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Effects of Fluid Shear Stress on a Distinct Population of Vascular Smooth Muscle Cells

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Abstract

Vascular smooth muscle cells (SMCs) are a major cell type involved in vascular remodeling. The various developmental origins of SMCs such as neural crest and mesoderm result in heterogeneity of SMCs, which plays an important role in the development of vascular remodeling and diseases. Upon vascular injury, SMCs are exposed to blood flow and subjected to fluid shear stress. Previous studies have shown that fluid shear stress inhibits SMC proliferation. However, the effect of shear stress on the subpopulation of SMCs from specific developmental origin and vascular bed is not well understood. Here we investigated how shear stress regulates human aortic SMCs positive for neural crest markers. DNA microarray analysis showed that shear stress modulates the expression of genes involved in cell proliferation, matrix synthesis, cell signaling, transcription and cytoskeleton organization. Further studies demonstrated that shear stress induced SMC proliferation and cyclin D1, downregulated cell cycle inhibitor p21, and activated Akt pathway. Inhibition of PI-3 kinase blocked these shear stress-induced changes. These results suggest that SMCs with neural crest characteristics may respond to shear stress in a different manner. This finding has significant implications in the remodeling and diseases of blood vessels.

Keywords

Smooth muscle cell; fluid shear stress; neural crest; proliferation

Introduction

Vascular smooth muscle cells (SMCs) populate the medial layer of a blood vessel and play important roles in the control of vasoactivity and the remodeling of the vessel wall. SMCs in the blood vessels are heterogeneous and have different developmental origins such as neural crest (NC) and mesoderm^{2, 11, 20, 22}. For example, SMCs of NC origin can be found in the aorta, and the right and left common carotid arteries¹². The heterogeneity of SMCs may play a role in the heterogeneous patterns of disease progression and recurrence among different human arterial beds^{8, 18, 20, 26}. Therefore, it is important to determine how a specific subpopulation of SMCs responds to vascular microenvironmental factors.

The phenotype and functions of vascular SMCs are regulated by biochemical and mechanical factors^{15, 16, 23, 33}. In a healthy vessel wall, the mature, terminally differentiated SMCs are in a quiescent, contractile phenotype. Under pathological conditions such as

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atherosclerosis and restenosis, SMCs demonstrate a proliferative phenotype and contribute to the narrowing of the lumen^{1, 19, 27}. Although there have been extensive studies on the roles of growth factors, cytokines and inflammatory factors in the development of atherosclerosis and restenosis, the effects of hemodynamic forces, especially fluid shear stress, on the phenotype of SMCs are less well understood.

The luminal surface of the blood vessel is constantly subjected to fluid shear stress, the tangential component of hemodynamic force in the blood flow direction. Under physiological conditions, only the endothelial monolayer is subjected to arterial levels of fluid shear stress (average ~10–20 dyn/cm²)^{9, 10}, while SMCs are embedded in a three-dimensional extracellular matrix (ECM) and experience very low shear stress due to interstitial fluid flow (average ~1 dyn/cm² by theoretical estimation)³⁶. Under pathological conditions such as atherosclerosis, angioplasty, or in-stent restenosis, endothelial cells (ECs) and the underlying elastic lamina are disrupted. As a result, SMCs migrate to the inner surface and are directly exposed to fluid shear stress due to blood flow. In the past, laminar shear stress has been shown to inhibit SMC proliferation^{13, 1428, 31, 35}. However, whether the subpopulation of SMCs from different developmental origins responds differently to shear stress is not known.

In this study, we investigated the effects of shear stress on human aortic SMCs with NC characteristics. DNA microarray analysis and the follow-up studies showed that shear stress promoted SMC proliferation, decreased cyclin-dependent kinase inhibitor 1A (p21), and activated Akt in a manner dependent on phosphoinositide 3-kinase (PI 3-kinase). These results suggest that the subpopulation of SMCs with NC characteristics respond to fluid shear stress differently.

Results

Characterization of SMCs with the Expression of NC Markers

Human aortic SMCs were stained for SMC and NC markers. As shown in Figure 1, SMCs were positive for contractile markers such as smooth muscle α -actin, SM-22 α and calponin-1 (Figure 1A–C), but were negative for smooth muscle myosin heavy chain (data not shown). These cells were also expandable, suggesting that these SMCs had proliferative instead of contractile phenotype. In addition, these cells were homogeneously positive for NC markers such as Sox10, Slug and nestin (Figure 1A, B and D), suggesting that these cells might be derived from NC origin.

Effects of Fluid Shear Stress on SMC Gene Expression

To determine the global effects of fluid shear stress on SMCs, Affymetrix DNA microarrays were used to profile the expression of ~14,500 genes. As exemplified in Table 1, fluid shear stress resulted in significant changes in a variety of genes involved in cell cycle and death, cell adhesion and ECM, cytoskeleton organization, cell-cell signaling, intracellular signaling, and transcription. After SMCs were subjected to shear stress at 12 dyn/cm² for 24 hours, a total of 1472 genes (10.2%) had statistically significant changes ($P \leq 0.05$) of at least 1.5 fold, with 243 genes (1.7%) being upregulated and 1229 genes (8.5%) being downregulated. A total of 615 genes (4.2%) exhibited statistically significant changes ($P \leq 0.05$) of at least 2 fold, with 111 genes (0.8%) being upregulated and 504 genes (3.5%) being downregulated. These results suggest that fluid shear stress significantly affect SMC function and induce global genetic changes in SMCs. Several genes were selected for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and confirmed the results from DNA microarray analysis (Figure 2A).

Specifically, shear stress significantly decreased the expression of p21 and cyclin-dependent kinase inhibitor 1C (p57), both molecules involved in negatively regulating cell cycle progression. Cyclin D1, a G1 cyclin that mediates cell cycle progression from the G1 phase to the proliferating S phase, was increased by shear stress. The changes in the gene expression of cyclin D1, p21 and p57 were verified by qRT-PCR (Figure 2A). In addition, we selectively verified the protein expression of p21 and cyclin D1. Shear stress upregulated cyclin D1 protein expression and downregulated p21 protein expression (Figure 2B), which mirrored the changes we observed in gene expression for these two molecules. These data suggested that fluid shear stress regulated the expression of cell cycle signaling molecules and might promote SMC proliferation.

Effects of Fluid Shear Stress on Human SMC Proliferation

To directly determine the effects of shear stress on SMC proliferation, we used 5-bromo-2'-deoxy-uridine (BrdU) labeling to detect the cells in S-phase. After 24 hours of exposure to shear stress, the percentage of BrdU-positive (BrdU+) SMCs increased approximately by 7 fold (~36%) compared to static control samples (~5%), as shown in Figure 3. This was consistent with gene and protein expression data (Figure 2).

Effects of Fluid Shear Stress on Akt Activation

Akt, or protein kinase B, is a serine/threonine kinase that is activated by phosphorylation at threonine (Thr)-308 and serine (Ser)-473 residues. Previous studies have shown that Akt is involved in regulating many cellular processes, including proliferation^{4,29}. Immunoblotting analysis revealed that fluid shear stress induced Akt activation at both phosphorylation sites after 16 and 24 hours without significantly affecting Akt expression (Figure 4A).

PI 3-Kinase Inhibition Blocks Fluid Shear Stress-Induced Akt Activation

Since PI 3-kinase is a major regulator of Akt activation, we used a specific PI 3-kinase inhibitor, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY 294002), to investigate the upstream regulation of Akt activation. As shown in Figure 4B, fluid shear stress induced Akt activation in the absence of LY 294002; however, the presence of LY 294002 blocked Akt activation. These results suggested that fluid shear stress activated Akt via PI 3-kinase.

PI 3-Kinase Inhibition Blocks Fluid Shear Stress-Induced SMC Proliferation

Then we examined whether PI 3-kinase inhibition affects SMC proliferation. As shown in Figure 5A, fluid shear stress induced a ~5 fold increase in the percentage of BrdU+ cells in the absence of LY294002. In the presence of LY 294002, there was a ~3 fold decrease in the percentage of BrdU+ cells compared to untreated static control samples. Addition of LY 294002 to the circulating media resulted in a ~ 4 fold decrease in the percentage of BrdU+ cells compared to sheared SMCs without LY 294002 treatment.

PI 3-Kinase Inhibition Blocks Shear Stress-Induced Downregulation of p21 Gene Expression

Previous studies on SMCs not involving mechanical force stimulation have shown that inhibition of Akt increases p21 expression, leading to a decrease in proliferation^{5, 21, 30, 32}. As shown in Figure 5B, shear stress downregulated the expression of p21 by ~ 2 fold compared to static control samples. However, in static samples treated with LY 294002, p21 expression was upregulated by ~2 fold compared to untreated static samples. In addition, LY 294002 upregulated the expression of p21 even in the presence of shear stress. These results suggested that p21 could be a downstream target of the PI 3-kinase/Akt pathway involving SMC proliferation in response to a fluid shear stress.

Discussion

A major finding of this study is that laminar fluid shear stress increases the proliferation of SMCs with NC characteristics. This is in contrary to previous reports^{31, 35}. The discrepancy may be explained by the different origins and subpopulations of SMCs used in different studies. In the previous reports, it is not clear whether SMC culture was heterogeneous, and the developmental origins of SMCs were not examined. Future investigations are needed to directly compare the proliferation and functions of SMCs with different developmental origins in response to shear stress. Consistent with this notion, a previous study demonstrated that SMCs in different phenotypic states exhibited opposite proliferative responses when subjected to the same pressure loading³. The differential responses of SMC subpopulations have significant implications in vascular biology and diseases. It suggests that, besides local hemodynamic factors, the composition of SMCs in the vessel wall is also an important determinant of vascular remodeling in response to injuries. For example, the regions with NC-like SMCs might be lesion prone regions.

It is worth noting that in vivo microenvironment is quite different from that in vitro. For example, the presence of ECs could modify the responses of SMCs to fluid shear stress through cell to cell signaling. By using a EC-SMC coculture model⁶, it has been shown that shear stress induces synthetic-to-contractile phenotypic modulation in SMCs via peroxisome proliferator-activated receptor activation by prostacyclin released by sheared ECs³⁴. Shear stress also regulates the crosstalk between ECs and SMCs through PDGF-BB and TGF- β ²⁵. Therefore, while in vitro experimental system provides well defined conditions for the dissection of the role of each stimulus, the in vivo responses of SMCs to shear stress will be the integration of the signaling from biochemical, mechanical and cellular factors in the microenvironment. For example, there is evidence that laminar shear stress and disturbed flow have differential effects on the expression of intercellular signaling molecules such as nitric oxide, bone morphogenetic protein-4, TGF- β and metalloproteinases in ECs⁷. These signaling molecules can regulate the proliferation and differentiation of SMCs with NC characteristics, resulting different responses of these cells at different locations of blood vessels.

At molecular level, we demonstrated that PI 3-kinase/Akt signaling pathway was involved in the regulation of SMC proliferation in response to fluid shear stress and that p21 was a downstream target of this pathway. Whether PI 3-kinase/Akt pathway is activated in SMCs with different origins and phenotypes is not clear. The activation of PI 3-kinase/Akt pathway may be dependent on the expression of specific cell surface receptors such as integrins, G protein-linked receptors and growth factor receptors. The role of PI 3-kinase/Akt pathway in SMC proliferation could also be modified by other signaling pathways activated or suppressed by shear stress.

Besides cell proliferation, shear stress regulates many other functions of SMCs. DNA microarray analysis showed that shear stress regulated the expression of collagens and MMPs, suggesting an important role of shear stress in matrix remodeling. In addition, shear stress either suppressed (e.g., BMP4) or induced (e.g. IL-11) the expression of growth factors and cytokines, which could result in paracrine signaling among SMCs and between SMCs and ECs. Consistently, Smad1 and Smad3 were downregulated, while inhibitory Smad7 was upregulated by shear stress, implicating the suppression of BMP and TGF- β signaling. The inhibition of p53 expression was also consistent with the activation of Akt and the decrease of p21 expression. All of these changes at molecular level need further investigations to generate a big picture of shear stress-regulated genetic network.

Overall, the results of this study advance the current understanding on the response of human vascular SMCs to an applied mechanical force, and provide a rational basis towards the development of novel therapeutic strategies to block intimal hyperplasia for the clinical treatment of cardiovascular diseases.

Materials & Methods

Cell Culture

Human aortic smooth muscle cells (SMCs) were obtained from Cell Systems, St. Katharinen, Germany. The cells were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 1 mM penicillin-streptomycin (Invitrogen Co.) in 100 mm tissue culture dishes. Cell cultures were maintained in a humidified 95% air-5% CO₂ incubator at 37°C, with culture media changed every 2 days. Cell purity and homogeneity was verified by immunofluorescent staining of SMC markers α -actin (Chemicon, Inc.) and calponin-1 (Santa Cruz Biotechnology, Inc.).

Fluid Shear Stress Experiments

Glass slides (75 mm $\times$ 38 mm) (Erie Scientific, Portsmouth, NH) were autoclaved and exposed to ultraviolet (UV) light for 1 hour for sterilization. Slides were then coated with 5 μ g/cm² of fibronectin (Sigma, Inc.), incubated for 2 hours at room temperature to allow for protein adsorption, and washed once with phosphate buffered saline (PBS) (Sigma-Aldrich Corp.). Following trypsinization, SMCs were initially seeded onto the slides at approximately 60–70 % confluency and allowed to attach and grow for 2 days prior to use in experiments.

A well-defined laminar fluid shear stress was applied to SMCs using a parallel plate flow chamber as previously described with minor modifications¹⁷. Briefly, the SMC-cultured glass slide was mounted in a rectangular flow channel created by sandwiching a silicone gasket between the glass slide and the polycarbonate flow chamber base. The base contains an inlet and an outlet for perfusing the cultured cells with the circulating medium. Initiation of fluid flow generated a laminar shear stress due to the hydrostatic pressure between two reservoirs, where the flow rate could be adjusted to obtain a specific physiological shear stress level based upon the following equation: $Q = \tau W h^2 / 6\mu$, where Q is the flow rate across the chamber, τ is the shear stress, μ is the viscosity of the medium (0.00755 dyn * seconds/cm² at 37°C), W is the inner width of the gasket (1.5 cm), and h is the gasket height (250 μ m). The flow system was set up in a humidified cell culture incubator (37°C, 5% CO₂). All shear stress experiments included paired static controls, i.e. SMCs cultured on slides not exposed to shear stress. SMCs were subjected to a shear stress of 12 dyn/cm². This level of shear stress is within the physiological range found in major human arteries and has been shown to regulate vascular cell functions *in vitro*.

BrdU Incorporation

For cell cycle analysis, following exposure to a shear stress of 12 dyn/cm² for 24 hours, both flow and static control samples were incubated with 10 μ M 5-bromo-2'-deoxyuridine (BrdU) (Amersham, Inc.) for 2 hours at 37 °C. Samples were then fixed with 4% paraformaldehyde, pretreated with 50% methanol, permeabilized with 0.5% Triton X-100, and then treated with 2N HCl. BrdU was stained by a mouse anti-BrdU antibody (BD Biosciences, Inc.) and a FITC-anti-mouse antibody (15 μ g/mL) (Jackson ImmunoResearch, Inc.). The cell nuclei were stained with 1 μ g/mL propidium iodide (Molecular Probes, Eugene, OR) for 5 minutes. After washing with PBS, samples were mounted in Vector-Shield antifade solution (Vector Laboratories, Inc.) and used for fluorescence microscopy.

The percentage of SMCs that incorporated BrdU correlated with the number of SMCs in the S phase of the cell cycle. Data were collected for each sample by averaging the percentage of BrdU-positive cells over 10 randomly selected viewing fields. Three independent experiments were carried out.

RNA Isolation and DNA Microarray

Flow and static control samples were lysed in RNA STAT-60 lysis buffer (Tel-Test "B", Inc.). RNA was extracted using chloroform and phenol. Isopropanol was added to precipitate the RNA and samples were centrifuged at 13,000 rpm and 4°C for 30 minutes. 75% ethanol was added to wash the RNA pellet and centrifuged at 7500 rpm for 5 minutes. The pellet was resuspended in DEPC-treated water and quantified using a RiboGreen® RNA quantification assay (Molecular Probes Inc.). The quality of the isolated RNA was monitored by the ratio of the absorption at 260 nm and 280 nm measured with a Bio-Rad spectrophotometer (Bio-Rad Laboratories, Inc.).

DNA microarray experiments were performed by using a high throughput array (HTA) system from Affymetrix (Affymetrix, Inc.). RNA samples (3 µg each) were loaded into the ArrayStation, and the signals from the array were collected by using the high throughput HTA scanner. The human DNA microarray chip contained ~400,000 probes, including about 14,500 genes on the chip. The probes are designed to maximize sensitivity, specificity, and reproducibility, allowing consistent discrimination between specific and background signals, and between closely related target sequences, by using the Perfect Match/Mismatch probe strategy. Each independent experiment was analyzed using a separate chip.

Data management and statistical analysis were performed by using GeneTraffic Microarray Data Management and Analysis software (Stratagene, Inc.). The ratios between shear stressed and control samples were calculated. The data was expressed as: the average of $\text{Log}_2(\text{ratios}) \pm \text{standard deviation (SD)}$ from 4 independent experiments. Student's t-test was used to discover the genes of which the expression levels had significantly changed ($P < 0.05$). The significant change of the genes in different categories gave us comprehensive information on SMC function changes in response to a well-defined laminar fluid shear stress.

Quantitative RT-PCR

To verify DNA microarray results for specific genes, two-step quantitative RT-PCR was performed using the ThermoScript RT-PCR system as previously described²⁴. Briefly, cDNA was made from equal amounts of total RNA from each sample, and real-time quantitative PCR was performed using SYBR-green kits and the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Inc.) in a 96-well format. Primers for qPCR were designed using the ABI Prism Primer Express™ software v.2.0 (Applied Biosystems, Inc.) and custom-made (Operon Technologies, Inc.). Primers used are as indicated in Table 2.

After each experiment, the melting temperature and the dissociation curve of PCR products was obtained to confirm product specificity. The amount of RNA for each gene was normalized with the amount of 18S rRNA in the same sample, and the ratio of the gene expression was calculated. We have shown that the expression of 18S rRNA does not change significantly in response to fluid shear stress by running qRT-PCR with the same amount of total RNA.

Immunoblotting Analysis

For immunoblotting analysis, flow and static control samples were lysed in a lysis buffer containing 25 mM Tris, pH 7.4, 0.5 M NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 1 mM PMSF, 10 µg/ml leupeptin and 1 mM Na₃VO₄. The lysates were centrifuged at 13,000 rpm for 15 minutes using a microcentrifuge, and the protein concentration of the supernatants was measured by a DC protein assay (Bio-Rad Laboratories, Inc.). Equal amounts of protein (15 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 2% SDS sample buffer containing 2-mercaptoethanol (Sigma-Aldrich Corp.). Samples were boiled for 3 minutes before being loaded into the gel. Proteins in the gel were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc.), and the membrane was blocked with 3% nonfat milk and washed in TBST buffer (25 mM Tris-HCl, pH 7.4, 60 mM NaCl, and 0.05% Tween 20) containing 0.1% bovine serum albumin (BSA). Membranes were incubated with primary antibodies. Following primary antibody incubation, membranes were incubated with the appropriate secondary IgG-horseradish peroxidase conjugate antibody (Santa Cruz Biotechnologies, Inc.). Proteins were then visualized with ECL detection reagents (Amersham Biosciences Co.). Primary antibodies against phospho-Akt(ser473) and phospho-Akt(thr308) were from Cell Signaling Technology, Inc.; antibodies against ERK2, p21, cyclin D1 and Akt were from Santa Cruz Biotechnologies, Inc.

Immunostaining and Microscopy

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 10 min. For immunostaining, the specimens were incubated with respective primary antibodies for 2 h, and with appropriate secondary antibodies for 1 h. Nuclei were stained by DAPI (Invitrogen Corp.) in blue. The following antibodies were used: Sox10 was from R&D Systems, Inc.; nestin, SM-22α and SM-MHC were from Abcam Inc.; smooth muscle α-actin was from Sigma-Aldrich Corp.; CNN1 was from Epitomics; Slug was from Santa Cruz Biotechnologies, Inc. The fluorescently stained samples were imaged by using a Nikon fluorescence microscope.

Statistical Analysis

For each group of data, the mean and standard deviation (SD) were calculated. For the comparison of more than 2 groups, analysis of variance (ANOVA) was performed to detect whether a significant difference ($P < 0.05$) existed between groups with different treatments, and a Holm's t-test was used as a multiple comparison procedure to find where the differences existed. For the comparison of two groups, Student's paired t-test was performed, while qRT-PCR data verifying DNA microarray results were analyzed by using a log transformed one sample t-test.

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References

1. Bauters C, Isner JM. The biology of restenosis. *Progress in Cardiovascular Diseases*. 1997; 40(2): 107–116. [PubMed: 9327827]
2. Bergwerff M, et al. Neural crest cell contribution to the developing circulatory system: implications for vascular morphology? *Circ Res*. 1998; 82(2):221–231. [PubMed: 9468193]
3. Cappadona C, et al. Phenotype dictates the growth response of vascular smooth muscle cells to pulse pressure in vitro. *Exp Cell Res*. 1999; 250(1):174–186. [PubMed: 10388531]

4. Chang F, et al. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia*. 2003; 17(3):590–603. [PubMed: 12646949]
5. Chang MW, et al. Adenovirus-mediated over-expression of the cyclin/cyclindependent kinase inhibitor, p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty. *J Clin Invest*. 1995; 96(5):2260–2268. [PubMed: 7593612]
6. Chiu JJ, et al. Mechanisms of induction of endothelial cell E-selectin expression by smooth muscle cells and its inhibition by shear stress. *Blood*. 2007; 110(2):519–528. [PubMed: 17371946]
7. Chiu JJ, Chien S. Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiological reviews*. 2011; 91(1):327–387. [PubMed: 21248169]
8. DeBaakey ME, Glaeser DH. Patterns of atherosclerosis: effect of risk factors on recurrence and survival-analysis of 11,890 cases with more than 25-year follow-up. *The American journal of cardiology*. 2000; 85(9):1045–1053. [PubMed: 10781750]
9. Fung, YC. *Biodynamics*. Springer-Verlag New York Inc.; 1984. p. 77-85.
10. Giddens DP, Zarins CK, Glagov S. The role of fluid mechanics in the localization and detection of atherosclerosis. *J Biomech Eng*. 1993; 115(4B):588–594. [PubMed: 8302046]
11. Hirschi KK, Majesky MW. Smooth muscle stem cells. *Anat Rec A Discov Mol Cell Evol Biol*. 2004; 276(1):22–33. [PubMed: 14699631]
12. Jiang X, et al. Fate of the mammalian cardiac neural crest. *Development*. 2000; 127(8):1607–1616. [PubMed: 10725237]
13. Kohler TR, et al. Increased blood flow inhibits neointimal hyperplasia in endothelialized vascular grafts. *Circ Res*. 1991; 69(6):1557–1565. [PubMed: 1954675]
14. Kraiss LW, et al. Shear stress regulates smooth muscle proliferation and neointimal thickening in porous polytetrafluoroethylene grafts. *Arterioscler Thromb*. 1991; 11(6):1844–1852. [PubMed: 1931886]
15. Kurpinski K, et al. Regulation of Vascular Smooth Muscle Cells and Mesenchymal Stem Cells by Mechanical Strain. *Mol Cell Biomech*. 2006; 3(1):21–34. [PubMed: 16711069]
16. Li C, Xu Q. Mechanical stress-initiated signal transductions in vascular smooth muscle cells. *Cell Signal*. 2000; 12(7):435–445. [PubMed: 10989277]
17. Li S, et al. Distinct roles for the small GTPases Cdc42 and Rho in endothelial responses to shear stress. *Journal of Clinical Investigation*. 1999; 103(8):1141–1150. [PubMed: 10207166]
18. Li S, et al. Innate diversity of adult human arterial smooth muscle cells: cloning of distinct subtypes from the internal thoracic artery. *Circulation Research*. 2001; 89(6):517–525. [PubMed: 11557739]
19. Libby P, Tanaka H. The molecular bases of restenosis. *Progress in Cardiovascular Diseases*. 1997; 40(2):97–106. [PubMed: 9327826]
20. Majesky MW. Developmental basis of vascular smooth muscle diversity. *Arteriosclerosis Thrombosis and Vascular Biology*. 2007; 27(6):1248–1258.
21. Martin KA, et al. The mTOR / p70 S6K1 pathway regulates vascular smooth muscle cell differentiation. *Am J Physiol Cell Physiol*. 2003
22. Nakamura T, Colbert MC, Robbins J. Neural crest cells retain multipotential characteristics in the developing valves and label the cardiac conduction system. *Circulation Research*. 2006; 98(12):1547–1554. [PubMed: 16709902]
23. Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev*. 1995; 75(3):487–517. [PubMed: 7624392]
24. Park J, et al. Differential effects of equiaxial and uniaxial strains on mesenchymal stem cells. *Biotechnology and Bioengineering*. 2004; 88(3):359–368. [PubMed: 15486942]
25. Qi YX, et al. PDGF-BB and TGF- β 1 on cross-talk between endothelial and smooth muscle cells in vascular remodeling induced by low shear stress. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108(5):1908–1913. [PubMed: 21245329]
26. Regan CP, et al. Molecular mechanisms of decreased smooth muscle differentiation marker expression after vascular injury. *J Clin Invest*. 2000; 106(9):1139–1147. [PubMed: 11067866]

27. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med.* 1999; 340(2):115–126. [PubMed: 9887164]
28. Shigematsu K, et al. Direct and indirect effects of pulsatile shear stress on the smooth muscle cell. *Int Angiol.* 2000; 19(1):39–46. [PubMed: 10853684]
29. Shiojima I, Walsh K. Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res.* 2002; 90(12):1243–1250. [PubMed: 12089061]
30. Stabile E, et al. Akt Controls Vascular Smooth Muscle Cell Proliferation In Vitro and In Vivo by Delaying G1/S Exit. *Circ Res.* 2003; 93(11):1059–1065. [PubMed: 14605018]
31. Sterpetti AV, et al. Shear stress modulates the proliferation rate, protein synthesis, and mitogenic activity of arterial smooth muscle cells. *Surgery.* 1993; 113(6):691–699. [PubMed: 8506528]
32. Tanner FC, et al. Differential effects of the cyclin-dependent kinase inhibitors p27(Kip1), p21(Cip1), and p16(Ink4) on vascular smooth muscle cell proliferation. *Circulation.* 2000; 101(17):2022–2025. [PubMed: 10790340]
33. Thyberg J. Differentiated properties and proliferation of arterial smooth muscle cells in culture. *Int Rev Cytol.* 1996; 169(183):183–265. [PubMed: 8843655]
34. Tsai MC, et al. Shear stress induces synthetic-to-contractile phenotypic modulation in smooth muscle cells via peroxisome proliferator-activated receptor α/δ activations by prostacyclin released by sheared endothelial cells. *Circulation Research.* 2009; 105(5):471–480. [PubMed: 19628794]
35. Ueba H, Kawakami M, Yaginuma T. Shear stress as an inhibitor of vascular smooth muscle cell proliferation. Role of transforming growth factor- β 1 and tissue-type plasminogen activator. *Arterioscler Thromb Vasc Biol.* 1997; 17(8):1512–1516. [PubMed: 9301628]
36. Wang DM, Tarbell JM. Modeling interstitial flow in an artery wall allows estimation of wall shear stress on smooth muscle cells. *J Biomech Eng.* 1995; 117(3):358–363. [PubMed: 8618390]

Figure 1. Characterization of SMCs. The cells were stained for the markers of SMCs (α -actin, calponin-1, SM-22 α) and NC cells (Sox10, Slug and nestin), followed by fluorescence microscopy. Scale bar=50 μ m.

Figure 2.

Effects of fluid shear stress on the gene expression in SMCs. SMCs were subjected to a shear stress of 12 dyn/cm² or kept as static controls for 24 hours. **(A)** The expression of selected genes from DNA microarray analysis was verified by qRT-PCR. Bars represent mean $\pm$ standard deviation (SD). Statistical significance was determined using a log-transformed one-sample t-test. * indicates $P < 0.05$ ($n=4$). **(B)** Representative immunoblotting images from three experiments showing the effects of fluid shear stress on the protein expression of p21 and cyclin D1. ERK-2 protein levels were used to demonstrate the equal loading of protein lysates.

Figure 3.

Effects of fluid shear stress on cell proliferation. SMCs were subjected to a fluid shear stress of 12 dyn/cm² for 24 hours or kept as static controls. (A) Fluorescent double-staining of cells in S-phase (BrdU+) and nuclei (propidium iodide). (B) Statistical analysis of cell proliferation. Bars represent mean $\pm$ SD of the percentage of BrdU+ cells quantified from 10 random viewing fields of three independent experiments. *Significant difference ($P < 0.05$, two-tailed paired t-test).

Figure 4.

Effects of fluid shear stress on Akt activation. SMCs were subjected to a fluid shear stress of 12 dyn/cm² for 16 or 24 hours or kept as static controls (n=3), followed by immunoblotting analysis. **(A)** Shear stress-induced Akt phosphorylation. **(B)** Effects of PI 3-kinase inhibition on fluid shear stress-induced Akt activation. The experiments were performed in the presence or absence of 20 μ M of LY 294002, a PI-3 kinase inhibitor.

Figure 5.

Effects of PI-3 kinase inhibition on SMC proliferation (**A**) and p21 gene expression (**B**). SMCs were subjected to a fluid shear stress of 12 dyn/cm² for 24 hours or kept as static controls, in the presence and absence of 20 μ M LY 294002. The cells were either fixed for proliferation analysis or qRT-PCR analysis. Bars represent mean $\pm$ SD. *Significant difference ($P < 0.05$, One-Way ANOVA and Holm's test for multiple comparisons).

Table 1
DNA microarray analysis of shear stress-regulation of gene expression in SMCs

Cells were subjected to a shear stress of 12 yn/cm^2 for 24 hours, followed by DNA microarray analysis (n=4). A list of genes showing more than 1.5 fold change ($P < 0.05$) in response to shear stress is included in the table. The data is presented as mean Log_2 ratio (shear stress/static control) and standard deviation (SD).

Gene Name	Gene Symbol	Log_2 Mean	SD
Cell Cycle and Apoptosis			
caspase 1, apoptosis-related cysteine protease	CASP1	-2.97	0.53
cyclin-dependent kinase inhibitor 1C (p57, Kip2)	CDKN1C	-1.9	1.01
hepatocyte growth factor (hepatopoietin A; scatter factor)	HGF	-1.9	0.07
cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	CDKN2C	-1.38	1.29
cell division cycle 25B	CDC25B	-1.23	0.22
cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-1	0.1
cyclin D1	CCND1	0.57	0.07
Cell Adhesion and ECM			
vascular cell adhesion molecule 1	VCAM1	-3.43	0.3
collagen, type IV, alpha 5	COL4A5	-2.38	0.52
collagen, type III, alpha 1	COL3A1	-1.61	0.97
collagen, type V, alpha 1	COL5A1	1.73	0.46
cadherin 2, type 1, N-cadherin (neuronal)	CDH2	1.95	0.23
tissue inhibitor of metalloproteinase 3	TIMP3	2.03	0.48
matrix metalloproteinase 3 (stromelysin 1, progelatinase)	MMP3	2.4	0.76
collagen, type VII, alpha 1	COL7A1	2.72	0.53
hyaluronan synthase 2	HAS2	4.04	0.64
Matrix metalloproteinase 10 (stromelysin 2)	MMP10	4.23	0.95
Cytoskeleton			
trophinin	TRO	-1.37	0.58
myosin, light polypeptide kinase	MYLK	-1.24	0.27
dystrophin	DMD	1.62	0.43
tropomyosin 1 (alpha)	TPM1	1.64	0.47
tensin	TNS	1.69	0.46
transgelin 3	TAGLN3	2.44	0.9
Soluble Factors, Receptors, and Membrane Proteins			
chemokine (C-C motif) ligand 2	CCL2	-4.16	0.37
gamma-aminobutyric acid (GABA) A receptor, beta 1	GABRB1	-3.63	0.44
bone morphogenetic protein 4	BMP4	-3.46	0.58
hepatocyte growth factor	HGF	-3.35	0.53
solute carrier family 14 (urea transporter), member 1	SLC14A1	-2.85	1.24
chemokine (C-X-C motif) ligand 1	CXCL1	-2.82	0.66

Gene Name	Gene Symbol	Log ₂ Mean	SD
secreted frizzled-related protein 1	SFRP1	-2.52	0.19
peroxisome proliferative activated receptor, gamma	PPARG	-2.42	0.7
transient receptor potential cation channel, subfamily A, member 1	TRPA1	-2.16	0.72
major histocompatibility complex, class II, DM alpha	HLA-DMA	-1.93	0.13
purinergic receptor P2Y, G-protein coupled, 5	P2RY5	-1.58	0.2
frizzled homolog 7	FZD7	-1.53	0.21
sodium channel, voltage-gated, type IX, alpha	SCN9A	1.61	0.28
inhibin, beta A (activin A)	INHBA	1.67	0.33
plasminogen activator, urokinase receptor	PLAUR	2.03	0.09
nerve growth factor, beta polypeptide	NGFB	2.27	0.53
heparin-binding EGF-like growth factor	HBEGF	2.32	0.13
a disintegrin and metalloproteinase domain 19 (meltrin beta)	ADAM19	2.43	0.17
solute carrier family 19 (thiamine transporter), member 2	SLC19A2	2.48	0.56
interleukin 13 receptor, alpha 2	IL13RA2	2.5	0.62
interleukin 6	IL6	2.66	0.59
G protein-coupled receptor 51	GPR51	2.67	0.19
heparin-binding EGF-like growth factor	HBEGF	2.68	0.26
angiopoietin-like 4	ANGPTL4	3.83	1.22
endothelial cell-specific molecule 1	ESM1	3.98	0.29
interleukin 11	IL11	4.42	0.99
Intracellular Signaling Molecules			
SMAD, mothers against DPP homolog 3	SMAD3	-3.18	0.21
phosphatidic acid phosphatase type 2B	PPAP2B	-2.5	0.33
phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)	PIK3R3	-1.95	0.56
tumor protein p53	TP53	-1.93	0.44
mitogen-activated protein kinase kinase 6	MAP2K6	-1.65	1.08
SMAD, mothers against DPP homolog 1	SMAD1	-1.65	0.61
dual specificity phosphatase 1	DUSP1	1.55	0.4
SMAD, mothers against DPP homolog 7	SMAD7	1.57	0.12
dual specificity phosphatase 5	DUSP5	1.83	0.47
Ras homolog gene family, member B	RHOB	2.3	0.24
Transcription Factors			
CCAAT/enhancer binding protein (C/EBP), delta	CEBPD	-3.58	0.33
mesenchyme homeo box 2 (growth arrest-specific homeo box)	MEOX2	-1.96	0.14
homeo box A5	HOXA5	-1.67	0.56
Kruppel-like factor 2	KLF2	2.27	0.26
basic helix-loop-helix domain containing, class B, 2	BHLHB2	3.52	0.55
Kruppel-like factor 4	KLF4	-0.77	0.72
Protein Translation, Processing, and Transport			

Gene Name	Gene Symbol	Log ₂ Mean	SD
serine (or cysteine) proteinase inhibitor, clade E (nexin)	SERPINE1	2.27	0.4
serine (or cysteine) proteinase inhibitor, clade B	SERPINB2	3.19	0.39

Table 2
List of qRT-PCR primers

Sequence Name	5' to 3' Sequence	RNA Name
18SrRNA-952F	CGCAGCTAGGAATAATGGAATAGG	18S
18SrRNA-1016R	CATGGCCTCAGTTCCGAAA	
CCND1-402F	CTGGAGGTCTGCGAGGAACA	Cyclin D1
CCND1-508R	TGCAGGCGGCTCTTTTC	
CDKN1A-491F	GCGGCAGACCAGCATGA	Cyclin dependent kinase
CDKN1A-563R	GGATTAGGGCTTCCTCTTGAG	inhibitor 1A (p21)
CDKN1C-1042F	GCGCGGCGATCAAGAA	Cyclin dependent kinase
CDKN1C-1132R	ACATCGCCCGACGACTTC	inhibitor 1C (p57)
KLF4-1021F	TCCTTCCTGCCCCGATCAG	Kruppel-like factor 4 (KLF4)
KLF4-1082R	GGCATGAGCTCTTGTAATGG	