+Boc: p=n.s.; n=6, Figure 7A). As the aorta is prone to develop atherosclerosis and behaving similar to the SV, we also compared MA VSMC with those from aorta.[10] Analogous to the observations in VSMC from bypass vessels, growth rates in VSMC from MA were lower than those from aorta (increase in cell number: MA: 6’250±2’592, aorta: 21’188±5’857; $p<0.05$; n=5). The growth differences were abolished by the caspase inhibitors (increase in cell number: MA+VAD: 22’750±4’111, aorta+VAD: 26’812±6’462; p=n.s.; MA+Boc: 17’625±2’866, aorta+Boc: 21’437±7’888; p=n.s.; MA vs. MA+VAD or MA+Boc: $p<0.05$; aorta vs. aorta+VAD or aorta+Boc: p=n.s.; n=5; Figure 7B). Thus, these data indicate that the different increase in cell number of VSMC from RA, MA, and SV is due to different caspase-dependent apoptosis rates.

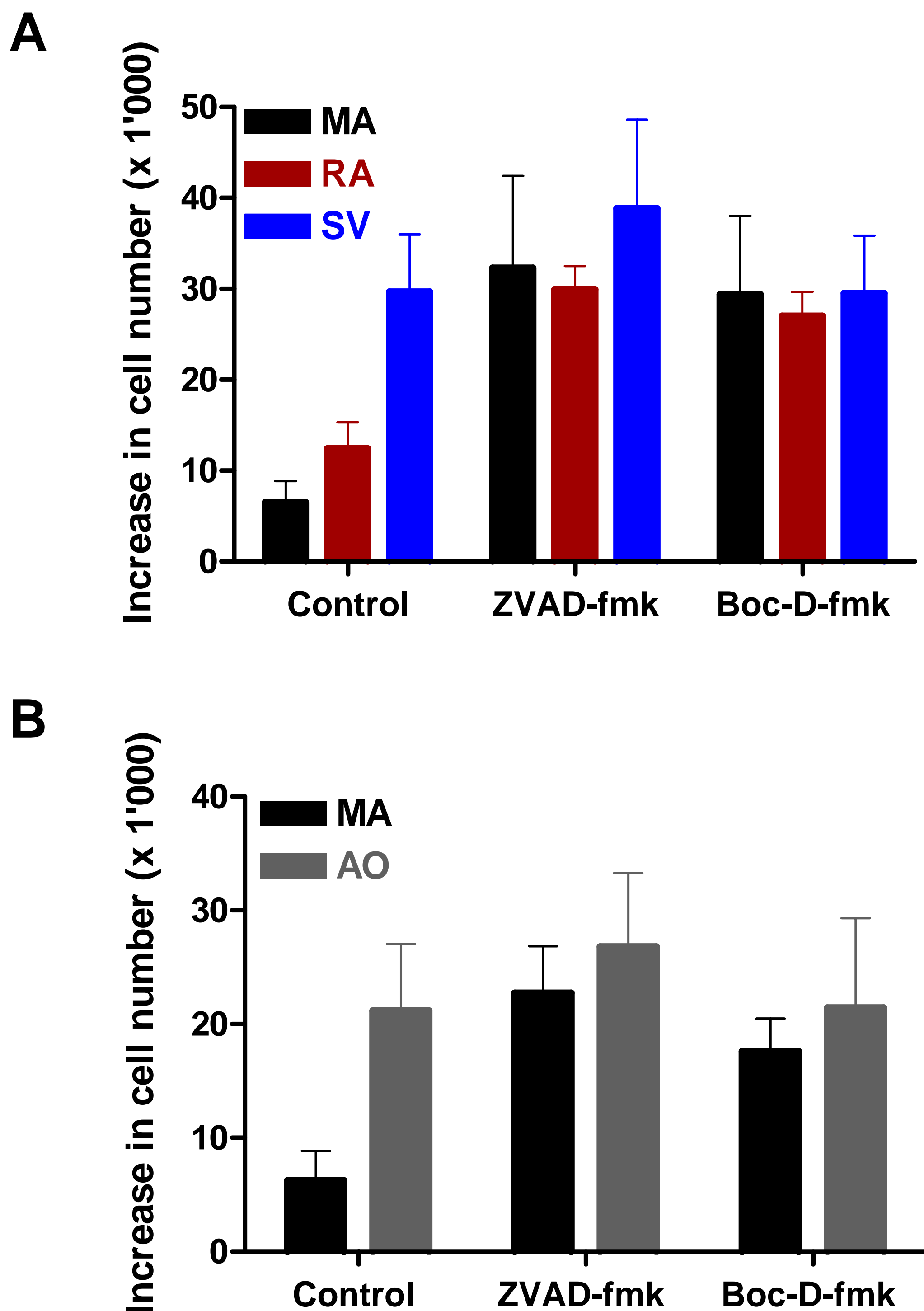


Figure 7. Caspase inhibitors abolish differences in cell number increase of vascular smooth muscle cells (VSMC). **A.** VSMC from radial artery (RA), internal mammary artery (MA) and saphenous vein (SV) were serum starved for 48 hours and then stimulated with 10 ng/ml PDGF-BB plus either the specific capase inhibitors Z-VAD-fmk (50 μM) or Boc-D-fmk (30 μM). Cell number was determined on day 4 after stimulation (n=6). **B.** VSMC from MA and aorta were serum starved for 48 hours. Stimulation was performed as described in A (n=5).

4.2.6. Akt Activity

Akt plays a key role in the cellular balance between cell proliferation and apoptosis.[11] Therefore, levels of total and activated Akt were determined by Western blotting, which revealed lower activation in VSMC. RA was higher than that of MA and lower than that of SV (n=4; Figure 8A). These data could be confirmed by an Akt activity assay determining the levels of phosphorylated GSK-3β; indeed, GSK3-β activation of RA was in between that from MA and SV (n=3, Figure 8B). Similar to caspase inhibitors, adenoviral transduction of VSMC with the constitutively active Akt mutant Ad-HA-m/p-PKB abrogated the differences in cell number increase of VSMC from RA, MA and SV.[11] RA VSMC number was in between that of MA than SV after PDGF-stimulation (cell number in % of MA: RA: 13.5±3.0, MA: 0±13.5, SV:70.2±11.2; MA vs. SV: p<0.01, RA vs. MA or SV: p=n.s.). Transduction with the Akt mutant Ad-HA-m/p-PKB revealed similar increase in cell number of VSMC in RA, MA, and SV (increase in cell number (%): RA+AdPKB: 72.0±13.3, MA+AdPKB: 80.3±10.6, SV+AdPKB: 69.2±14.8; p=n.s.; MA vs. MA+AdPKB: p<0.001; RA vs. RA+AdPKB: p=n.s., SV vs. SV+AdPKB: p=n.s.). No effect of the control vector was observed (RA+AdGFP: 10.4±4.6, MA+AdGFP: 11.9±8.6, SV+AdGFP: 68.4±12.3; p<0.01; MA vs. MA+AdGFP and SV vs. SV+ AdGFP: p=n.s.; n=4; Figure 8C). These data confirm that different levels of Akt determine the different apoptosis rates of RA, MA, and SV.

4.2.7. Celecoxib treatment

Recently it was shown that celecoxib, a specific cyclooxygenase-2 inhibitor, reduces neointima formation in rat VSMC by inhibiting Akt activation.[12] Therefore, levels of total and activated Akt after pre-treatment of SV VSMC with celecoxib (10 µM) was determined by Western blotting. Whereas celecoxib had no effect on total Akt levels, it reduced PDGF-induced Akt activation (optical density of p-Akt in percent of total Akt: control: 16.4±8.2, PDGF: 111.4±43.8, celecoxib: 11.6±3.8, PDGF plus Celecoxib: 40.2±8.0; p=n.s.; n=4; Figure 9A+B).

Cell death was as analysed by LDH-release. Stimulation with PDGF-BB reduced LDH release as compared to control conditions (LDH release in % of Triton X-100: PDGF: 7.7±0.9, control:

15.9±1.8; p<0.001; n=5). In contrast, cell death was increased by pre-treatment of SV VSMC with celecoxib in both, unstimulated and PDGF-stimulated cells (control plus celecoxib: 27.1±3.3, PDGF plus celecoxib: 25.1±5.5; p=n.s.; n=5). LDH release of celecoxib-treated cells was significantly higher as compared to untreated cells (control vs. celecoxib+control: p<0.02, PDGF vs. celecoxib plus PDGF: p=0.01; n=5).

Thus, despite of identical PDGF-receptor surface expression and identical cell cycle regulation, a cell population with higher death rates, such as MA VSMC, will exhibit slower proliferation than a population with lower death rates, such as SV VSMC. RA VSMC seem to act similar to MA. Different apoptosis rates due to different activation levels of Akt determine increase in cell number of VSMC from RA, MA, and SV coronary artery bypass vessels (Figure 10).

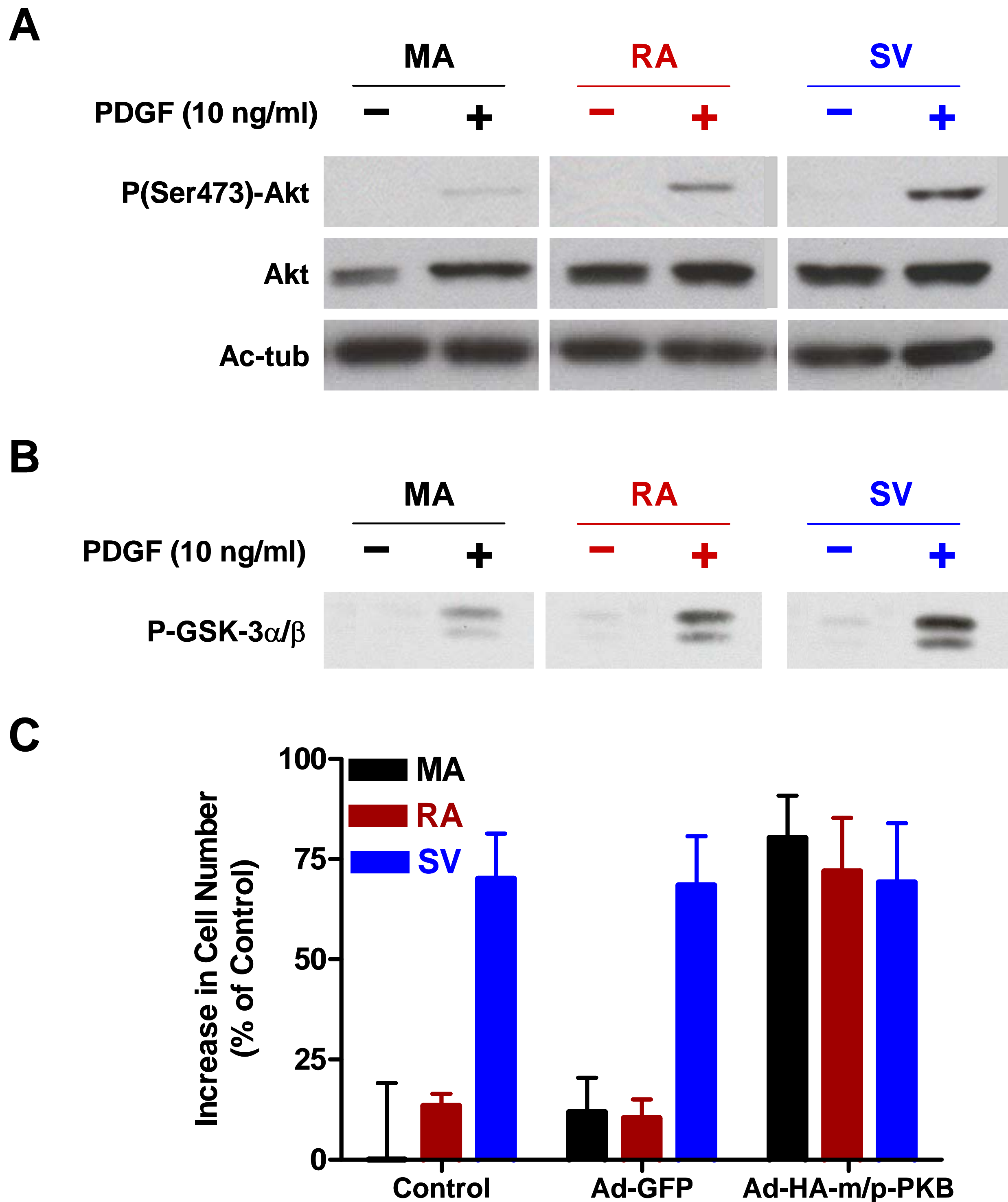


Figure 8. Different levels of Akt activation lead to different apoptosis rates of human vascular smooth muscle cells (VSMC). **A.** VSMC from radial artery (RA), internal mammary artery (MA), and saphenous vein (SV) were serum starved for 48 hours, and then stimulated with 10 ng/ml PDGF-BB for 30 hours. Cells were lysed and Akt levels were determined by Western Blotting (n=4). **B.** VSMC from RA, MA, and SV were serum starved for 48 hours, and then stimulated with 10 ng/ml PDGF-BB for 30 hours. Cells were lysed and Akt activity was determined by Western Blotting, measuring GSK3-β levels (n=3). **C.** VSMC from RA, MA and SV were serum starved for 48 hours, and then transfected with 800 pfu of an adenoviral mutant, expressing a constitutive active Akt (Ad-HA-m/p-PKB) or a control virus (Ad-GFP) and stimulated with 10 ng/ml PDGF-BB. Cell number was determined 4 days after stimulation (n=4).

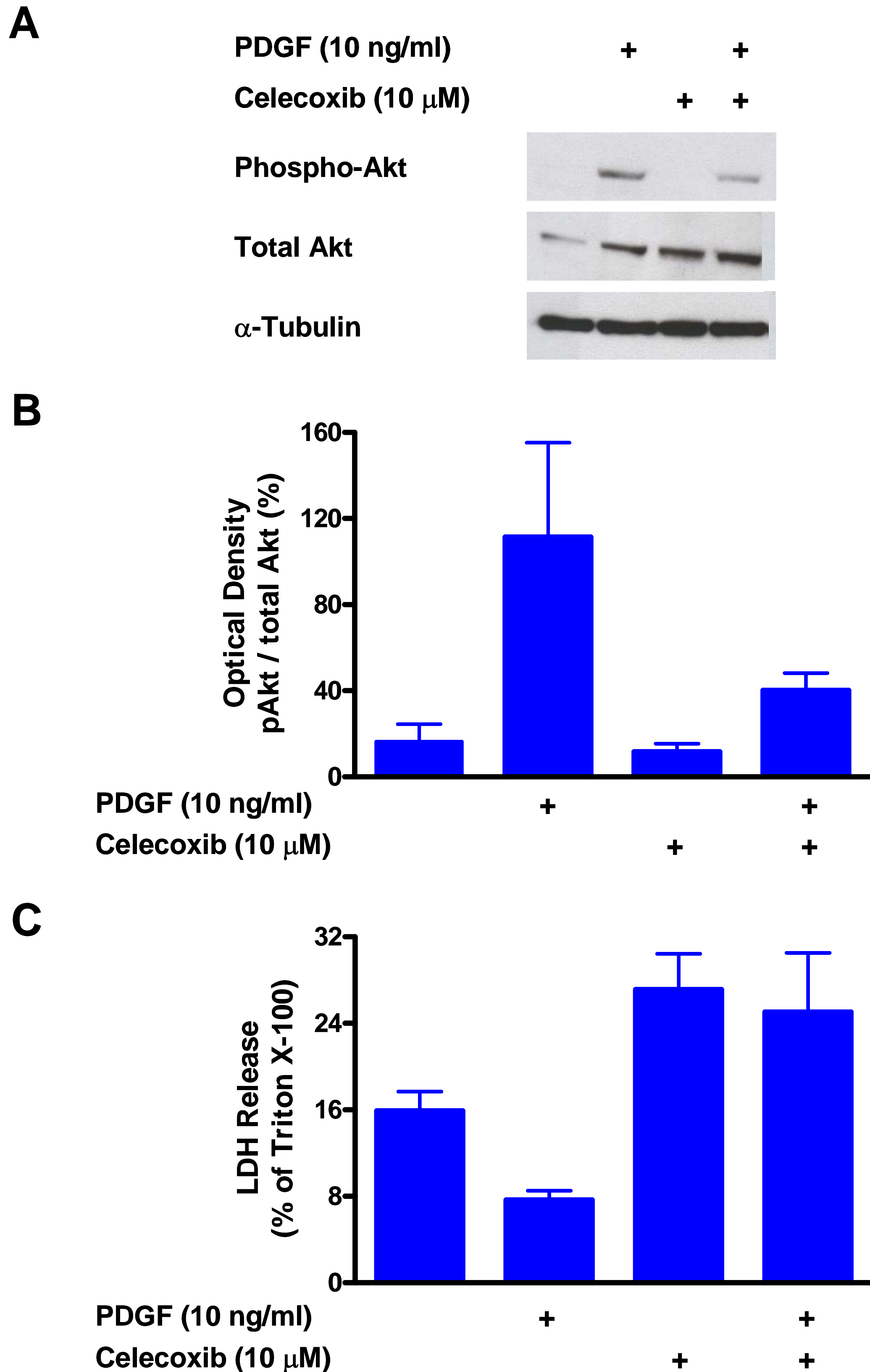


Figure 9. Celecoxib induces cell death in vascular smooth muscle cells (VSMC) from saphenous vein (SV) by inhibiting the activation of Akt. SV VSMC were serum starved for 48 hours, preincubated with 10 μM celecoxib for 1 hour and then stimulated with PDGF-BB for 30 hours. A. Cells were lysed and levels of activated and total Akt were determined by Western blotting. B. Optical densitiy was determined (n=4). C. LDH release was analysed by ELISA-Assay.

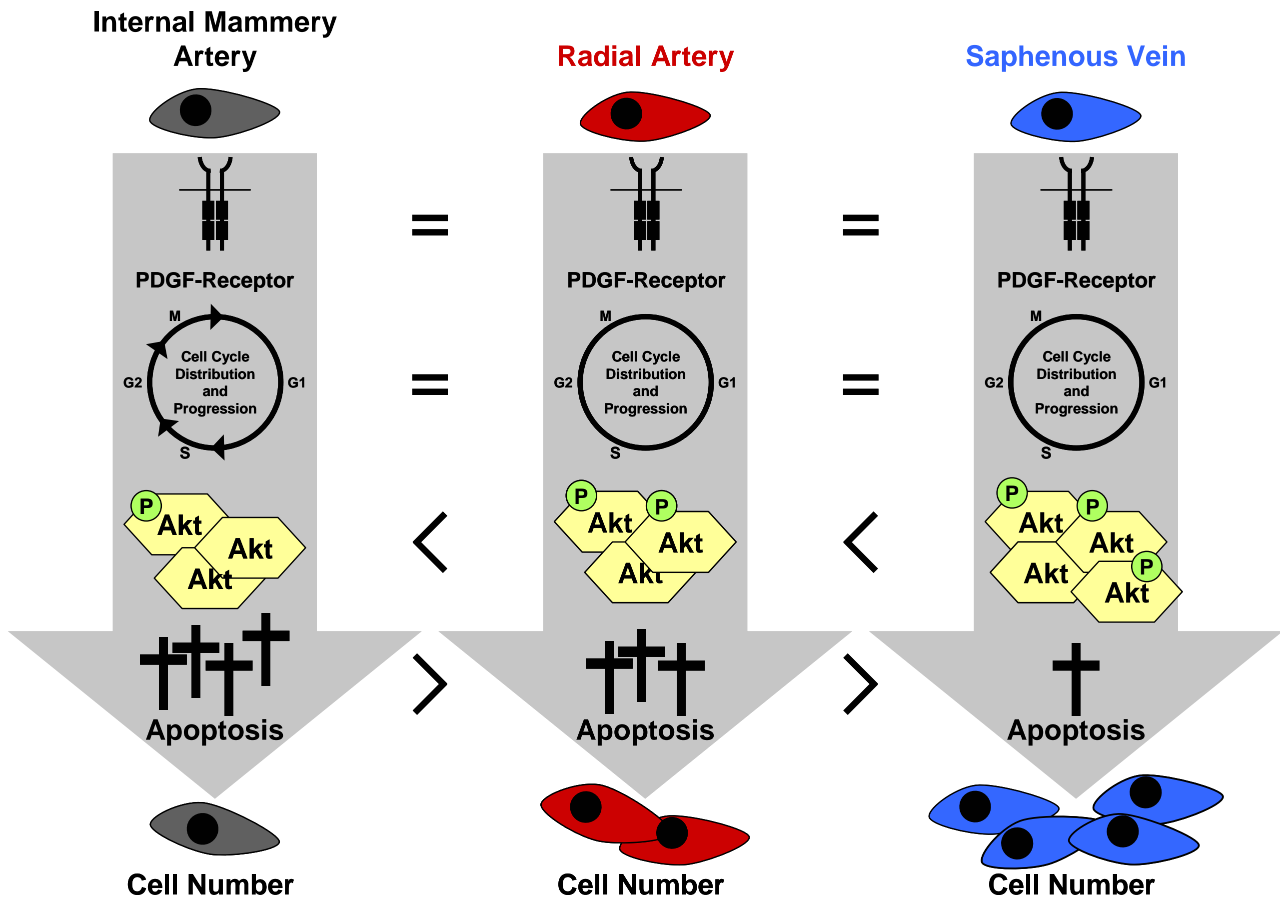


Figure 10. Different apoptosis levels due to different Akt activation rather than different PDGF-receptor expression, cell cycle distribution and progression accounts for the different increase in cell number of vascular smooth muscle cells from radial artery, internal mammary artery, and saphenous vein.

4.3. Discussion

This study demonstrates that VSMC from human vessels used for coronary artery bypass grafting exhibit differences in increase in cell number, indicating that there is an intrinsic heterogeneity of VSMC from RA, MA, and SV. The increase in cell number of RA is slightly higher than in MA but lower than in SV, which is consistent with the different propensity to accelerated atherosclerosis in these vessels. The difference is neither related to PDGF receptor expression nor PDGF-BB induced cell cycle progression. Therefore, the difference in the increase in cell number is rather determined by different apoptosis rates due to different Akt activation.

VSMC from RA and MA grew slower in response to FCS or PDGF-BB as compared to those from SV. Again, this observation compares well with clinical data demonstrating that patency rates of RA are slightly lower than those from MA, but higher than those from SV.[2,3] Moreover, the differences in cell number increase must be related to different intrinsic properties of VSMC, as they were not only observed during explant outgrowth, but also in isolated cells. Increase in cell number of RA VSMC was in between that of MA and SV after stimulation with FCS, while it was as low as that of MA in response to PDGF-BB. The PDGF induced pattern corresponds well to RA long-term patency rates. This observation suggests that PDGF-induced VSMC proliferation is a key phenomenon in bypass graft disease and influences long-term patency rates.

PDGF receptor α surface expression was similar in VSMC from RA, MA, and SV, while expression of the β receptor tended to be higher in RA as compared to the other vessels. This pattern is not consistent with the differences in increase in cell number observed in response to PDGF-BB suggesting that neither α nor ß receptor expression can account for the different increase in cell number. Hence, the reason for the differences in increase in cell number must be located below the membrane level. Irrespective of whether the differences in increase in cell number is due to signal transduction or cell cycle regulation, differences in cell cycle distribution will be present. As mitogenic signals are integrated in G1 phase, these differences will be apparent in G1 progression.[13] The latter is related to the activity of protein complexes consisting of a cyclin and a

cyclin-dependent kinase (cdk).[14] Indeed, cyclin E and cdk2 mediate late G1 progression and S phase entry. Endogenous protein inhibitors of cyclin-cdk complexes (CKI) can modulate cdk2 activity; the members of the p21 family of CKI inhibit cell cycle progression in such a potent manner that they have been used for reducing neointima formation in restenosis models.[15] Therefore, regulation of G1 progression in VSMC from RA, MA, and SV was examined by assessing expression and activity of cyclin E, cdk2, p21, p27, p57. The expression pattern of cell cycle proteins upon mitogenic stimulation was consistent with the literature. Indeed, cdk2 is threonine phosphorylated and hence activated in response to mitogenic stimulation.[16] p21 is slightly induced in response to mitogens suggesting that it regulates kinase activity in proliferating cells.[17] p27 is downregulated in response to mitogens indicating that it controls kinase activity in arrested cells and its downregulation is a prerequisite for proliferation.[18,19,20] The role of p57 in VSMC is not well defined yet; it was not regulated in response to PDGF-BB, which points to a specialized role coming into play in a restricted manner only. In a previous report, a lower degree of p27 downregulation by PDGF-BB was observed in MA as compared to SV.[21] However, I was not able to confirm this observation; in all experiments performed, there was a tendency towards a higher p27 downregulation in MA as compared to SV. A transient downregulation of p27 in SV within the 30 hours window can be excluded, as preliminary experiments demonstrated a continuously increasing downregulation over 30 hours. The difference to my observation is most probably related to the fact that the protein lysates of MA and SV were made from different patients in the previous paper, while they originated from the same patient in my experiments.

As demonstrated by measurement of fragmented DNA, LDH release, and Hoechst staining, cell death due to apoptosis rather than cell cycle progression determines the difference in increase of cell number of VSMC from RA, MA, and SV. VSMC apoptosis is an important mechanism in atherosclerosis; indeed, apoptotic VSMC have been demonstrated in atherectomy specimens from human coronary and peripheral plaques.[8,9,22,23,24] It therefore seems likely that apoptosis of VSMC is an important pathogenic event in bypass graft disease. Akt is an important signaling molecule, which plays a key role in cell survival. Indeed, it also acts anti-apoptotic effects after stimulation

with growth factors such as PDGF-BB.[11] In this study, levels of activated and total Akt revealed that activation of RA VSMC was in between that of MA and SV, whereas transduction of a constitutively active Akt mutant abrogated the differences in cell number increase. This emphasizes the crucial role of Akt as a balance between cell survival and apoptosis in VSMC of coronary artery bypass grafts.

Traditionally, the fundamental contribution of VSMC to atherosclerosis has been ascribed mainly to proliferation, leading to neointima formation.[25] More recent studies, however, revealed an important role of VSMC apoptosis in the development of atherosclerosis.[8,9] Apoptosis in the vessel wall is considered to be a two-sided sword depending on the stage of atherosclerosis.[26] VSMC in advanced atherosclerotic lesions exhibit an increased susceptibility towards undergoing apoptosis, which may result in plaque instability favoring the occurrence of acute coronary syndromes and myocardial infarction. On the other hand, VSMC apoptosis may counteract proliferation, thereby slowing neointima formation and the development of bypass graft disease. Therefore, VSMC apoptosis seems to be beneficial regarding the patency of vessels after bypass surgery. Indeed, VSMC from MA coronary artery bypass vessels have higher patency rates than those from SV, and MA VSMC undergo apoptosis more often than those from SV. RA patency rates correspond with VSMC apoptosis as well. Therefore, VSMC apoptosis protects the RA and even more so the MA from bypass graft disease.

4.4. References

1. Loop FD, Lytle BW, Cosgrove DM, Stewart RW, Goormastic M, Williams GW, Golding LA, Gill CC, Taylor PC, Sheldon WC. Influence of the internal-mammary artery graft on 10-year survival and other cardiac events. *N Engl J Med*. 1986;314:1-6
2. Acar C, Jebara VA, Portoghese M, Beyssen B, Pagny JY, Grare P, Chachques JC, Fabiani JN, Deloche A, Guermonprez JL. Revival of the radial artery for coronary artery bypass grafting. *Ann Thorac Surg*. 1992;54:652-659
3. Acar C, Ramsheyi A, Pagny JY, Jebara V, Barrier P, Fabiani JN, Deloche A, Guermonprez JL, Carpentier A. The radial artery for coronary artery bypass grafting: clinical and angiographic results at five years. *J Thorac Cardiovasc Surg*. 1998;116:981-989
4. Possati G, Gaudino M, Prati F, Alessandrini F, Trani C, Glieca F, Mazzari MA, Luciani N, Schiavoni G. Long-term results of the radial artery used for myocardial revascularization. *Circulation*. 2003;108:1350-1354.
5. Loscalzo J. Vascular matrix and vein graft failure. *Circulation*. 2000;101:221-223
6. Ross R. Platelets, platelet-derived growth factor, growth control, and their interactions with the vascular wall. *J Cardiovasc Pharmacol*.1985;7:186-190
7. Rössig L, Dimmeler S, Zeiher AM. Apoptosis in the vascular wall and atherosclerosis. *Basic Res Cardiol*. 2001;96:11-22
8. Han DK, Haudenschild CC, Hong MK, Tinkle BT, Leon MB, Liau G. Evidence for apoptosis in human atherogenesis and in a rat vascular injury model. *Am J Pathol*. 1995;147:267-277
9. Isner JM, Kearney M, Bortmann S, Passeri J. Apoptosis in human atherosclerosis and restenosis. *Circulation*. 1995;91:2703-2711
10. Braunwald: Heart Disease: A Textbook of Cardiovascular Medicine, Chapter 45. 5th ed.1997
11. Brazil DP, Hemmings BA. Ten years of protein kinase B signaling: a hard Akt to follow. *Trends Biochem Sci*. 2001;26:657-664

12. Yang HM, Kim HS, Park KW, You HJ, Jeon SI, Youn SW, Kim SH, Oh BH, Lee MM, Park YB, Walsh K. Celecoxib, a cyclooxygenase-2 inhibitor, reduces neointimal hyperplasia through inhibition of Akt signaling. *Circulation*. 2004;110:301-308

13. Sherr CJ. G1 phase progression: cycling on cue. Cell. 1994;79:551-555

14. Morgan DO. Principles of CDK regulation. *Nature*. 1995;374:131-134

15. Tanner FC, Boehm M, Akyürek LM, San H, Yang ZY, Nabel GJ, Nabel EG. Differential effects of the cyclin-dependent kinase inhibiors p27Kip1, p21Cip1, and p16Ink4 on vascular smooth muscle cell proliferation. *Circulation*. 2000;101:2022-2025

16. Gu Y, Rosenblatt J, Morgan DO. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. *EMBO J*. 1992;11:3995-4005.

17. Zhang H, Hannon GJ, Beach D. p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev*. 1994;8:1750-1758

18. Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin–cdk protein kinase activity, is related to p21. *Cell*. 1994;78:67-74

19. Nourse J, Firpo E, Flanagan WM, Coats S, Polyak K, Lee MH, Massague J, Crabtree GR, Roberts JM. Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature*. 1994;372:570-573

20. Coats S, Flanagan WM, Nourse J, Roberts JM. Requirement of p27Kip1 for restriction point control of the fibroblast cell cycle. *Science*. 1996;272:877-880

21. Yang Z, Oemar BS, Carrel T, Kipfer B, Julmy F, Lüscher TF. Different proliferative properties of smooth muscle cells of human arterial and venous bypass vessels. *Circulation*. 1998;97:181-187

22. Bennett MR. Apoptosis in the cardiovascular system. *Heart*. 2002;87:480-487

23. Bennett MR. Vacular smooth muscle cell apoptosis – a dangerous phenomenon in vascular disease. *J Clin Basic Cardiol*. 2000;3:63-65

24. Kockx MM, De Meyer GR, Muhring J, Jacob W, Bult H, Herman AG. Apoptosis and related proteins in different stages of human atherosclerotic plaques. *Circulation*. 1998;97:2307-2315

25. Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science*. 1973;180:1332-1339

26. Kockx MM, Herman AG. Apoptosis in atherosclerosis: beneficial or detrimental? *Cardiovasc Res*. 2000;45:736-746

5. Part Two

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

5.1. Introduction

Vascular smooth muscle cell (VSMC) proliferation and migration play a crucial role in the development of atherosclerotic lesions.[1,2] Therefore, these processes are also involved in the pathogenesis of bypass graft disease.[3,4] In contrast to SV grafts, RA and MA conduits develop significantly less atherosclerotic lesions, even years after surgery.[5,6] This seems to be due to different intrinsic properties of the three vessels.[7,8] As shown in part one of this thesis, proliferation rates of RA are similar to that of MA, but lower than those from SV. In this part of my studies I analyzed migration of VSMC from RA, MA and SV.

5.2. Results

5.2.1. Migration

In RA VSMC migration was 18.7±7.8% in response to 1 ng/ml, 38.7±7.1% to 3 ng/ml, and 55.7±9.7% to 10 ng/ml PDGF-BB. Migration of MA was lower as compared to RA, reaching 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 (Figure 1A). In SV VSMC, migration was more pronounced as compared to the other vessels and reached 44.7±8.5% in response to 1 ng/ml, 85.4±6.9% to 3 ng/ml, 100.0±1.0% to 10 ng/ml PDGF-BB (Figure 1B). There was a significant difference between MA and SV at all three concentrations examined (10 ng/ml: p=0.0001, 3 ng/ml: p<0.0001, 1 ng/ml: p=0.01; n=5). Between RA and SV, there was a significant difference at 10 ng/ml (p<0.005; n=5) and 3 ng/ml (p<0.001; n=5), but not at 1 ng/ml (p= n. s.), whereas there were no significances at any concentration between RA and MA. No difference in attachment of VSMC from RA, MA, and SV was observed (number of attached cells (x 1'000): RA: 25.6±2.7, MA: 24.4±5.7, SV: 23.5±5.2; p=n.s., n=6; Figure 2A). Similarly, chemokinesis of all three vessels was identical (migrated cells / well: RA: 14.7±4.3, MA: 17.8±3.2, SV: 17.5±2.0; p=n.s.; n=5; Figure 2B).

5.2.2. PDGF-receptor expression

The different migration rates in response to PDGF-BB may be related to different expression of PDGF-receptors. Therefore, as shown in part one of this study, surface expression of PDGF-receptor α and β was determined by FACS analysis and Western blotting. Expression of PDGF-receptor α was similar from all three vessels, whereas PDGF-receptor β expression was higher in RA as compared to MA or SV (Part One - Figure 3). Thus, the pattern of PDGF-receptor expression did not correspond to the different migration rates of VSMC from RA, MA and SV.

.

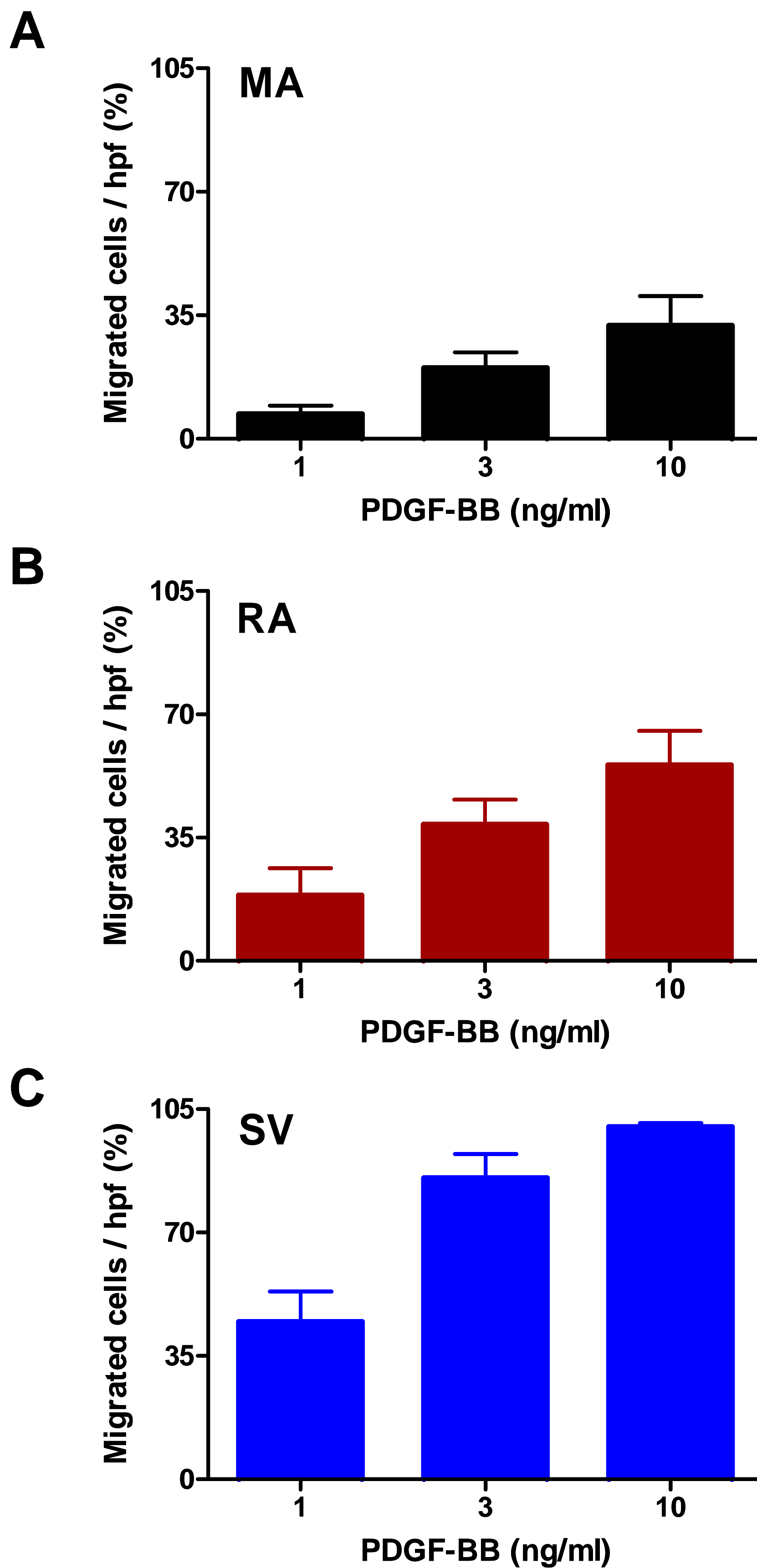


Figure 1. Different migration of vascular smooth muscle cells (VSMC) from human coronary artery bypass vessels. VSCM of **A.** internal mammary artery (MA), **B.** radial artery (RA) and **C.** saphenous vein (SV) were stimulated with different concentrations of PDGF-BB. Migration was determined using a modified Boyden chamber (n=5).

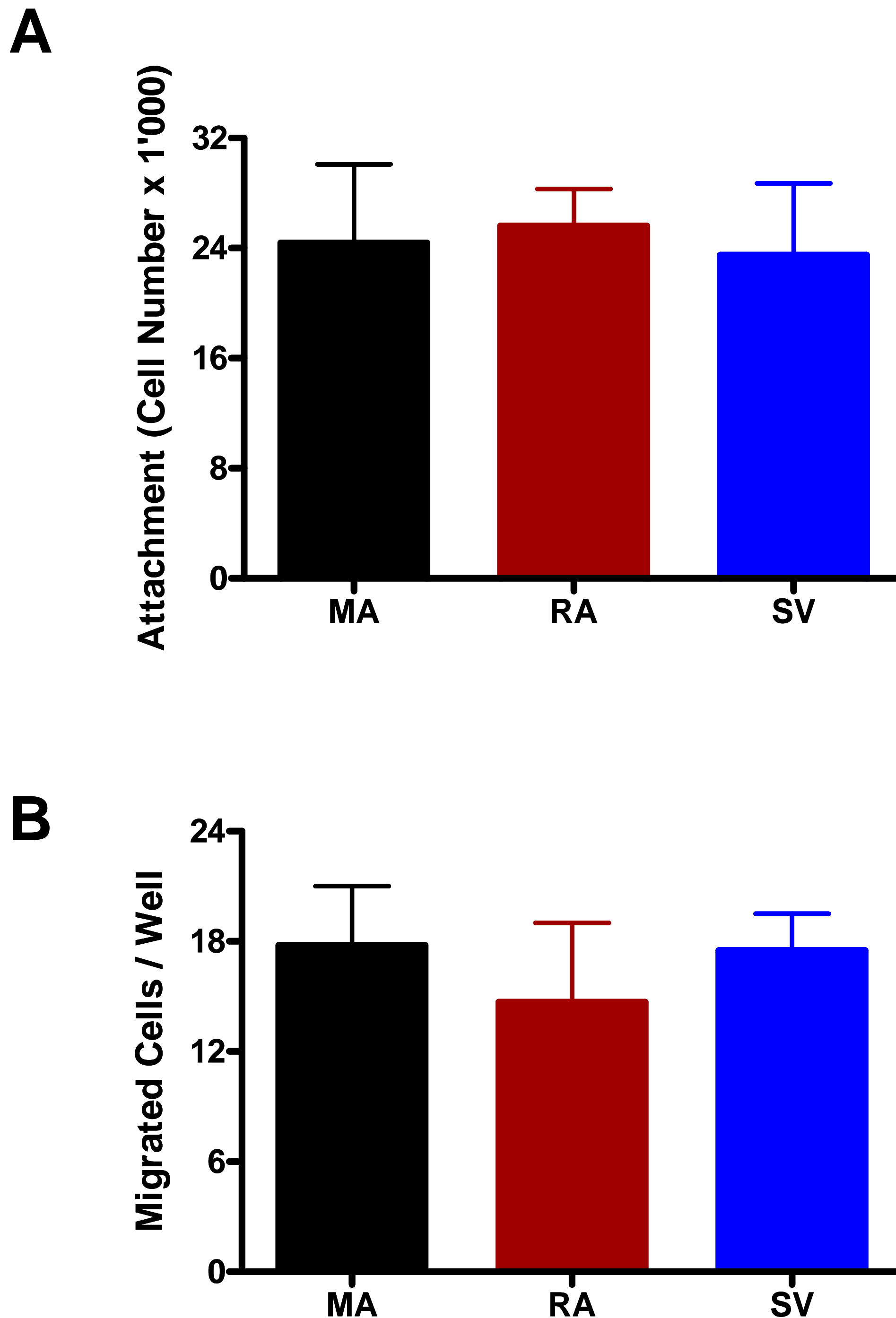


Figure 2. Attachment and chemokinesis of vascular smooth muscle cells (VSMC) from radial artery (RA), internal mammary artery (MA), and saphenous vein (SV). **A.** Newly seeded VSMC were incubated with PDGF-BB for 5 hours. Cells were washed and number of attached cells was determined (n=6). **B.** Chemokinesis of VSMC was assessed after 5 hours of incubation in a Boyden chamber (n=5).

5.2.3. Signal transduction

The differences in migration of VSMC from RA, MA, and SV may be due to differences in signal transduction. The Rho / ROCK pathway is known to be involved in VSMC migration.[4] Therefore, we examined the role of this pathway on migration of VSMC from RA, MA, and SV. Rho A activity in response to PDGF-BB stimulation was determined by Rho A pulldown. After PDGF-BB stimulation, Rho A activity of VSMC from RA and MA was similar, whereas it was strongly increased in SV (RA or MA vs. control: p=n.s.; SV vs. control: p<0.05; n=4). There was a significant difference between the three vessels (RA vs. SV: p<0.001; MA vs. SV: p=0.001; MA vs. RA: p=n.s.; n=4; Figure 3A). In contrast, Rho A expression was similar in all three vessels and not affected by PDGF-BB (p=n.s.; n=4; data not shown). Levels of active Rho A in percentage of total Rho A were similar in VSMC from RA and MA; moreover there was no significant induction of activity in response to PDGF-BB (RA: control: 21±12%, PDGF: 37±7%; MA: control: 17±9%, PDGF: 44±10%; p=n.s. for RA vs. MA and for control vs. PDGF of RA and MA; n=4; Figure 3B). In contrast, SV VSMC stimulation with PDGF-BB significantly increased active Rho A as compared to control conditions (control: 44±20%, PDGF: 100±1%; p<0.05; n=4; Figure 3B). Rho A activity of unstimulated VSMC was similar in RA, MA, and SV.

A necessary role of Rho A for the different migration of VSMC from the three vessels was confirmed by preincubating cells with Rho / ROCK pathway inhibitors before PDGF-BB stimulation. As statins are known to inhibit Rho A, the effect of rosuvastatin (10 µM) on VSMC migration of RA, MA, and SV was analyzed. Rosuvastatin inhibited migration of VSMC from RA, MA and SV in response to PDGF-BB (inhibition in % of control: RA: 84.6±3.7, MA: 84.7±5.8%, SV: 74.4±6.5%; p=n.s.; RA, MA, and SV: PDGF vs. PDGF+rosuvastatin: p<0.001; n=4; Figure 4A). These data could be confirmed by addition of the Rho-Kinase (ROCK) inhibitor hydroxyfasudil (10 µM). It almost completely abolished the differences in migration of RA, MA, and SV (inhibition in % of control: RA: 91.9±1.8, MA: 87.3±8.3%; SV: 87.4±5.9%; p=n.s.; RA, MA, and SV: PDGF vs. PDGF+rosuvastatin: p<0.001; n=4; Figure 4B). Toxicity of rosuvastatin and hydroxyfasudil was

tested by LDH release; no significant increase in LDH release was observed in the presence of either rosuvastatin nor hydorxyfasudil (data not shown).

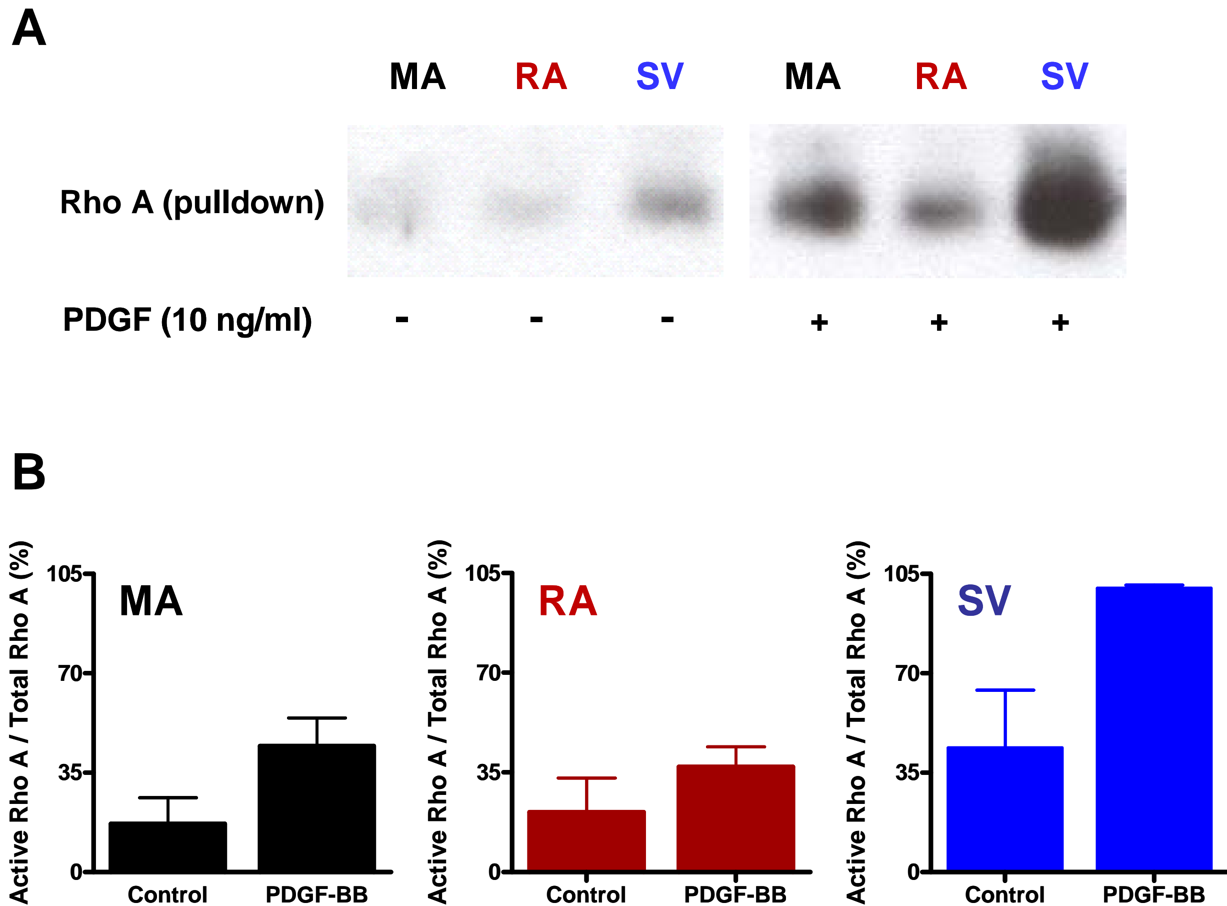


Figure 3. Different Rho A activity in vascular smooth muscle cells (VSMC) from radial artery (RA), internal mammary artery (MA), and saphenous vein (SV). **A.** Rho A activity was assessed by Rho A pulldown, 30 minutes after stimulation of VSMC with 10 ng/ml PDGF-BB (n=4). **B.** Active Rho A is expressed in percentage of Total Rho A levels (n=4).

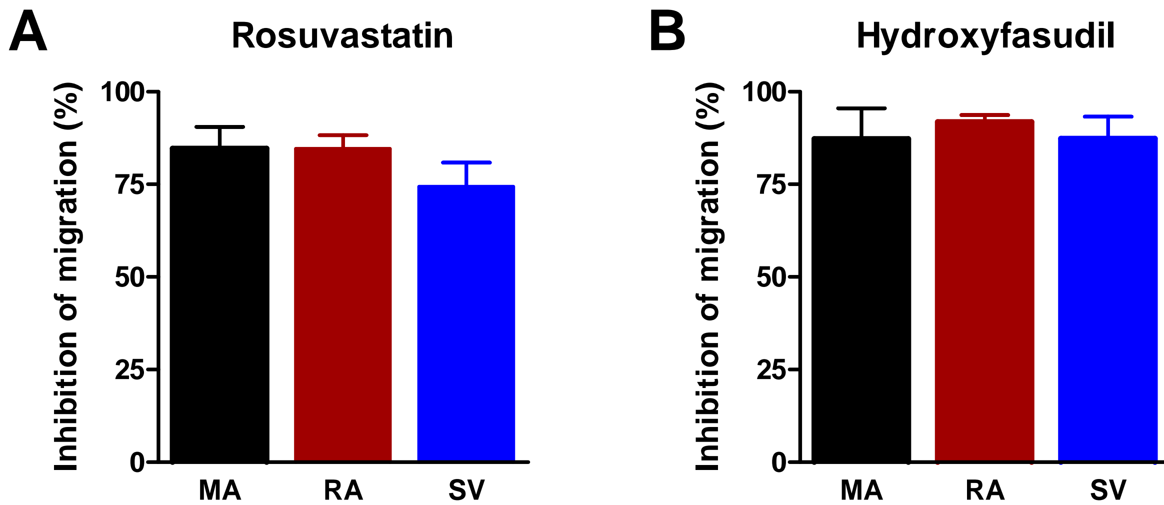


Figure 4. Inhibition of vascular smooth muscle cell (VSMC) migration of internal mammary artery (MA), radial artery (RA) and saphenous vein (SV) after Rho inhibition. Cells were preincubated with rosuvastatin (**A**) or hydroxyfasudil (**B**) for 30 minutes before incubating 5 hours in Bodyden chamber. Chemokinesis was subtracted from number of migrated VSMC in response to PDGF-BB and results were expressed in percentage of chemotaxis of SV VSMC in response to PDGF-BB.

5.3. Discussion

This study demonstrates that VSMC from human coronary artery bypass vessels exhibit differences in migration, indicating that there are differences in the intrinsic properties of VSMC from RA, MA and SV. Migration of VSMC from RA was slightly higher than that of MA but lower than that of SV. Thus, migration corresponds to patency rates of the according vessels. The differences are not releated to PDGF-receptor expression but are due to different Rho A activation. Rho A plays an important role in VSMC migration.[4,9] Statins were shown to inhibit the Rho pathway leading to reduction of VSMC migration. Therefore, statins have been implicated in the treatment of a variety of cardiovascular disorders, including vasospasm, hypertension, and atherosclerotic vascular disease.[10,11,12] Differences of migration in VSMC from RA, MA and SV were related to differences in Rho A activity. Migration of VSMC was inhibited by adding rosuvastatin; therefore statins might have beneficial effects on patency rates of coronary artery bypass vessels by inhibition of VSMC migration. Further the ROCK inhibitor hydroxyfasudil prevents intimal hyperplasia by inhibiting VSMC migration as well.[13] These data suggest that hydoxyfasudil might have similar applications as statins in preventing coronary artery bypass graft disease.

Taken together these data demonstrate that a) the Rho A / ROCK pathway is essential for migration of human VSMC, b) the difference in migration of VSMC from RA, MA, and SV is related to different Rho A activity, c) both statins and hydroxyfasudil may have beneficial effects on patency rates of coronary artery bypass vessels via inhibition of VSMC migration.

5.4. References

1. Schwartz SM. Smooth muscle migration in atherosclerosis and restenosis. *J Clin Invest*. 1997;100:S87-S89
2. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801-809
3. Johnson JL, van Eys GJ, Angelini GD, George SJ. Injury induces dedifferentiation of smooth muscle cells and increased matrix-degrading metalloproteinase activity in human saphenous vein. *Arterioscler Thromb Vasc Biol*. 2001;21:1146-1151
4. Porter KE, Naik J, Turner NA, Dickinson T, Thompson MM, London NJ. Simvastatin inhibits human saphenous vein neointima formation via inhibition of smooth muscle cell proliferation and migration. *J Vasc Surg*. 2002;36:150-157
5. Loop FD, Lytle BW, Cosgrove DM, Stewart RW, Goormastic M, Williams GW, Golding LA, Gill CC, Taylor PC, Sheldon WC. Influence of the internal-mammary-artery graft on 10-year survival and other cardiac events. *N Engl J Med*. 1986;314:1-6
6. Barner HB, Barnett MG. Fifteen- to twenty-one-year angiographic assessment of internal thoracic artery as a bypass conduit. *Ann Thorac Surg*. 1994;57:1526-1528
7. Dobrin P, Canfield T, Moran J, Sullivan H, Pifarre R. Coronary artery bypass. The physiological basis for differences in flow with internal mammary artery and saphenous vein grafts. *J Thorac Cardiovasc Surg*. 1977;74:445-454
8. Canham PB, Finlay HM, Boughner DR. Contrasting structure of the saphenous vein and internal mammary artery used as coronary bypass vessels. *Cardiovasc Res*. 1997;34:557-567
9. Negre-Aminou P, van Erck M, van Leeuwen RE, Collard JG, Cohen LH. Differential effect of simvastatin on various signal transduction intermediates in cultured human smooth muscle cells. *Biochem Pharmacol*. 2001;61:991-998
10. Shimokawa H. Rho-kinase as a novel therapeutic target in treatment of cardiovascular diseases. J *Cardiovasc Pharmacol*. 2002;39:319-327

11. Eto M, Kozai T, Cosentino F, Joch H, Luscher TF. Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. *Circulation*. 2002;105:1756-1759

12. Nakagami H, Jensen KS, Liao JK. A novel pleiotropic effect of statins: prevention of cardiac hypertrophy by cholesterol-independent mechanisms. *Ann Med.* 2003;35:398-403

13. Negoro N, Hoshiga M, Seto M, Kohbayashi E, Ii M, Fukui R, Shibata N, Nakakoji T, Nishiguchi F, Sasaki Y, Ishihara T, Ohsawa N. The kinase inhibitor fasudil (HA-1077) reduces intimal hyperplasia through inhibiting migration and enhancing cell loss of vascular smooth muscle cells. *Biochem Biophys Res Commun*. 1999;262:211-215

6. Part Three

Thrombin Potentiates PDGF-Induced Human Vascular Smooth Muscle Cell Proliferation via PI3 Kinase Activation Leading to p27 Downregulation

6.1. Introduction

Vascular smooth muscle cells (VSMC) accumulation in the subendothelial space of grafted vessels is a major mediator of neointima formation, and hence plays an important role for long-term patency.[1] Not only VSMC proliferation, but also thrombus formation is a key event in pathogenesis of bypass graft disease. At the site of thrombus formation both aggregating platelets and the coagulation cascade play an important role, therefore mediators from both parts may interact at the level of VSMC proliferation. Aggregating platelets release growth factors such as platelet-derived growth factor (PDGF), which initiates VSMC to proliferate, to migrate to the intima, and to produce extracellular matrix.[2] PDGF may interact with thrombin, which is a mediator of the coagulation cascade catalyzing the formation of fibrin (Figure 1).[3] In addition, thrombin has been reported to stimulate proliferation of VSMC.[4,5] However, with respect to the effect of thrombin on proliferation of human VSMC contradictory observations have been made.[6,7]

Mitogenic signals are integrated in G1 phase. Activity of cell cycle proteins regulating G1 progression is related to complexes of cyclins and cyclin-dependent kinases (cdk).[8] Further, cell cycle progression is negatively regulated by cyclin-dependent kinase inhibitors (CKI); indeed, CKI of the Kip/Cip family are potent inhibitors of VSMC proliferation.[9,10,11] Consistent with this observation, upregulation of CKI p27 and p21 coincides with the decline in proliferation after balloon dilatation, and adenoviral overexpression of these genes reduces neointima formation.[11,12]

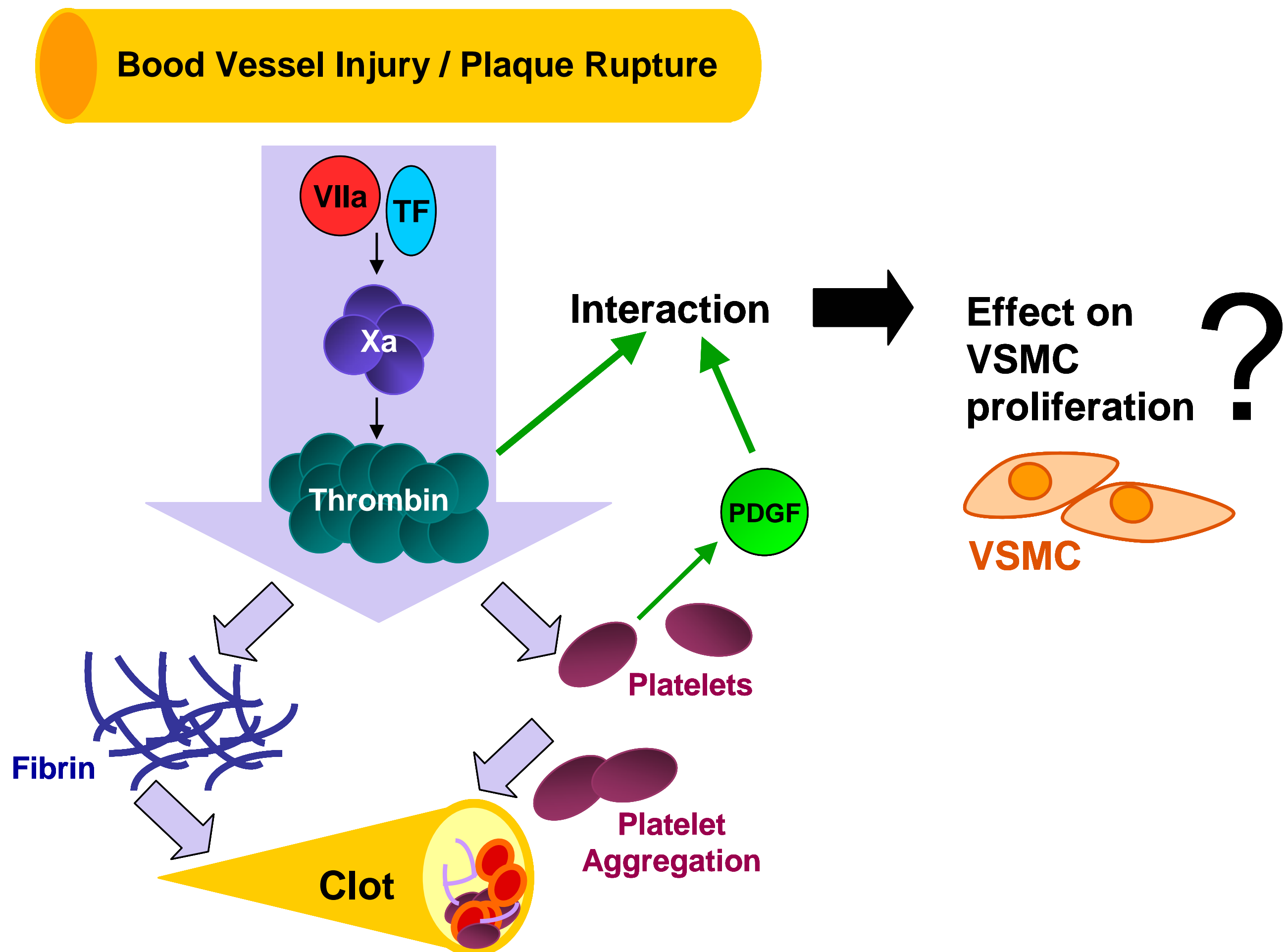


Figure1. After blood vessel injury, mediators from both platelets such as platelet-derived growth factor (PDGF) and the coagulation system such as thrombin can affect vascular smooth muscle cell (VSMC) proliferation. Therefore, the interaction of PDGF and thrombin at the level of signal transduction and cell cycle regulation of human saphenous vein VSMC was examined.

Similar to other cell types, p27 and p21 are known to enter a complex with cdk2 in VSMC; this causes inhibition of cdk2 activity, which negatively regulates VSMC proliferation and neointima formation.[13] A recent study from our laboratory demonstrated, that intrinsic differences in expression and/or activity of cell cycle proteins may indeed profoundly affect VSMC proliferation.[14] VSMC from hypertensive rats (SHR) exhibit a higher proliferation as those from normotensive controls (WKY); this difference is related to a higher expression and a faster induction of both cyclin E and cyclin A in response to mitogenic stimulation, resulting in a higher cdk2 kinase activity in the hypertensive cells (Figure 2).

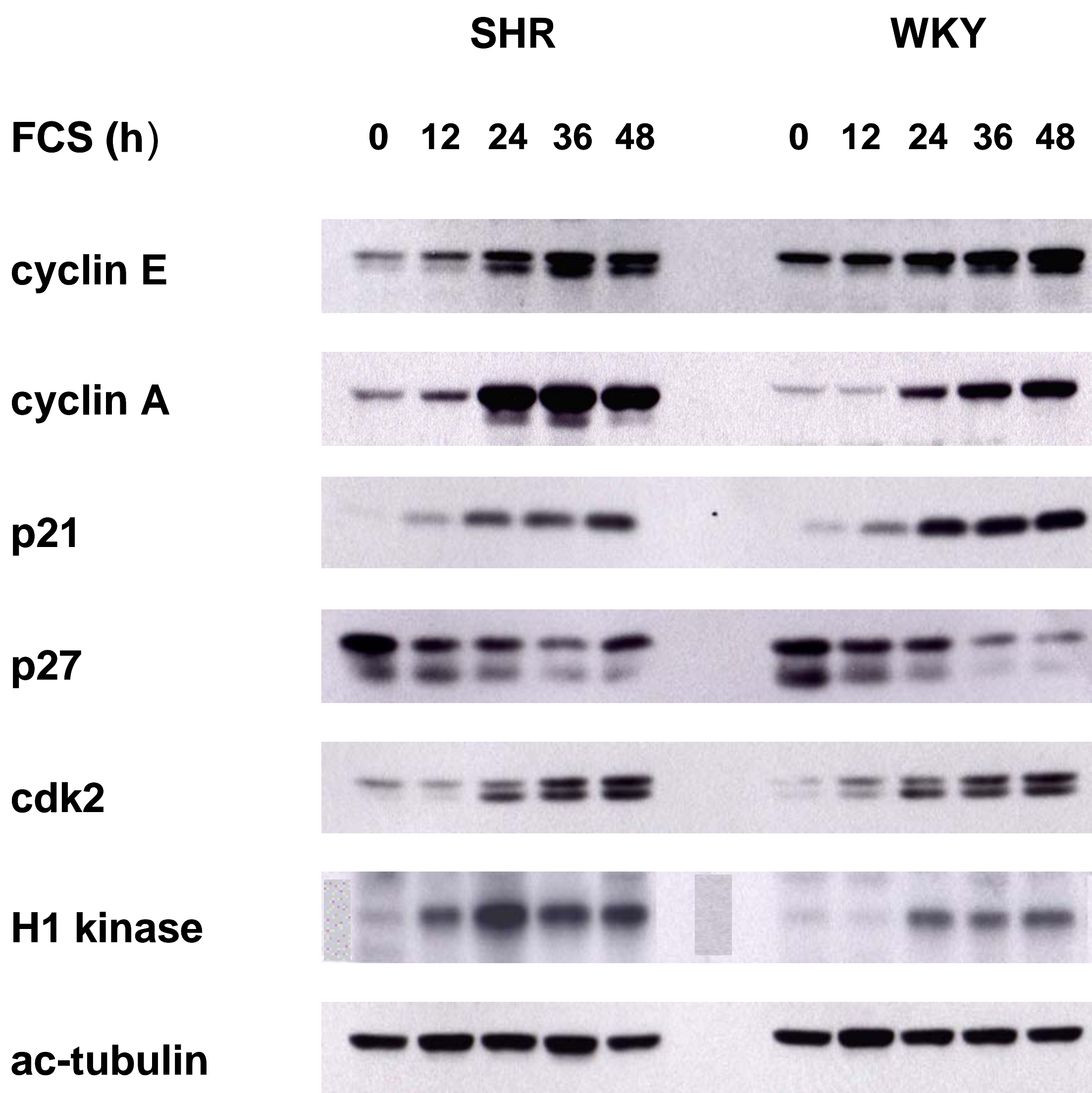


Figure 2. G1 phase progression in vascular smooth muscle cells from hypertensive rats (SHR) and normotensive control rats (Wistar-Koyoto rats; WKY) (n=3).

Activation of PI3 kinase generates phosphorylated inositol lipids, which serve as second messengers for cell survival, proliferation, and metabolism.[15,16] The PI3 kinase pathway seems to be a major regulator of VSMC proliferation, as it is both necessary and sufficient for proliferation in response to several mitogens.[17] The mammalian target of rapamycin (mTOR) belongs to a family of phosphatidylinositol kinase-like kinases. In the context of cell proliferation, the best known function of mTOR is regulation of translation initiation.[18] Indeed, inhibition of mTOR by rapamycin impairs cell cycle progression in human VSMC.[19] In some cell lines, cell cycle inhibition by rapamycin has been related to p27; however, the latter was not affected by rapamycin in rat VSMC.[20,21] In human VSMC, the mitogen-activated protein (MAP) kinase pathway is involved in the response to different mediators. MAP kinases are a family of protein serine/threonine kinases which are part of the regulation of cell survival and proliferation.[22] However, PDGF strongly activated MAP kinase, while thrombin had only weak effects.[7,23,24]

6.2. Results

6.2.1. Proliferation rates

Preliminary experiments with human SV VSMC demonstrated that 1% FCS and 1 ng/ml PDGF-BB induced a threshold proliferation, while higher concentrations evoked a significant increase in cell number (n=4; data not shown). In contrast, thrombin did not induce a significant proliferation even at concentrations as high as 9 U/ml (increase in cell number: control: 16'750±228, thrombin: 16'313±1159; n=4; p=n.s.). Thrombin concentrations of 3 U/ml evoke a maximal endothelium-dependent relaxation of isolated vessels, therefore, this concentration was chosen to study interactions with FCS and PDGF-BB.[25] Hence, VSMC were stimulated with either a maximal concentration of thrombin (3 U/ml), a threshold concentration of FCS (1%), or both. Stimulation with thrombin alone or FCS alone did not significantly affect cell number over 4 days (increase in cell number: thrombin alone: 4'375±2'105; FCS alone 5'937±2'018; n=6; p=n.s. for thrombin or FCS vs. control). In contrast, thrombin potentiated threshold concentrations of FCS, leading to a significant increase in cell number (16'344±3'216; n=6; p<0.01 for thrombin or FCS vs. thrombin plus FCS; Figure 3A). Similarly, thrombin potentiated threshold concentrations of PDGF-BB (1 ng/ml) (increase in cell number: thrombin alone: 4'375±2'105; PDGF alone: 5'375±2'057; thrombin plus PDGF: 18'688±1'711; n=6; p=n.s. for thrombin or PDGF vs. control; p<0.0002 for thrombin or PDGF vs. thrombin plus PDGF; Figure 3B). ^{3}H-thymidine incorporation revealed that thrombin potentiates VSMC proliferation to PDGF-BB in a concentration-dependent manner from 0.1 to 3 U/ml. Indeed, a maximal effect already occurred with 0.3 U/ml thrombin (DPM/1'000 cells: thrombin alone: 101±29; PDGF alone: 96±15; thrombin plus PDGF: 201±29; n=6; p<0.01 for thrombin or PDGF vs. thrombin plus PDGF; Figure 4).

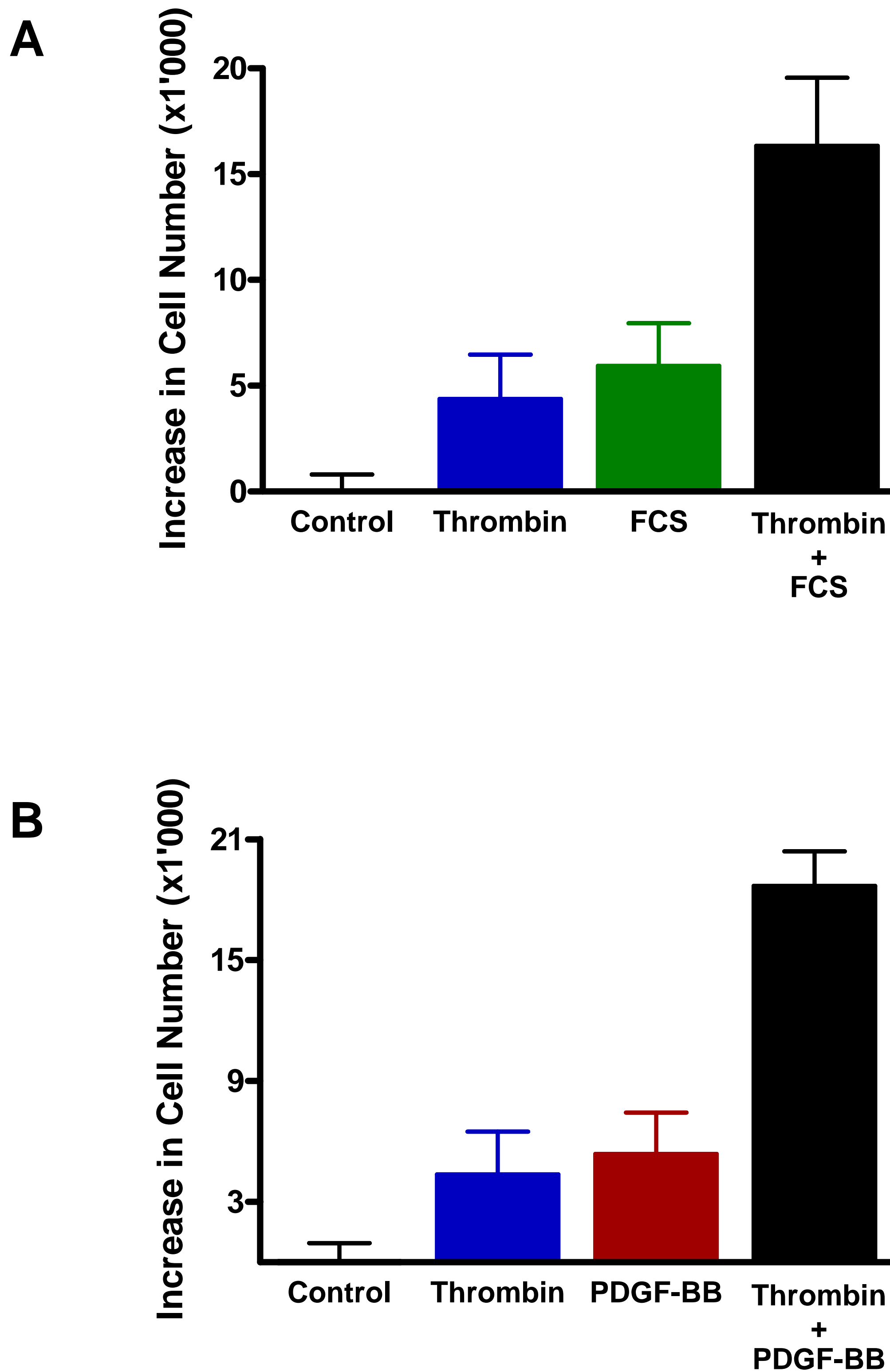
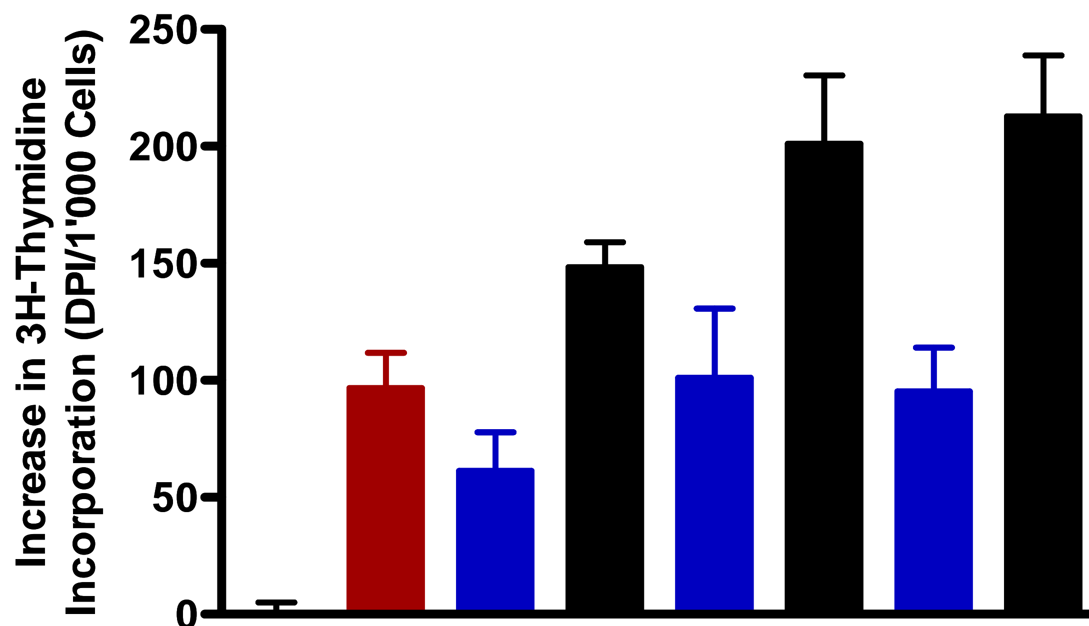


Figure 3. Thrombin potentiates proliferation of human vascular smooth muscle cells (VSMC) to threshold concentrations of FCS and PDGF-BB. **A.** VSMC were serum starved for 48 hours and then stimulated with either thrombin (3 U/ml), FCS (1%) or thrombin (3 U/ml) plus FCS (1%). Cell number was determined on day 4 after stimulation (n=4). **B.** VSMC were serum starved for 48 hours and then stimulated with either thrombin (3 U/ml), PDGF-BB (1 ng/ml), or thrombin (3 U/ml) plus PDGF-BB (1 ng/ml). Cell number was determined on day 4 after stimulation (n=4).

Potentiation was completely prevented by the direct thrombin inhibitor hirudin (3 U/ml), while proliferation in response to PDGF-BB was not affected (DPM/1000 cells: thrombin alone: 65±7; thrombin plus hirudin: 29±4, PDGF alone: 70±11; PDGF plus hirudin: 70±7; thrombin plus PDGF: 155±23, thrombin plus PDGF plus hirudin: 83±8; n=6; p<0.01 for thrombin or PDGF vs. thrombin plus PDGF; p=n.s. for thrombin or PDGF vs. thrombin plus PDGF plus hirudin; Figure 5).



T (U/ml)	-	-	0.1	0.1	0.3	0.3	1.0	1.0
P (ng/ml)	-	1	-	1	-	1	-	1

Figure 4. Thrombin (T) potentiates proliferation of human vascular smooth muscle cells (VSMC) in a concentration-dependent manner. Arrested VSMC were stimulated with a threshold concentration (1 ng/ml) of PDGF-BB (P) and increasing concentrations of thrombin (0.1 to 1 U/ml). Exit from G0/G1 phase was measured 30 hours after stimulation by quantification of 3H-thymidine incorporation (n=4).

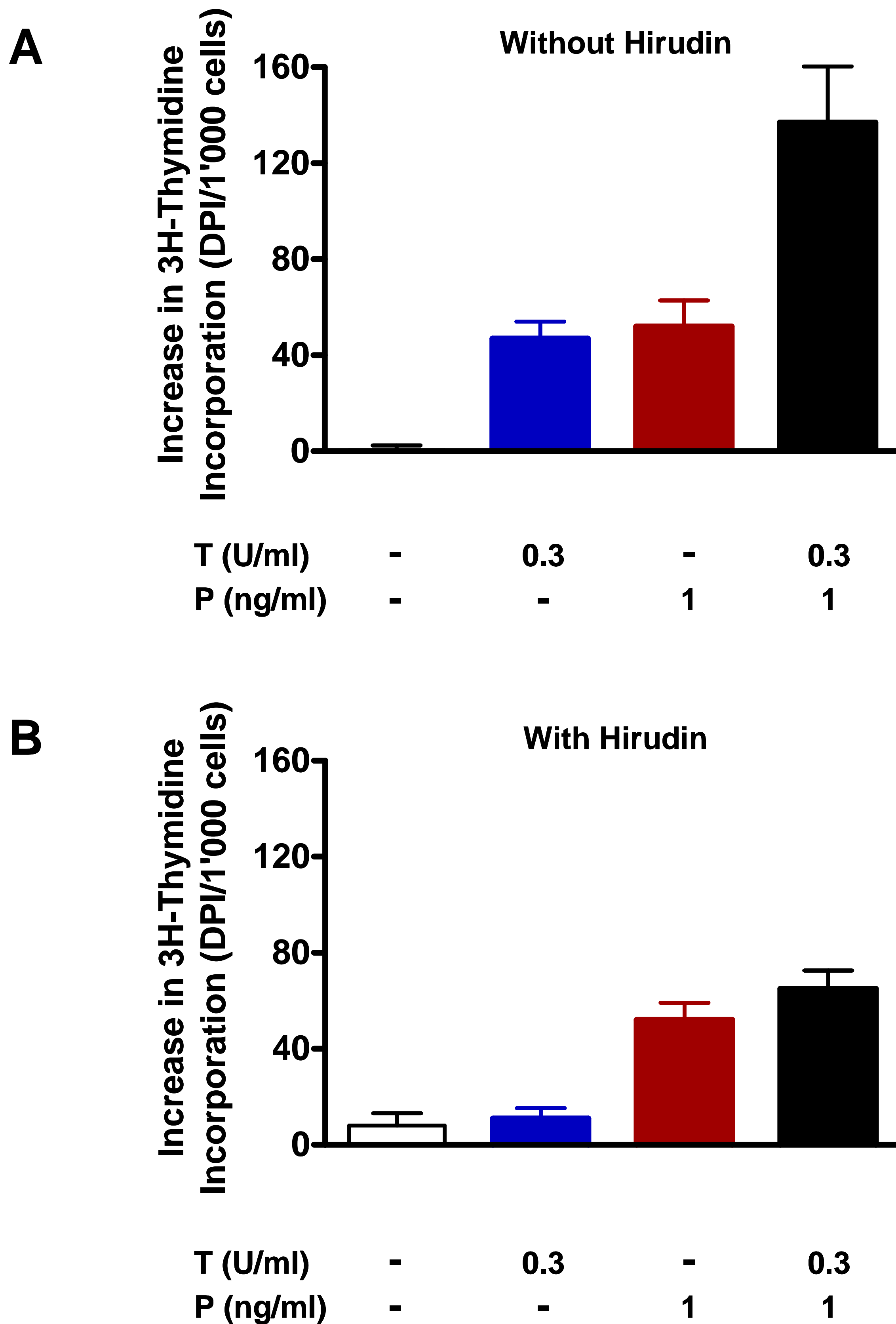


Figure 5. Hirudin prevents potentiation of thrombin. Arrested VSMC were stimulated with either thrombin (T, 0.3 U/ml), PDGF-BB (P, 1 ng/ml) or thrombin (0.3 U/ml) plus PDGF-BB (1 ng/ml) in the absence (**A**) or presence (**B**) of hirudin. Exit from G0/G1 phase was measured 30 hours after stimulation by quantification of 3H-thymidine incorporation (n=4).

6.2.2. Receptor expression

The thrombin induced potentiation of VSMC proliferation to PDGF-BB may be related to induction of PDGF receptor expression. Therefore, the effect of thrombin on PDGF receptor surface expression was determined by FACS analysis. PDGF receptor α expression was not significantly affected by exposure to 3 U/ml thrombin over 30 hours (fluorescence intensity in % of shift to isotype: control: 100.0±3.5; thrombin, 15 hours: 100.2±3.3, thrombin, 30 hours: 87.6±6.8; n=4; p=n.s.; Figure 6A). Similar observations were made for PDGF receptor β expression (control: 100.0±16.3; thrombin, 15 hours: 95.8±13.1; thrombin, 30 hours: 93.4±14.5; n=4; p=n.s.; Figure 6A). Therefore, thrombin induced potentiation of VSMC proliferation to PDGF-BB was not due to alteration of PDGF receptor surface expression.

Thrombin receptor activator peptide (TRAP) corresponds to the tethered ligand sequence of the human thrombin receptor and can therefore mimic specific thrombin receptor activation. ^{3}H-thymidine incorporation revealed that, similar to thrombin, TRAP (100 µM) potentiates threshold concentrations of PDGF-BB (1 ng/ml) on VSMC proliferation (DPM/1’000 cells: thrombin alone: 71±34; TRAP alone: 32±9; thrombin plus PDGF: 254±97; TRAP plus PDGF: 232±89; n=4; p=n.s.; Figure 6B). These data suggest that thrombin potentiates PDGF-induced VSMC proliferation via thrombin receptor activation.

6.2.3. Cell cycle

To determine if cell cycle progression was affected by thrombin induced potentiation, expression of cell cycle proteins regulating G1 progression was determined by Western blotting analysis for up to 30 hours of mitogenic stimulation. The cyclin-dependent kinase inhibitors p21, p27, and p57 were neither affected by thrombin (0.3 U/ml) nor PDGF-BB (1 ng/ml) alone. In contrast, thrombin plus PDGF-BB caused p27 downregulation, while p21 was slightly induced, and p57 remained unaffected. Expression of cyclin-dependent kinase 2 (cdk2) and cyclin E was not affected under all three conditions (Figure 7A). Consistent with these observations, cdk2 activity was neither affected

by thrombin (0.3 U/ml) nor PDGF-BB (1 ng/ml) alone, but enhanced by thrombin plus PDGF-BB. The increase in cdk2 activity was prevented by hirudin (Figure 7B).

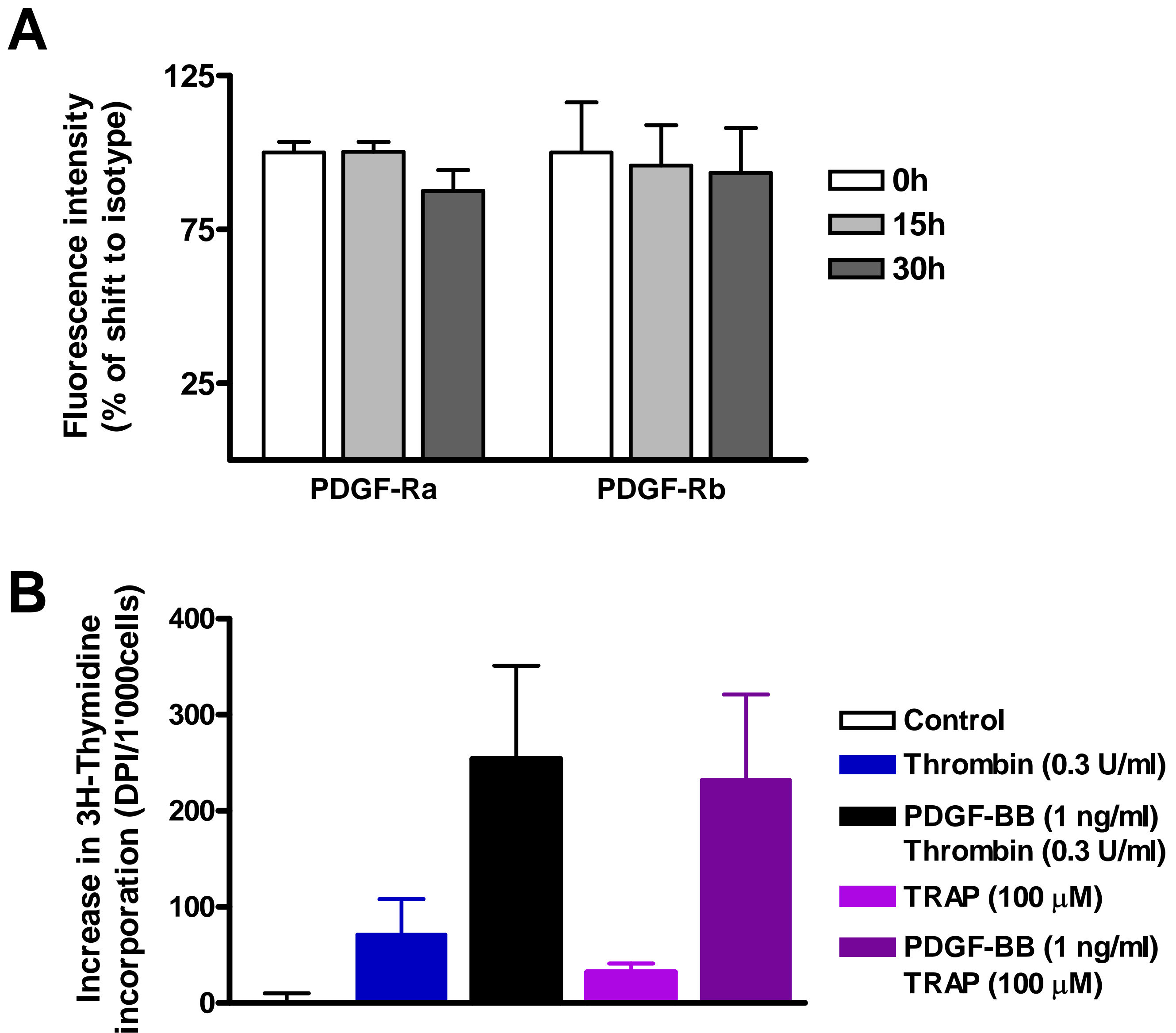


Figure 6. Thrombin does not change PDGF receptor surface expression **A**. Vascular smooth muscle cells (VSMC) were serum starved for 48 hours and then stimulated with thrombin (3 U/ml) for 30 hours. PDGF receptor α and β surface expression was analyzed by FACS and quantified by determining the fluorescence intensity shift (n=4). **B**. Similarly to thrombin, thrombin receptor activator peptide (TRAP) potentiates proliferation of VSMC. Arrested VSMC were stimulated with threshold concentration of PDGF-BB (1 ng/ml) and either thrombin (3U/ml) or TRAP (100μM). Exit from G0/G1 phase was measured 30 hours after stimulation by quantification of ^{3}H-thymidine incorporation.

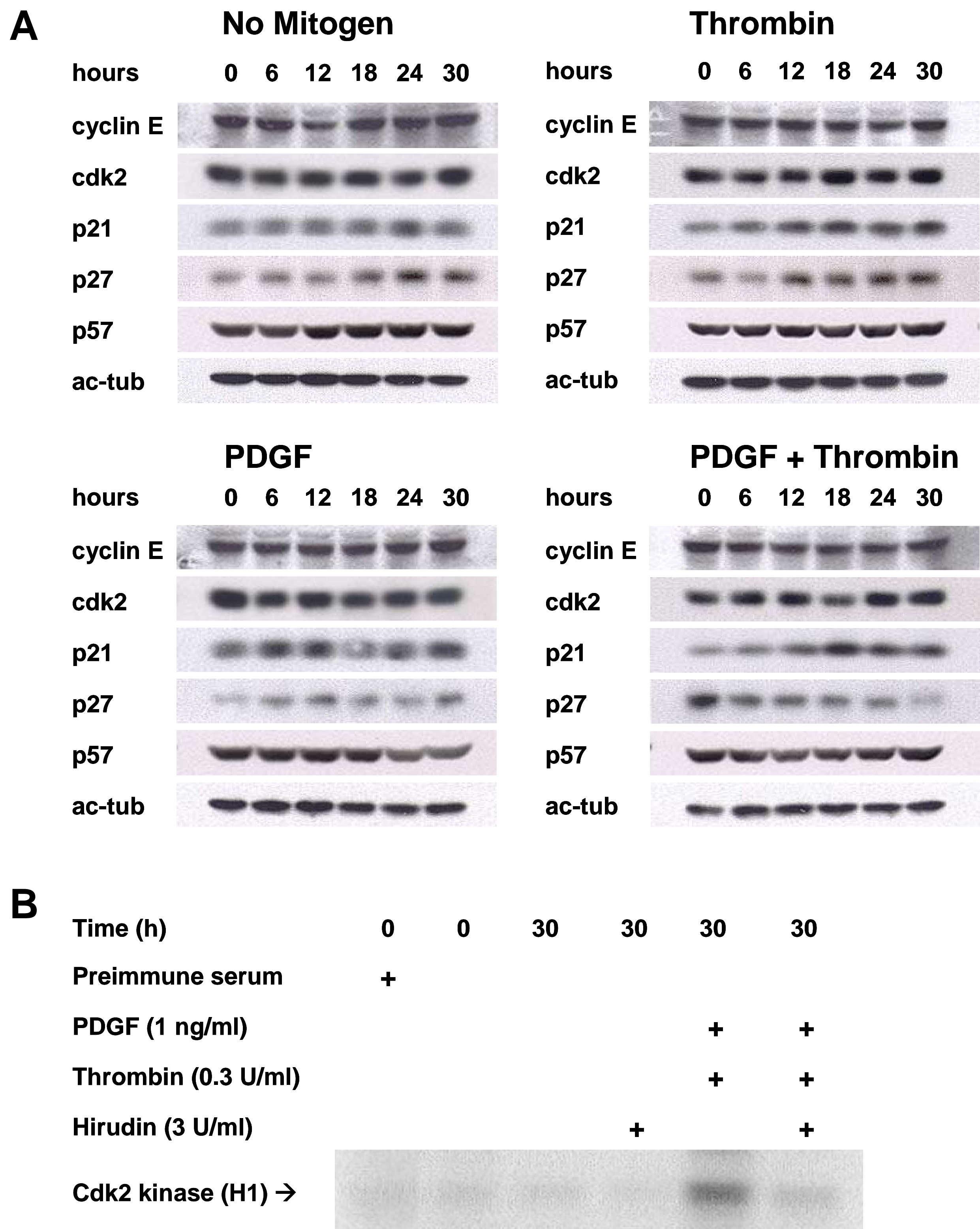
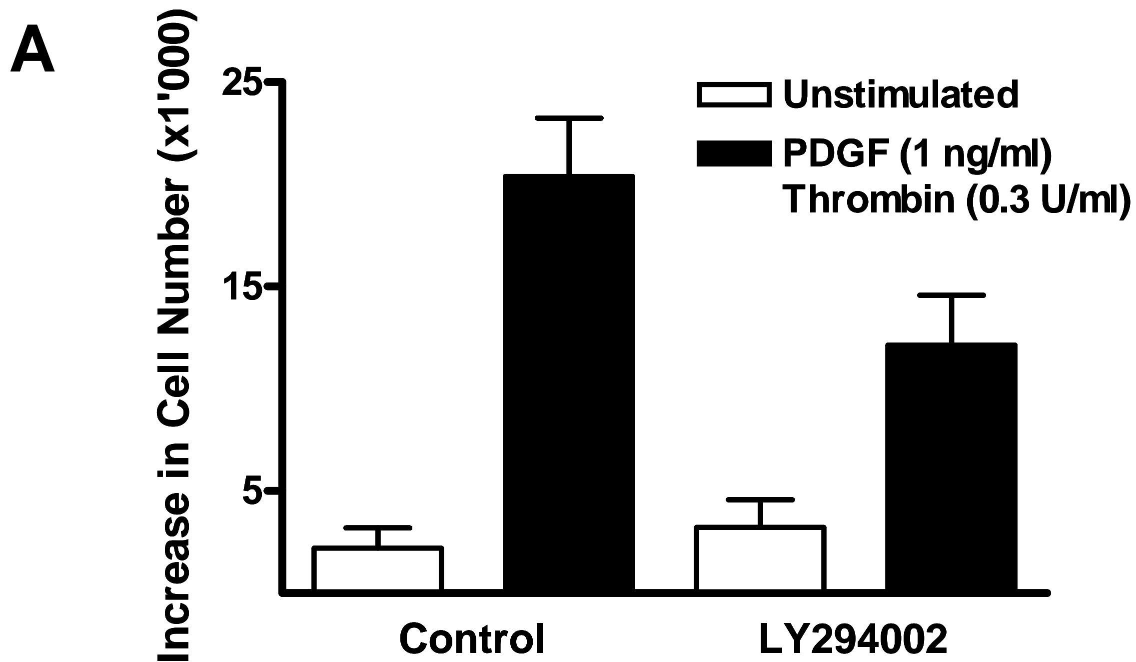


Figure 7. Cell cycle regulation and thrombin-induced potentiation of human vascular smooth muscle cell (VSMC) proliferation. **A.** VSMC were serum starved for 48 hours and then stimulated with either thrombin (0.3 U/ml), PDGF-BB (1 ng/ml) or thrombin (0.3 U/ml) plus PDGF-BB (1 ng/ml) for the indicated times. Cells were lysed and cell cycle regulatory protein levels were determined by Western blotting (n=4). **B.** VSMC were serum starved for 48 hours and then stimulated with thrombin (0.3 U/ml) plus PDGF-BB (1ng/ml) with and without hirudin (3 U/ml). Cells were lysed and cdk2 kinase activity was determined by H1 kinase assay (n=2).

6.2.4. Signal transduction

To determine whether proliferation and p27 downregulation in response to thrombin (0.3 U/ml) plus PDGF-BB (1 ng/ml) was mediated by PI3 kinase, VSMC were treated with one of the specific PI3 kinase inhibitors LY294002 or wortmannin.[26,27] Consistent with our previous observation, thrombin (0.3 U/ml) plus PDGF-BB (1 ng/ml) stimulated proliferation (increase in cell number: thrombin plus PDGF-BB: 20’375±2’858; control: 2’188±1’000; n=4; p<0.001 for thrombin plus PDGF-BB vs. control). This effect was abolished by LY294002 (50 μM) (thrombin plus PDGF-BB with LY294002: 3’500±1’540; control with LY294002: 1’156±393; n=4; p=n.s. for thrombin plus PDGF-BB with LY294002 vs. control with LY294002; p<0.005 for thrombin plus PDGF-BB vs. thrombin plus PDGF-BB with LY294002; p=n.s. for control vs. control with LY294002; Figure 8A). Toxicity of LY294002 was tested by LDH release. No significant increase in LDH release was observed in the presence of LY294002 (absorption: control: 0.075±0.014; LY294002: 0.128±0.023; n=5; p=n.s.). Consistent with the effect of LY294002 on cell number, p27 downregulation in response to thrombin plus PDGF-BB was abolished after inhibition of PI3 kinase (Figure 8B). Similar data were obtained with wortmannin (1 μM). Proliferation to thrombin plus PDGF-BB (increase in cell number: thrombin plus PDGF-BB: 12’850±1’160; control: 878±185; n=4; p<0.001 for thrombin plus PDGF-BB vs. control) was completely blocked by wortmannin (thrombin plus PDGF-BB with wortmannin: 2’910±1’264; control with wortmannin: 393±448; n=4; p=n.s. for thrombin plus PDGF-BB with wortmannin vs. control with wortmannin; p<0.001 for thrombin plus PDGF-BB vs. thrombin plus PDGF-BB with wortmannin; p=n.s. for control vs. control with wortmannin; Figure 9A). Consistent with the effect of wortmannin on cell number, p27 downregulation in response to thrombin plus PDGF-BB was abolished after inhibition of PI3 kinase (Figure 9B).

A



B

PDGF BB (1 ng/ml) + +

Thrombin (3 U/ml) + +

LY294002 (50 µM) + +

p27

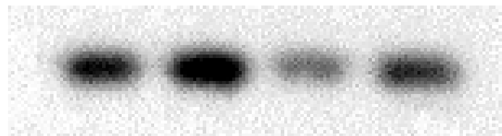
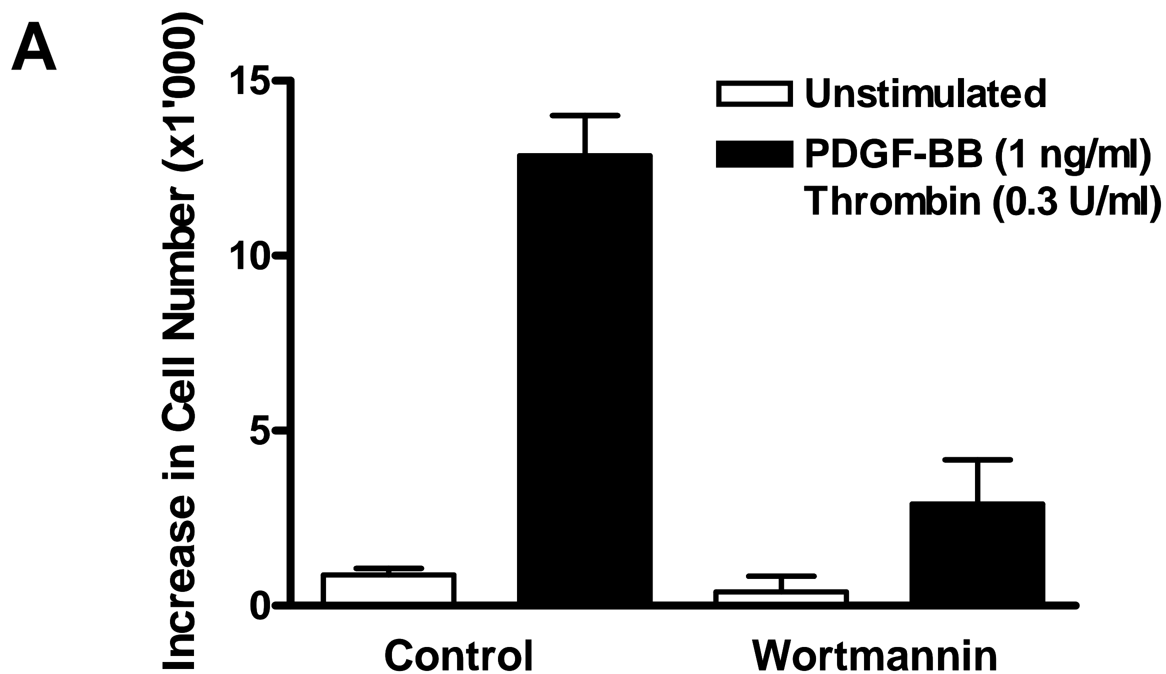


Figure 8. Signal transduction and thrombin-induced potentiation of human vascular smooth muscle cell (VSMC) proliferation. VSMC were serum starved for 48 hours and pretreated with the PI3 kinase inhibitor LY294002 (50 µM). **A**. VSMC were stimulated with thrombin (3 U/ml) plus PDGF-BB (1 ng/ml) and cell number was determined on day 4 after stimulation (n=4). **B**. VSMC were lysed after 30 hours of stimulation and p27 protein levels were determined by Western blotting analysis (n=4).



B

PDGF BB (1 ng/ml) + +
Thrombin (3 U/ml) + +
Wortmannin (1 µM) + +
p27

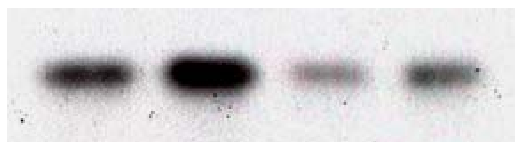


Figure 9. Signal transduction and thrombin-induced potentiation of human vascular smooth muscle cell (VSMC) proliferation. VSMC were serum starved for 48 hours and pretreated with the PI3 kinase inhibitor wortmannin (1 µM). **A**. VSMC were stimulated with thrombin (3 U/ml) plus PDGF-BB (1 ng/ml) and cell number was determined on day 4 after stimulation (n=4). **B**. VSMC were lysed after 30 hours of stimulation and p27 protein levels were determined by Western blotting analysis (n=4).

The mammalian target of rapamycin (mTOR) is located downstream of PI3 kinase in some cell types; however, it is not clear whether it is involved in regulation of p27 expression in VSMC. We therefore examined whether mTOR mediates downregulation of p27 in response to thrombin plus PDGF-BB. Proliferation to thrombin plus PDGF-BB (increase in cell number: thrombin plus PDGF-BB: 15'500±1'447; control: 1'063±300; n=4; p<0.0001 for thrombin plus PDGF vs. control) was only marginally inhibited by rapamycin (thrombin plus PDGF-BB with rapamycin: 9'313±900; control with rapamycin: 1'625±690; n=4; p<0.001 for thrombin plus PDGF-BB with rapamycin vs. control with rapamycin; p<0.05 for thrombin plus PDGF-BB vs. thrombin plus PDGF-BB with rapamycin; p=n.s. for control vs. control with rapamycin; Figure 10A). Toxicity of rapamycin was tested by LDH release. No significant increase in LDH release was observed in the presence of rapamycin (absorption: control: 0.080±0.024; rapamycin: 0.063±0.011; n=4; p=n.s.). Consistent with the effect of rapamycin on cell number, no difference in p27 expression was observed after inhibition of mTOR (Figure 10B).

To assess the contribution of the MAP kinase cascade, VSMC were exposed to PD98059 (30 μmol/L), which specifically inhibits activation of MEK-1/MEK-2.[28,29] Proliferation to thrombin plus PDGF-BB (increase in cell number: thrombin plus PDGF-BB: 20'375±2'858; control: 2188±1000; n=4; p<0.001 for thrombin plus PDGF-BB vs. control) was not significantly affected by PD98059, although a tendency towards a reduced cell number was observed as compared to control conditions (thrombin plus PDGF-BB with PD98059: 12'125±2'446; control with PD98059: 3'219±1'351; n=4; p<0.05 for thrombin plus PDGF-BB with PD98059 vs. control with PD98059; p=n.s. for thrombin plus PDGF-BB vs. thrombin plus PDGF-BB with PD98059; p=n.s. for control vs. control plus PD98059; Figure 11A). Toxicity of PD98059 was tested by LDH release. No significant increase in LDH release was observed in the presence of PD98059 (absorption: control: 0.052±0.004; PD98059: 0.092±0.026; n=4; p=n.s.). Consistent with the effect of PD98059 on cell number, no difference in p27 expression was observed after inhibition of the MAP kinase cascade (Figure 11B). These data indicate that thrombin potentiates PDGF-BB induced VSMC proliferation

via activation of PI3 kinase leading to downregulation of p27, while both mTOR and the MAP kinase pathway contribute only weekly to proliferation under these conditions.

6.2.5. Potency of potentiation

To determine the potency of thrombin (0.3 U/ml) in potentiating threshold concentrations of PDGF-BB (1 ng/ml), the increase in cell number was compared to the effect of 10 ng/ml PDGF-BB or 10% FCS. Preliminary experiments revealed that a PDGF-BB concentration of 10 ng/ml resulted in a maximal effect on proliferation (n=4; data not shown). Thrombin-induced potentiation of PDGF-BB threshold concentration was as potent as 10 ng/ml PDGF-BB alone (increase in cell number: thrombin plus PDGF-BB (1 ng/ml): 10’438±2’441; PDGF-BB alone (10 ng/ml): 7’313±2’002; n=4; p=n.s.; Figure 12A). In contrast, 10% FCS was twice as potent (increase in cell number: 23’063±4’286; n=4; FCS vs. thrombin plus PDGF-BB or PDGF-BB alone: $p<0.05$; Figure 12A). Similarly, expression of cell cycle regulatory proteins in response to thrombin (0.3 U/ml) plus PDGF-BB (1 ng/ml) was compared to PDGF-BB alone (10 ng/ml) and to FCS (10%). p27 protein level was reduced to a similar degree in all three groups (Figure 12B). In contrast, cdk2 activation, as revealed by the lower band of the doublet representing threonine phosphorylation, was more pronounced after FCS stimulation (Figure 12B). These data indicate that cdk2 phosphorylation, but not p27 downregulation, is responsible for the FCS induced additional increase in cell number as compared to thrombin (0.3 U/ml) plus PDGF-BB (1 ng/ml) or the maximal concentration of PDGF-BB alone (10 ng/ml).

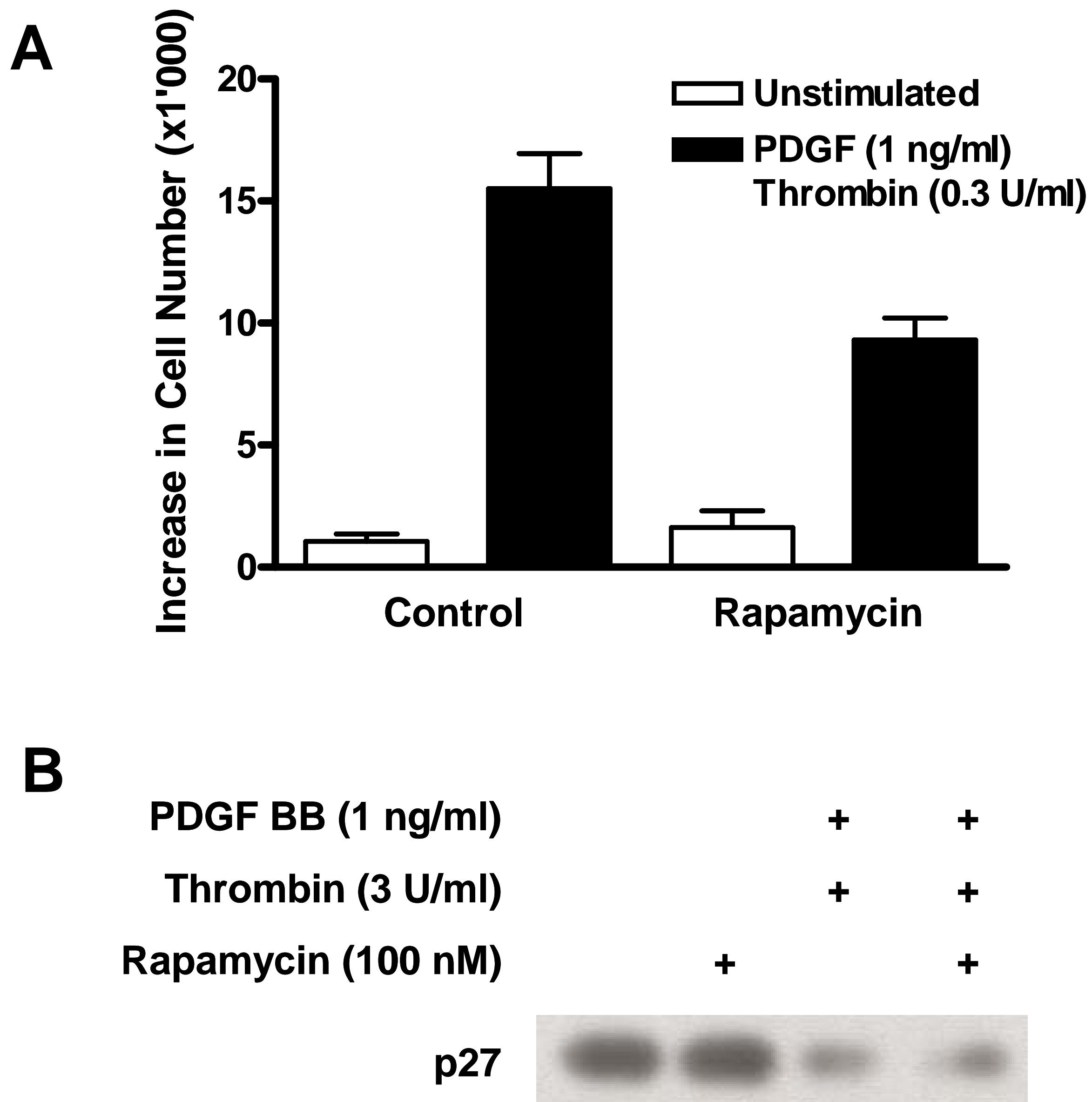
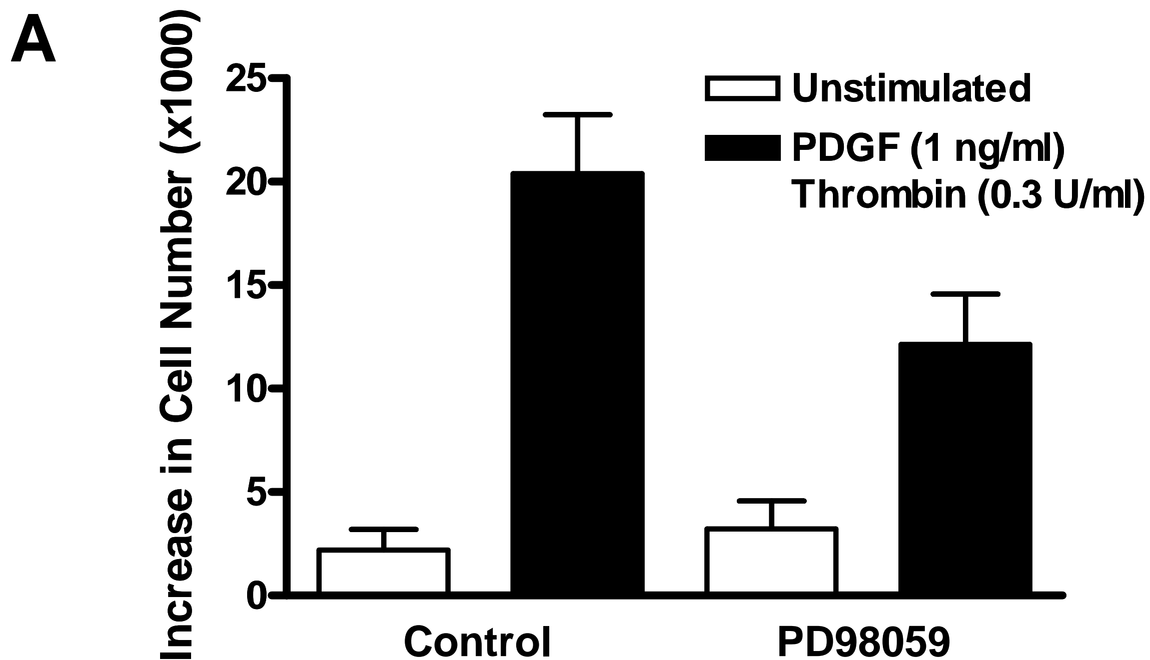


Figure 10. Signal transduction and thrombin-induced potentiation of human vascular smooth muscle cell (VSMC) proliferation. VSMC were serum starved for 48 hours and pretreated with the mTOR inhibitor rapamycin (100 nM). **A**. VSMC were stimulated with thrombin (3 U/ml) plus PDGF-BB (1 ng/ml) and cell number was determined on day 4 after stimulation (n=4). **B**. VSMC were lysed after 30 hours of stimulation and p27 protein levels were determined by Western blotting analysis (n=4).



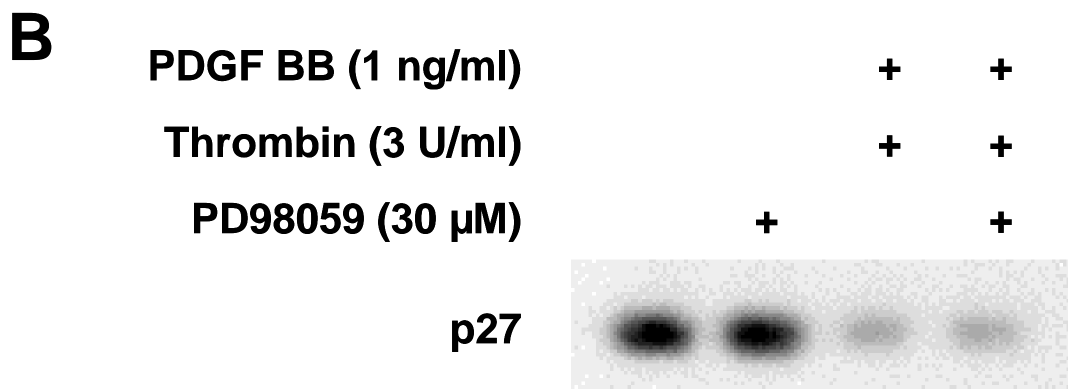


Figure 11. Signal transduction and thrombin-induced potentiation of human vascular smooth muscle cell (VSMC) proliferation. VSMC were serum starved for 48 hours and pretreated with the MAP kinase inhibitor PD98059 (30 μM). **A**. VSMC were stimulated with thrombin (3 U/ml) plus PDGF-BB (1 ng/ml) and cell number was determined on day 4 after stimulation (n=4). **B**. VSMC were lysed after 30 hours of stimulation and p27 protein levels were determined by Western blotting analysis (n=4).

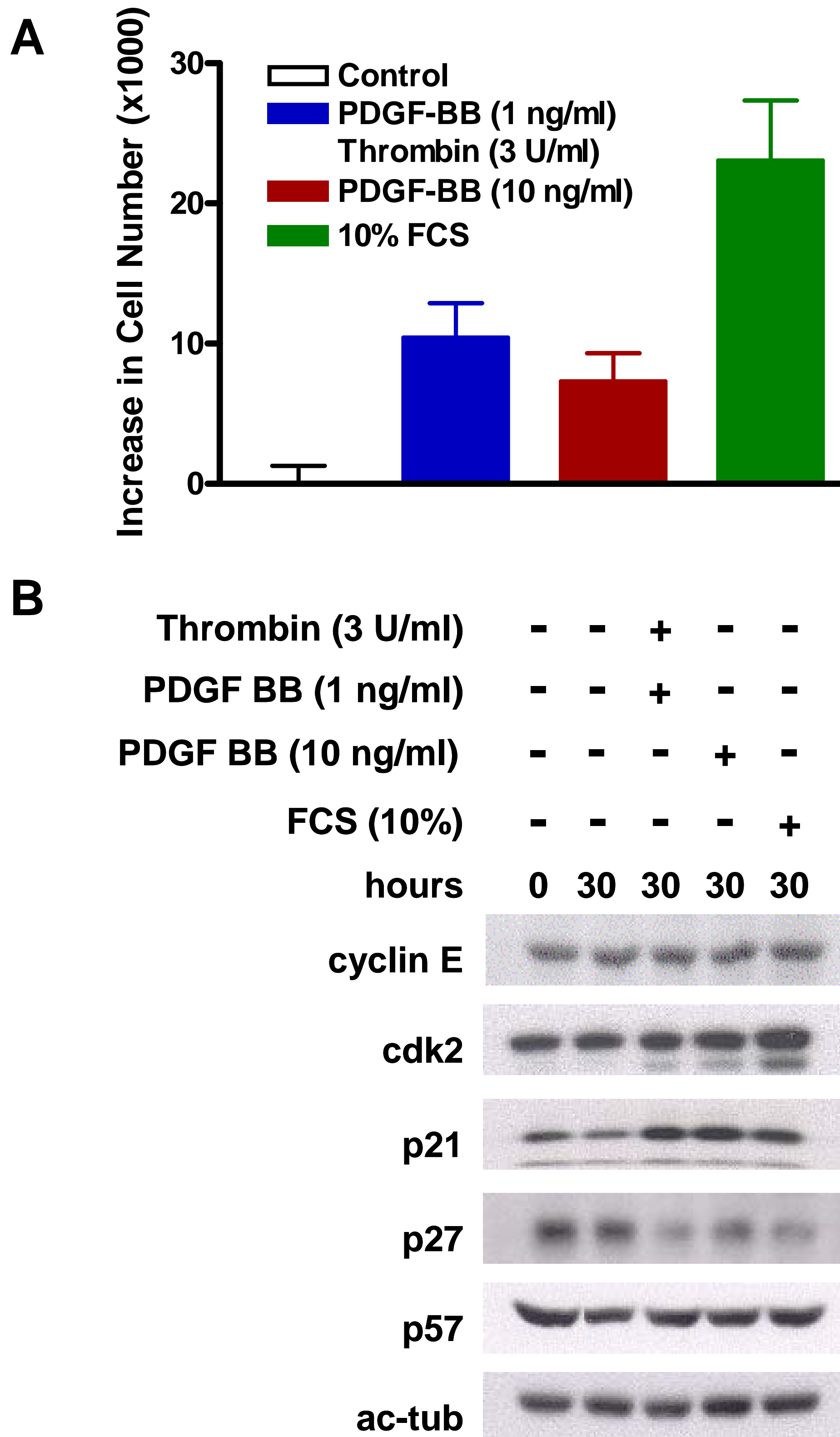


Figure 12. Potency of thrombin-induced potentiation of human vascular smooth muscle cell (VSMC) proliferation. **A.** VSMC were serum starved for 48 hours and then stimulated either with thrombin (3 U/ml) plus PDGF-BB (1 ng/ml) or PDGF-BB alone (10 ng/ml) or FSC (10%) Cell number was determined on day 4 after stimulation (n=4). **B.** The experiment was performed as described in A. Cells were lysed after 30 hours of stimulation and cell cycle regulatory protein levels were determined by Western blotting analysis (n=4).

6.3. Discussion

This study demonstrates that thrombin potentiates proliferation of human SV VSMC to PDGF-BB in a concentration-dependent manner, while it is not mitogenic by itself. The effect of thrombin is not related to induction of PDGF receptor expression, but is mediated by PI3 kinase activation leading to p27 downregulation. Both mTOR and the MAP kinase cascade only marginally contribute to proliferation; consistent with this observation, they are not involved in p27 downregulation.

Thrombin-induced potentiation was not only observed with PDGF-BB, but also with FCS; hence, it occurs with different mitogens and likely is a general phenomenon. Moreover, as mitogen concentrations in vivo are low, potentiation of proliferation may occur in many instances under these conditions. Therefore, the thrombin-induced potentiation may have a major stimulating effect on proliferation in vivo. By inhibiting thrombus formation, hirudin should reduce the release of PDGF-BB from platelets; moreover, by inhibiting the thrombin-induced potentiation of proliferation, it should affect the action of PDGF-BB as well.[30] The latter would represent a new beneficial effect of hirudin coming into play at sites of thrombus formation and occurring in addition to the direct thrombin inhibition.

Surface expression of PDGF receptor α and β was not affected by stimulation with thrombin; therefore, the latter did not potentiate proliferation via upregulation of PDGF receptor expression, whereas stimulation of cells with TRAP plus PDGF clearly showed that thrombin receptor is involved in potentiation. Based on this observation, thrombin would be expected to interact with PDGF-BB below the membrane level. Irrespective of whether potentiation is due to altered signal transduction leading to cell cycle progression or whether it is mediated by a primary cell cycle event, a difference in cell cycle distribution should take place. Further, as mitogenic signals are integrated in G1 phase, this difference should be related to G1 progression.[8] The latter is mediated by the activity of protein complexes consisting of a cyclin and a cdk.[9] Cyclin E and cdk2 are responsible for late G1 progression and S phase entry. Cdk2 activity is affected by CKI such as p21, p27, and p57; indeed, members of the p21 family have been shown to be such potent

inhibitors of cell cycle progression that they reduce neointima formation in restenosis models.[10,11,13] Therefore, expression and activity of cyclin E, cdk2, p21, p27, and p57 was examined in response to thrombin plus PDGF-BB. p27 was downregulated indicating that this CKI controls kinase activity of arrested cells and that its downregulation permits G1 progression.[31] p21 was slightly induced, which is consistent with regulation of kinase activity in proliferating cells.[32] p57 was not regulated, suggesting that it plays a specialized role in the cell cycle of VSMC. Expression of cyclin E and cdk2 was not affected by thrombin plus PDGF-BB. Therefore, major alterations in cell cycle protein expression after stimulation with thrombin plus PDGF-BB could be determined for p27 only. Indeed, downregulation of p27 has been shown to be a prerequisite for proliferation and is consistent with an enhanced cdk2 activity.[31,33,34] Thus, at the level of G1 progression, the potentiating effect of thrombin on PDGF-induced VSMC proliferation is specifically related to p27 downregulation.

Cell proliferation was blunted by pretreatment with the PI3 kinase inhibitors LY294002 and wortmannin indicating that the thrombin induced potentiation of VSMC proliferation to PDGF-BB is mediated by activation of PI3 kinase. Consistent with this observation, p27 downregulation in response to thrombin plus PDGF-BB was inhibited by pretreatment with the PI3 kinase inhibitors. Since PI3 kinase inhibition prevented both proliferation and p27 downregulation, and the latter was the only alteration observed in late G1 progression, the PI3 kinase pathway might fully account for proliferation in response to thrombin plus PDGF-BB. Consistent with a link between PI3 kinase activation and p27 downregulation in human primary VSMC, regulation of p27 expression by the PI3 kinase pathway has been observed in some cell lines.[35]

Rapamycin impairs proliferation and cell cycle progression of human and rat VSMC, while it does not prevent p27 downregulation in human VSMC and rat carotid artery.[19,21] Proliferation of human VSMC after stimulation with thrombin plus PDGF-BB was only slightly impaired by rapamycin, and p27 expression remained unaffected under these conditions. The inhibitory effect of rapamycin on proliferation to thrombin plus PDGF-BB was weaker than that observed in a previous publication; however, the difference is relatively small and may be related to the fact that proliferation was

stimulated by a different mitogen.[19] The missing effect of rapamycin on p27 downregulation is consistent with a previous observation, where proliferation was induced by several growth factors.[21] Thus, mTOR is involved in regulating proliferation to different mitogens including thrombin plus PDGF-BB, but its effect is relatively weak and is not related to downregulation of p27. Presumably, mTOR activation contributes to cell cycle progression by promoting translation of cyclins and cyclin-dependent kinases, while the major signal for cell cycle progression in response to thrombin plus PDGF-BB is p27 downregulation due to PI3 kinase activation.[21]

After stimulation with thrombin plus PDGF-BB, neither VSMC proliferation nor p27 expression was significantly affected by the MAP kinase inhibitor PD98059. The effect on cell number is consistent with observations in rabbit aortic VSMC demonstrating that the MAP kinase pathway barely contributes to VSMC proliferation.[36] Further, the absent effect of the MAP kinase pathway on p27 expression is in line with known roles of this pathway in inducing expression of cyclin D and promoting assembly of cyclin D with cdk4; indeed, generation of cyclin D cdk4 complexes results in sequestration of p27 leading to some degree of cell cycle progression irrespective of p27 protein level.[37]

The potency of thrombin in potentiating VSMC proliferation to PDGF-BB was determined by comparing proliferation and cell cycle regulation in response to thrombin plus PDGF-BB with either a maximal concentration of PDGF-BB (10 ng/mL) or 10% FCS. Analysis of cell number revealed that thrombin plus PDGF-BB was a as potent stimulus as the maximal concentration of PDGF-BB, while 10% FCS was twice as potent. p27 protein levels were similar in the three stimulation groups, but the lower band of the cdk2 doublet, representing threonine phosphorylation, was more pronounced after stimulation with FCS as compared to the maximal concentration of PDGF-BB or thrombin plus PDGF-BB.[38] Therefore, cdk2 activation seems to be responsible for the additional mitogenic potential of 10% FCS as compared to the maximal concentration of PDGF-BB or thrombin plus PDGF-BB.

Thus, thrombin potentiates proliferation of human VSMC to PDGF-BB via PI3 kinase activation leading to p27 downregulation (Figure 13). This interaction of thrombin and PDGF-BB may lead to

increased VSMC proliferation and accelerate the pathogenesis of vascular disease. As rapamycin only weakly inhibited proliferation to thrombin plus PDGF-BB, such an effect may be especially relevant for patients with restenosis after implantation of a rapamycin coated stent.[39] Indeed, the beneficial effects of rapamycin on in-stent-restenosis may primarily be related to inhibition of inflammatory aspects of stent-induced restenosis.[40] Therefore, direct thrombin inhibitors may prove an effective accessory therapy after implantation of a rapamcyin coated stent. Moreover, inhibition of PI3 kinase may represent a particularly promising therapeutic strategy under these conditions.

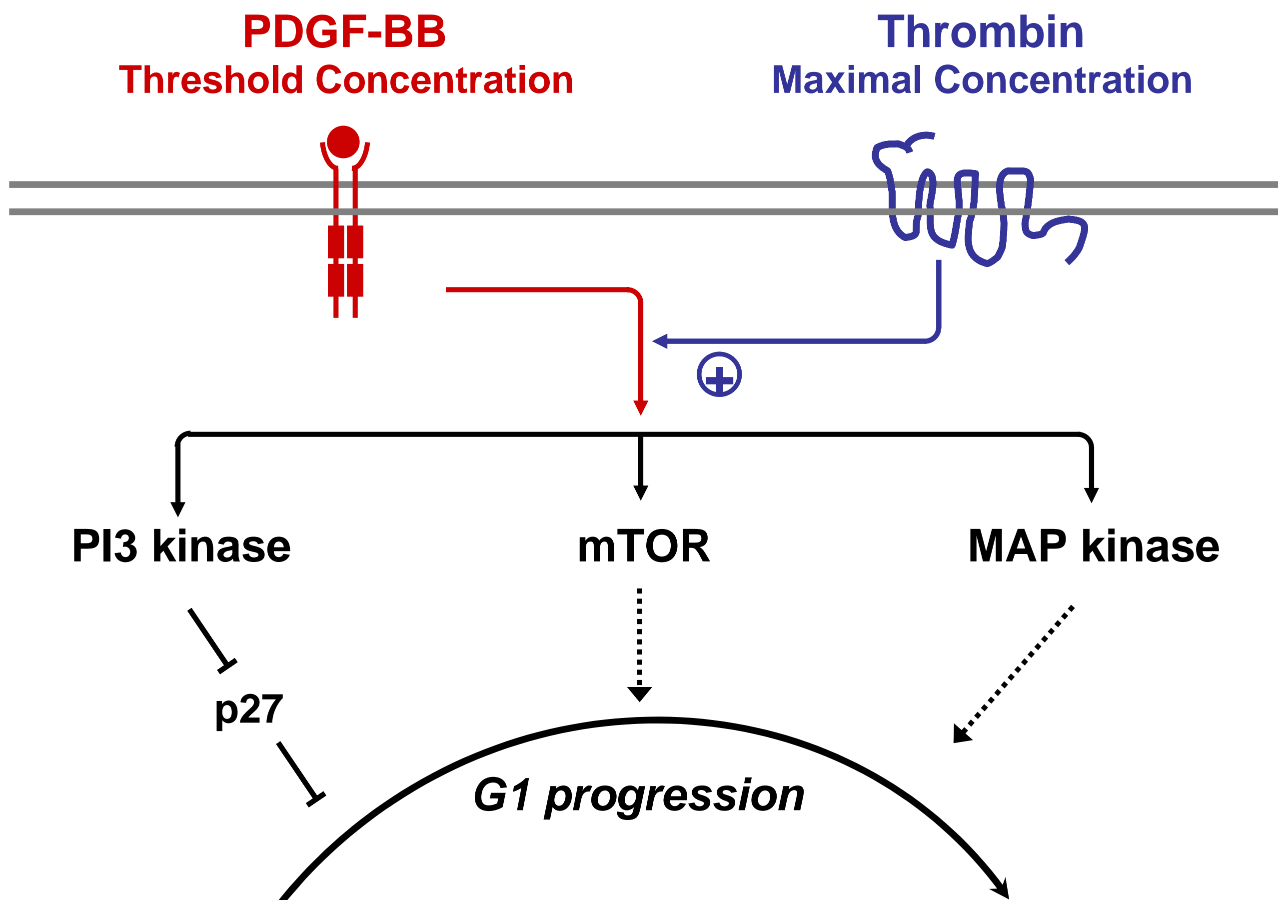


Figure 13. Schematic representation of the interaction of thrombin and PDGF-BB. Thrombin potentiates PDGF-induced vascular smooth muscle cell proliferation via PI3 kinase activation leading to p27 downregulation and cell cycle progression. The mammalian target of rapamycin (mTOR) and the MAP kinase pathway only weakly contribute to proliferation and are not involved in p27 downregulation.

6.4. References

1. Bryan AJ, Angelini GD. The biology of saphenous vein graft occlusion: etiology and strategies for prevention. *Curr Opin Cardiol*. 1994;9:641-649
2. Ross R. Platelets, platelet-derived growth factor, growth control, and their interactions with the vascular wall. *J Cardiovasc Pharmacol*. 1985;7:186-190
3. Lefkovits J, Topol EJ. Direct thrombin inhibitors in cardiovascular medicine. *Circulation*. 1994;90:1522-1536.
4. Zucker TP, Bonisch D, Muck S, Weber AA, Bretschneider E, Glusa E, Schrör K. Thrombin-induced mitogenesis in coronary artery smooth muscle cells is potentiated by thromboxane A2 and involves upregulation of thromboxane receptor mRNA. *Circulation*. 1998;97:589-595
5. McNamara CA, Sarembock IJ, Gimple LW, Fenton JW II, Coughlin SR, Owens GK. Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. *J Clin Invest*. 1993;91:94-98
6. Reusch HP, Zimmermann S, Schaefer M, Paul M, Moelling K. Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. *J Biol Chem*. 2001;276:33630-33637
7. Yang Z, Ruschitzka F, Rabelink TJ, Noll G, Julmy F, Joch H, Gafner V, Aleksic I, Althaus U, Lüscher TF. Different effects on thrombin receptor activation on endothelium and smooth muscle cells of human coronary bypass vessels. *Circulation*. 1997;95:1870-1876
8. Sherr CJ. G1 phase progression: cycling on cue. *Cell*. 1994;79:551-555
9. Morgan DO. Principles of CDK regulation. *Nature*. 1995;374:131-134
10. Sherr CJ, Roberts JM. Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev*. 1995;9:1149-63
11. Tanner FC, Boehm M, Akyürek LM, San H, Yang ZY, Tashiro J, Nabel GJ, Nabel EG. Differential effects of the cyclin-dependent kinase inhibiors p27Kip1, p21Cip1, and p16Ink4 on vascular smooth muscle cell proliferation. *Circulation*. 2000;101:2022-2025

12. Tanner FC, Yang ZY, Duckers E, Gordon D, Nabel GJ, Nabel EG. Expression of cyclin-dependent kinase inhibitors in vascular disease. *Circ. Res*. 1998;82:396-403

13. Chen D, Krasinski K, Chen D, Sylvester A, Chen J, Nisen PD, Andrés V. Downregulation of cyclin-dependent kinase 2 activity and cyclin A promoter activity in vascular smooth muscle cells by p27Kip1, an inhibitor of neointima formation in the rat carotid artery. *J. Clin. Invest*. 1997;99:2334-2341

14. Tanner FC, Greutert H, Barandier C, Frischknecht K, Luscher TF. Different cell cycle regulation of vascular smooth muscle in genetic hypertension. *Hypertension*. 2003;42:184-188

15. Hawkins PT, Jackson TR, Stephens LR. Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase. *Nature*. 1992;358:157-159

16. Auger KR, Serunian LA, Soltoff SP, Libby P, Cantley LC. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell*. 1989;57:167-75

17. Goncharova EA, Ammit AJ, Irani C, Carroll RG, Eszterhas AJ, Panettieri RA, Krzmskaya VP. PI3K is required for proliferation and migration of human pulmonary vascular smooth muscle cells. *Am J Physiol Lung Mol Physiol*. 2002;283:L354-L363

18. Chen J, Fang Y. A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. *Biochem Pharmacol*. 2002;64:1071-1077

19. Marx SO, Jayaraman T, Go LO, Marks AR. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. *Circ Res*. 1995;76:412-417

20. Luo Y, Marx SO, Kiyokawa H, Koff A, Massagué J, Marks AR. Rapamycin resistance tied to defective regulation of P27Kip1. *Mol Cell Biol*. 1996;16:6744-6751.

21. Braun-Dullaeus, Mann MJ, Seay U, Zhang L, von der Leyen HE, Morris RE, Dzau VJ. Cell cycle expression in vascular smooth muscle cells in vitro and in vivo is regulated through phosphatidylinositol 3-kinase and mammalian target of rapamycin. *Arterioscler Thromb Vasc Biol*. 2001;21:1152-1158

22. Chang L, Karin M. Mammalian MAP kinase signaling cascades. *Nature*. 2001;410:37-40

23. Yang Z, Barry SO, Carrel T, Kipfer B, Julmy F, Lüscher TF. Different proliferative properties of smooth muscle cells of human arterial and venous bypass vessels. *Circulation*. 1998;97:181-187

24. White PJ, Kumari R, Porter KE, London NJ, Ng LL, Boarder MR. Antiproliferative effect on UTP on human arterial and venous smooth muscle cells. *Am J Physiol Heart Circ Physiol*. 2000;279:H2735-H2742

25. Tanner FC, Noll G, Boulanger CM, Luscher TF. Oxidized low density lipoproteins inhibit relaxations of porcine coronary arteries. Role of scavenger receptor and endothelium-derived nitric oxide. *Circulation*. 1991;83:2012-20

26. Vlahos CJ, Matter VF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem*. 1994;269:5241-5248

27. Okada T, Sakuma L, Fukui Y, Hazeki O, Ui M. Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J Biol Chem*. 1994;269:3563-3567

28. Sekulic A, Hudson CC, Homme JL, Yin P, Otterness DM, Karnitz LM, Abraham RT. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. *Cancer Res*. 2000;60:3504-3513

29. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD098059 is a specific inhibitor of the activation of mitogen-acitvated protein kinase kinase in vitro and in vivo. *J Biol Chem*. 1995;270:27489-27494

30. Heras M, Chesebro JH, Webster MW, Mruk JS, Grill DE, Penny WJ, Badimon L, Fuster V. Hirudin, heparin and placebo during deep arterial injury in the pig. The in vivo role of thrombin in platelet-mediated thrombosis. *Circulation*. 1990;82:1476-1484

31. Nourse J, Firpo E, Flanagan WM, Coats S, Polyak K, Lee MH, Massague J, Crabtree GR, Roberts JM. Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature*. 1994;372:570-573

32. Zhang H, Hannon GJ, Beach D. p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev*. 1994;8:1750-1758

33. Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin–cdk protein kinase activity, is related to p21. *Cell*. 1994;78:67-74

34. Coats S, Flanagan WM, Nourse J.Roberts JM. Requirement of p27Kip1 for restriction point control of the fibroblast cell cycle. *Science*. 1996;272:877-880

35. Medema RH, Kops GJ, Bos JL, Burgering BM. AFX-like forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27Kip1. *Nature*. 2000;404:782-787

36. Cospedal R, Lobo M, Zachary I. Differential regulation of extracellular signal-regulated protein kinases (ERKs) 1 and 2 by cAMP and dissociation of ERK inhibition from anti-mitogenic effects in rabbit vascular smooth muscle cells. *Biochem J*. 1999;342:407-414

37. Cheng M, Sexl V, Sherr CJ, Roussel MF. Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). *Proc Natl Acad Sci USA*. 1998;95:1091-1096

38. Gu Y, Rosenblatt J, Morgan DO. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. *EMBO J*. 1992;11:3995-4005

39. Colombo A, Orlic D, Stankovic G, Corvaja N, Spanos V, Montorfano M, Liistro F, Carlino M, Airoldi F, Chieffo A, Di Mario C. Preliminary observations regarding angiographic pattern of restenosis after rapamycin-eluting stent implantation. *Circulation*. 2003;107:2178-2180

40. Inoue T, Uchida T, Yaguchi I, Sakai Y, Takayanagi K, Morooka S. Stent-induced expression and activation of the leucocyte integrin Mac-1 is associated with neointimal thickening and restenosis. *Circulation*. 2003;107:1757-1763

7. Conclusions and Outlook

In these studies presented, biology of vascular smooth muscle cells (VSMC) from coronary artery bypass vessels was investigated.

- In the first part proliferation of VSMC from radial artery (RA) was compared to that of internal mammary artery (MA) and saphenous vein (SV). Hypothesis 1 and 2 were confirmed.

Corroboration of Hypothesis 1:

In response to PDGF-BB, proliferation of VSMC from RA is in between that of MA and SV. Differences correlate with patency rates of the corresponding vessel.

Corroboration of Hypothesis 2:

Intrinsic differences in proliferation exist, this differences neither depend on different receptor expression nor different cell cycle distribution or regulation, but are due to different VSMC apoptosis rates.

As proliferation of RA VSMC after mitogenic stimulation is nearly as low as that of MA and much lower than that of SV; these data encourage the clinical use of RA and MA as bypass vessels. Moreover, the data indicate that increasing apoptosis rates in the SV might improve patency rates of this bypass graft. This goal might be achieved by reducing activation of the survival factor Akt. Indeed, celecoxib inhibits Akt activation of SV VSMC and thereby induces cell death; thus, celecoxib is a potential inhibitor of neointima formation and bypass graft disease.

- In the second part, migration of VSMC from RA, MA and SV was compared. Hypothesis 3 and 4 were confirmed.

Corroboration of Hypothesis 3:

Migration of VSMC of RA is in between that of MA and SV. Migration rates correspond to patency rates of the according vessels.

Corroboration of Hypothesis 4:

The lower migration of RA and MA as compared to SV is not due to different PDGF receptor expression, but due to lower Rho A activation. Differences in migration can be abolished by addition of Rho / ROCK pathway inhibitors.

Thus, development of bypass graft disease could be impaired by inhibition of VSMC migration of RA, MA and expecially SV by rosuvastation and hydroxyfasudil.

- In the third part the interaction of mediators from aggregating platelets and the coagulation cascade on SV VSMC were investigated. Hypothesis 5 and 6 were confirmed.

Corroboration of Hypothesis 5:

Thrombin and PDGF interact at the level of VSMC. Thrombin potentiates thresehold concentrations of PDGF in a concentration-dependent manner on VSMC proliferation.

Corroboration of Hypothesis 6:

The higher proliferation rates of VSMC stimulated with PDGF plus thrombin as compared to PDGF only or thrombin only, are not due to different PDGF-receptor expression, but are based on different cell cycle regulation, due to different p27 downregulation by the PI3 kinase pathway.

Thus, interaction of mediators from aggregating platelets and the coagulation cascade, such as PDGF and thrombin, increase VSMC proliferation, leading to a decreased patency rate. Hirudin can abolish this effect, which represents a potential novel clinical application of this drug.

In summary, regulation of A) VSMC proliferation by different apoptosis rates due to different Akt activity and B) different VSMC migration due to different Rho A activity are determinants of the different patency rates of coronary artery bypass vessels. Thereby, interaction of mediators such as thrombin and PDGF-BB may be as important as the action of PDGF-BB alone. Since proliferation and migration of VSMC from coronary artery bypass vessels differ according to long-term patency rates, intrinsic differences in the biological properties of VSMC may influence the propensity to atherosclerosis in these vessels. The improved understanding of the biology of these vessels may influence the current treatment of patients and thus translate into clinical applications.

CURRICULUM VITAE

PERSONAL DATA

Name:	Karin Frischknecht
Birth date:	15th September 1975
Birth place:	Frauenfeld, Switzerland
Nationality:	Switzerland

EDUCATION

1982 – 1988	Primarschule, Frauenfeld
1988 – 1991	Sekundarschule, Frauenfeld
1991 – 1996	Wirtschaftsgymnasium, Frauenfeld
1996	Cambridge Advanced Certificate, Eastbourne (England)
1996 – 2001	Federal Institute of Technology, Zürich, Studies of Biochemistry and Molecular Biology
2001	Graduation to “Dipl. nat. ETH“
2001 – 2002	University Bern, Institute of Cardiovascular Research, Ph.D. student in the lab of Prof. Thierry P. Carrel (Cardiovascular Surgery, Inselspital Bern) under supervision of Prof. Felix C. Tanner (Institute of Cardiovascular Research, Inselspital, Bern) and Prof. Daniel Schümperli (Institute of Cellular Biology, University Bern)
2002 – 2004	University Zürich, Institute of Cardiovascular Research, Ph.D. student in the lab of Prof. Thomas F. Lüscher (Institute of Cardiology, University Hospital Zürich) under supervision of Prof. Felix C. Tanner (Institute of Cardiovascular Research) and Prof. Beat Suter (Institute of Cellular Biology, University Bern)

AWARDS

2003 1st prize, Poster Award, Cardiology Update 2003, Davos, Switzerland

2003 Prize of the Schweizerische Gesellschaft für Thorax-, Herz- und Gefässchirurgie

WORK EXPERIENCE

1999 – 2000 Semester and diploma work at the Institute of Experimental Immunology, Zürich, under supervision of Prof. Hans Hengartner and Prof. Rolf M. Zinkernagel

LIST OF PULBLICATIONS

Original research articles

OR1. Tanner FC, Greutert H, Barandier C, Frischknecht K, Lüscher TF. Different Cell Cycle Regulation of Vascular Smooth Muscle in Genetic Hypertension. *Hypertension.* 2003;42:184-188.

OR2. Greutert H, Mei S, Graf P, Stalder M, Frischknecht K, Künzli A, Lüscher TF, Carrel TP, Tanner FC. Absence of Histamine-Induced Nitric Oxide Release in the Human Radial Artery - Implications for Vasospasm of Coronary Artery Bypass Grafts (*Circulation – in revision*)

OR3. Frischknecht K, Greutert H, Weisshaupt C., Carrel TP, Yang Z, Lüscher TF, Tanner FC. Vascular Smooth Muscle Cell Apoptosis protects the Internal Mammary Artery from Bypass Graft Disease. (*Submitted to Circulation*)

OR4. Weiss S, Frischknecht K, Greutert H, Jaschko A, Lüscher TF, Carrel TP, Tanner FC. Different Migration of Vascular Smooth Muscle Cells from Human Coronary Artery Bypass Vessels - Role of RhoA-ROCK pathway. (*Submission to Circulation*)

OR5. Frischknecht K, Greutert H, Carrel TP, Tanner FC. Thrombin Potentiates PDGF-Induced Vascular Smooth Muscle Cell Proliferation via PI3 kinase Activation Leading to p27 Downregulation. (*Submission to Cardiovasc Res*)

OR6. Frischknecht K, Weiss S, Greutert H, Jaschko A, Lüscher TF, Carrel TP, Tanner FC. Radial Artery Vascular Smooth Muscle Cell Proliferation and Migration. (*Submission to J Thorac Cardiovasc Surg*)

Abstracts

A1. Frischknecht K, Greutert H, Carrel TP, Tanner FC. Thrombin potentiates PDGF-induced vascular smooth muscle cell proliferation by downregulation of the cyclin-dependent kinase inhibitor p27. *Kardiovask Med*. 2002;5(suppl.4):24

A2. Frischknecht K, Greutert H, Carrel TP, Tanner FC. Different proliferation of vascular smooth muscle cells from human aortocoronary bypass vessels: Implications for patency of radial artery grafts. *Kardiovask Med*. 2002;5(suppl.4):26

A3. Frischknecht K, Greutert H, Carrel TP, Tanner FC. Thrombin potentiates PDGF-induced vascular smooth muscle cell proliferation via PI3 kinase activation and p27 downregulation. *J Am Coll Cardiol*. 2003:41 (suppl.A):232A

A4. Frischknecht K, Greutert H, Carrel TP, Tanner FC. Different cell death rates determine growth of vascular smooth muscle cells from human aortocoronary bypass vessels: Implications for patency of radial artery grafts. *J Am Coll Cardiol*. 2003:41 (suppl.A):241A

A5. Frischknecht K, Greutert H, Carrel TP, Tanner FC. Thrombin potentiates PDGF-induced vascular smooth muscle cell proliferation via PI3 kinase activation leading to p27 downregulation. *Kardiovask Med.*2003;6(suppl.5):50

A6. Frischknecht K, Greutert H, Carrel TP, Tanner FC. Different cell death rates determine growth of vascular smooth muscle cells from human aortocoronary bypass vessels: Implications for patency of radial artery grafts. *Eur Heart J*. 2003;24(suppl):333

A7. Tanner FC, Greutert H, Barandier C, Frischknecht K, Lüscher TF. Different cell cycle regulation of vascular smooth muscle in genetic hypertension. *Circulation*. 2003;108(suppl.4):89

A8. Frischknecht K, Greutert H, Carrel TP, Lüscher TF, Tanner FC. Different apoptosis rates of vascular smooth muscle cells from human coronary artery bypass vessels. *Kardiovask Med.*2004;7(suppl.6):6

A9. Weiss S, Frischknecht K, Greutert H, Jaschko A, Lüscher TF, Tanner FC. Different migration rates of vascular smooth muscle cells from human coronary artery bypass vessels. *Kardiovask Med.*2004;7(suppl.6):40

A10. Frischknecht K, Greutert H, Weisshaupt C, Carrel TP, Lüscher TF, Tanner FC. Different apoptosis rates of vascular smooth muscle cells from human coronary artery bypass vessels. *Eur Heart J.* 2004;25(suppl):41

A11. Weiss S, Frischknecht K, Greutert H, Jaschko A, Lüscher TF, Tanner FC. Different migration rates of vascular smooth muscle cells from human coronary artery bypass vessels. *Eur Heart J.* 2004;25(suppl):51