Development and application of a flow system to study shear stress-induced genes in primary endothelial cells

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Thesis submitted for the degree of Doctor of Philosophy PhD

Department of Bioengineering
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August 2012
Declaration of Originality

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Place, Date

Bonn, 22.08.2012

Signature

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Abstract

Atherosclerosis is a chronic lipid-modulated inflammatory disease, which develops preferentially in arterial regions, where blood flow is disturbed. Shear stress is exerted on the endothelium and it has been shown that the tangential force can influence intracellular signalling cascades, modulating transcriptional activation or repression of mechanosensitive genes. The exact mechanisms of mechanotransduction are not entirely understood, but the shear-dependent transcription factor Kruppel-like factor 2 (KLF2) has been considered as the master switch in shear-controlled endothelial gene expression. Induction of its target genes triggers anti-inflammatory and anti-thrombotic signalling cascades, determining an atheroprotective phenotype. Endothelial KLF4 has also recently been shown to be shear-sensitive and that it can compensate for reduced KLF2 transcripts. Another member of the KLF family, KLF6, is known to be present in the endothelium, but has not been further investigated. This suggests that KLF2 might not be a single master regulator in the process of shear-dependent atheroprotection.

Since KLFs have been demonstrated to form circuitries in other tissues, the hypothesis of the present study was that shear stress invokes a KLF2-KLF4-KLF6 network, which regulates the atheroprotective transcriptional program in the endothelium. The work aimed at understanding shear-dependent signalling mechanisms to potentially reveal therapeutic targets. Firstly, a flow device allowing the in vitro exposure of primary porcine aortic endothelial cells (PAECs) to shear stress was designed. Gene expression patterns were examined with the consideration of parameters associated with time, shear stress magnitude and interference with short inhibitory ribonucleic acid (siRNA). These studies were carried out using quantitative polymerase chain reaction (qPCR), genome-wide microarray analysis, protein detection and computational modelling. The findings revealed an oscillatory expression pattern for KLF2 and KLF4 in high shear regimes of 20 dyne/cm². siRNA-mediated silencing of KLF4 resulted in a significant downregulation of KLF2 and this dependency was shown for the first time. Global genome analysis identified a number of novel shared, but also individual downstream targets, suggesting that the transcription factors have important non-compensatory regulatory functions. In conclusion, this work contributes to the understanding of the individual roles of KLF2 and KLF4 and their functional overlap in the mediation of atheroprotection. Further investigations are required to assess whether KLFs could potentially be useful in preventing atherosclerosis or identifying atherosclerosis treatments.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
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<tbody>
<tr>
<td>2NR2</td>
<td>Nuclear receptor subfamily 2</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>ALCAM</td>
<td>Activated leukocyte cell adhesion molecule</td>
</tr>
<tr>
<td>AMPK</td>
<td>5' adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANG</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>ASK</td>
<td>Apoptosis signal-regulating kinase</td>
</tr>
<tr>
<td>ASS</td>
<td>Argininosuccinate synthase</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>BHF</td>
<td>British Heart Foundation</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CASP</td>
<td>Caspase</td>
</tr>
<tr>
<td>CBP/p300</td>
<td>Co-activator cyclic AMP response element-binding protein</td>
</tr>
<tr>
<td>CCNB</td>
<td>Cyclin B</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CECs</td>
<td>Coronary endothelial cells</td>
</tr>
<tr>
<td>CFD</td>
<td>Computational fluid dynamics</td>
</tr>
<tr>
<td>CM DMEM</td>
<td>Complete Media Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>CNP</td>
<td>C-type natriuretic peptide</td>
</tr>
<tr>
<td>CNPase</td>
<td>2',3'-cyclic nucleotide 3' phosphodiesterase</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP-responsive element</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>ctr</td>
<td>Control</td>
</tr>
<tr>
<td>CTSB</td>
<td>Cathepsin b</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DAP</td>
<td>Death-associated protein</td>
</tr>
<tr>
<td>DAVID</td>
<td>Database for Annotation, Visualization and Integrated Discovery</td>
</tr>
<tr>
<td>DCBLD2</td>
<td>LCCL and coagulation factor V/VIII-homology domains protein 1</td>
</tr>
<tr>
<td>DGEA</td>
<td>Differential gene expression analysis</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DUSP</td>
<td>Dual specificity phosphatase</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic age</td>
</tr>
<tr>
<td>ECGF</td>
<td>Endothelial cell growth factor</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECs</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ENG</td>
<td>Endoglin</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ES cells</td>
<td>Embryonic stem cells</td>
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<tr>
<td>EScr</td>
<td>Enrichment score</td>
</tr>
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<td>ET-1</td>
<td>Endothelin 1</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FM</td>
<td>Freezing media</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box O</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl-pyrophosphate</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>G protein</td>
<td>Guanine nucleotide-binding protein</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GATA2</td>
<td>GATA binding protein 2</td>
</tr>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GGPP</td>
<td>Geranyl-geranyl-pyrophosphate</td>
</tr>
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<td>GIMAP</td>
<td>GTPase IMAP family member</td>
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<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipid protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>Hydroxyethyl piperazineethanesulfonic acid</td>
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<td>HGNC</td>
<td>Human Gene Nomenclature Committee</td>
</tr>
<tr>
<td>His</td>
<td>Histidines</td>
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<tr>
<td>HMG-CoA reductase</td>
<td>3-Hydroxy-3-Methylglutaryl-Coenzym-A-Reductase</td>
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<tr>
<td>HO</td>
<td>Hemeoxygenase</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HSS</td>
<td>High shear stress</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical cord vein cells</td>
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<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>IECs</td>
<td>Iliac artery endothelial cells</td>
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<tr>
<td>IFI16</td>
<td>Interferon gamma-inducible protein 16</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of kappa light polypeptide gene enhancer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity Systems Pathway analysis</td>
</tr>
<tr>
<td>ISO</td>
<td>International Organization for Standardization</td>
</tr>
<tr>
<td>ITGB8</td>
<td>Integrin b 8</td>
</tr>
<tr>
<td>JAZF</td>
<td>Juxtaposed with another zinc finger gene</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
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<tr>
<td>KEAP</td>
<td>Kelch-like ECH-associated protein</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>KLFs</td>
<td>Kruppel-like factors</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>LRPAP</td>
<td>Low density lipoprotein receptor-related protein associated protein</td>
</tr>
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<td>LSS</td>
<td>Low shear stress</td>
</tr>
<tr>
<td>M</td>
<td>Mock siRNA</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
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<td>M-CSF</td>
<td>Macrophage colony stimulation factor</td>
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<td>MEF2</td>
<td>Myocyte enhancer factor-2</td>
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<tr>
<td>NFATc3</td>
<td>Nuclear factor of activated T cells cytoplasmic-dependent</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-kappa-B-inducing kinase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NOV</td>
<td>Nephroblastoma over-expressed</td>
</tr>
<tr>
<td>NPC</td>
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<tr>
<td>NPR1</td>
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</tr>
<tr>
<td>NPRB</td>
<td>B-type natriuretic receptor</td>
</tr>
<tr>
<td>NR3C2</td>
<td>Mineralocorticoid receptor 1</td>
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<tr>
<td>Nrf2</td>
<td>Nuclear factor (erythroid-derived)-like 2</td>
</tr>
<tr>
<td>NTC</td>
<td>Negative controls containing no template</td>
</tr>
<tr>
<td>OTE</td>
<td>Off-target effect</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidised low density lipid</td>
</tr>
<tr>
<td>P</td>
<td>Passage</td>
</tr>
<tr>
<td>PAECs</td>
<td>Porcine aortic endothelial cells</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PAR-1</td>
<td>Protease-activated receptors</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Principle component analysis</td>
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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>PCAF</td>
<td>P-300/cAMP-response element-binding protein-binding protein-associated factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>PEGAM</td>
<td>Phosphoglycerate mutase family member</td>
</tr>
<tr>
<td>PG2</td>
<td>Prostaglandin 2</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>PIK3R3</td>
<td>Phosphoinositide-3-kinase, regulatory subunit 3</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PM</td>
<td>Perfect match</td>
</tr>
<tr>
<td>PPM1D</td>
<td>Protein phosphatase, Mg2+/Mn2+ dependent</td>
</tr>
<tr>
<td>PTGDS</td>
<td>Prostaglandin D2 synthase</td>
</tr>
<tr>
<td>PTK2</td>
<td>Protein tyrosine kinase 2</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>Re</td>
<td>Reynolds number</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RMA</td>
<td>Robust multi-array analysis</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RVSS</td>
<td>Reversing shear stress</td>
</tr>
<tr>
<td>SAM</td>
<td>Significance analysis of microarrays</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERBP1</td>
<td>Serpine1 mRNA binding protein 1</td>
</tr>
<tr>
<td>Shc</td>
<td>SHC-transforming protein 1</td>
</tr>
<tr>
<td>SHP</td>
<td>Small heterodimeric partner</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short inhibitory ribonucleic acid</td>
</tr>
<tr>
<td>SMURF-1</td>
<td>Smad specific E3 ubiquitin protein ligase 1</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>SOM</td>
<td>Self-organised maps</td>
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<tr>
<td>SOPs</td>
<td>Standard operating procedures</td>
</tr>
<tr>
<td>Sox7</td>
<td>Sex determining region Y-box 7</td>
</tr>
<tr>
<td>SP1</td>
<td>Specificity protein 1</td>
</tr>
<tr>
<td>SS</td>
<td>Stock solution</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>SSRE</td>
<td>Shear stress response elements</td>
</tr>
<tr>
<td>STAT6</td>
<td>Signal transducer and activator of transcription 6</td>
</tr>
<tr>
<td>STK</td>
<td>Serine threonine kinase</td>
</tr>
<tr>
<td>SUMO</td>
<td>SMT3 suppressor of mitf two 3 homolog</td>
</tr>
<tr>
<td>Ta</td>
<td>Annealing temperature</td>
</tr>
<tr>
<td>TCFA</td>
<td>Thin cap fibroatheroma</td>
</tr>
<tr>
<td>Tf</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TFIIIA</td>
<td>Protein transcription factor IIIA</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>THBS</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>Tie</td>
<td>Tie-like receptor tyrosine kinase</td>
</tr>
<tr>
<td>TLR-2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion protein 1</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>vs</td>
<td>Versus</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>VWF</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>WHHL</td>
<td>Watanabe hereditary hypercholesterolemic</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WS</td>
<td>Working solution</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
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1 CHAPTER INTRODUCTION

The first section of the introduction provides background information on the epidemiology and risk factors of cardiovascular diseases (CVDs), focusing later on the progression and development of atherosclerosis. Hereby the role of shear stress and mechanisms of mechanosensation are detailed. As the shear-dependent KLF transcription factors (TFs) play a major role in atheroprotection, their structure and function in the vascular endothelium are further examined. The chapter concludes with the presentation of the interference RNA (RNAi) and microarray technology as these methods were used to explore KLF function in a genome-wide context.

1.1 Cardiovascular diseases

1.1.1 Epidemiology

In September 2011 the World Health Organisation (WHO) published the Global atlas on cardiovascular disease prevention and control. The report reveals that CVDs are the number one cause of death globally: more people die annually from CVDs than from any other reason. An estimated 17.3 million people died from CVDs in 2008, representing 30% of all global deaths. The prognosis for 2030 predicts an increase of mortality to 23.6 million and approximately 80% of these occur in low-, and middle-income countries (1).

Coronary heart disease with 7.3 million and cerebrovascular disease (stroke) with 6.2 million mortalities per year are the main forms of CVDs. These acute events occur when blood vessels to the heart muscle and the brain respectively are blocked, resulting in deficient blood supply. The underlying disease in the blood vessels is known as atherosclerosis and the different types of CVDs can be classified as shown in Table 1.

Table 1: Classification and types of cardiovascular diseases.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Cardiovascular disease</th>
</tr>
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<tbody>
<tr>
<td>Dependent on atherosclerosis</td>
<td>Ischaemic heart disease or coronary artery disease</td>
</tr>
<tr>
<td></td>
<td>Cerebrovascular disease (e.g. stroke)</td>
</tr>
<tr>
<td></td>
<td>Diseases of the aorta and arteries</td>
</tr>
<tr>
<td>Independent of atherosclerosis</td>
<td>Congenital heart disease</td>
</tr>
<tr>
<td></td>
<td>Rheumatic heart disease</td>
</tr>
<tr>
<td></td>
<td>Cardiomyopathies</td>
</tr>
<tr>
<td></td>
<td>Cardiac arrhythmias</td>
</tr>
</tbody>
</table>
1.1.2 Risk factors

Over the past two decades, cardiovascular mortality rates have declined substantially in high-income countries. The WHO report shows clear evidence that population-wide primary prevention and individual healthcare intervention strategies have both contributed to these declining mortality trends (1). A large percentage of CVDs could be prevented through the reduction of risk factors, which can be classified as behavioural, metabolic, personal and environmental determinants, as outlined in Table 2.

Table 2: Risk factors of cardiovascular disease.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Risk factors of CVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behavioural</td>
<td>Tobacco smoking</td>
</tr>
<tr>
<td></td>
<td>Physical inactivity</td>
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<td></td>
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In 2009, the prevalence of daily tobacco smoking varied between 31% in the WHO European Region and 10% in the WHO African Region. Smoking is estimated to cause nearly 10% of all CVDs and there is a large body of evidence that non-smokers and people, who try to avoid passive smoking, are at reduced risk (2). As a totally avoidable risk factor smoking is a lifestyle choice, which can be made by each individual.

Choosing a physically active lifestyle, which is defined as more than 5x 30 min of moderate activity per week or more than 3x 20 min of vigorous activity per week, is also important. The 2011 WHO report summarises scientific studies, showing that physical activity reduced risk of death from coronary heart disease and reduced risk of overall CVDs in a dose–response fashion. Physically active people are therefore at reduced risk of CVD (3).

Drinking alcohol presents another behavioural risk factor, as the WHO estimated that approximately 2.8% of all CVD mortalities are related to excessive alcohol consumption. It is hereby important to distinguish the level and the pattern of alcohol intake. Epidemiological studies show that while excessive consumption and the pattern of binge drinking increase the risk, moderate consumption has an overall protective effect (4).
An unbalanced diet with a high intake of salt, saturated fat and trans-fat cholesterol and low intake of fruits, vegetables and fish is linked to cardiovascular risk. Salt is known to modulate blood pressure level, which is a risk factor of CVD. Unfortunately, it has been shown that the current average consumption of 9–12 grams/day is much higher than the level of 5 grams/day recommended by the WHO. Therefore, reduction of the daily salt intake, which is an individual choice, would be beneficial to lower the risk of CVDs.

Adequate consumption of fresh fruits and vegetables also reduces the risk of CVD, while high-energy foods, such as processed foods, have the opposite effect. They often consist of saturated fats and trans-fatty acids, which have shown to be important factors that elevate plasma low density lipoprotein (LDL) levels.

LDL is one of the five major groups of lipoproteins, which enables the transport of lipids from the liver to other tissues in the body through the bloodstream. It is sometimes referred to as the "bad cholesterol" as it can deposit any excess on artery walls and in tissues. In contrast, high density lipoprotein (HDL), which transports cholesterol to the liver for excretion, has an antagonistic role (5). In humans LDL levels of 3.35 mmol/L–4.10 mmol/L are considered to be borderline high and the lipid hypothesis associates elevated LDL blood levels with atherosclerosis (6). This theory has become controversial as new findings revealed that one of the key factors in atherogenesis is not total cholesterol, but oxidized cholesterol, which has been damaged by reactive oxygen molecules (7).

Nevertheless, the INTER-HEART study on behalf of the WHO and the World Heart Federation concluded that 45% of heart attacks in Western Europe are caused by abnormal blood lipid levels and that those with such blood levels are at three times higher risk of CVD in comparison to people that have normal blood lipid levels (8). A high fat diet often also contributes to a higher body mass index (BMI), which in conjunction with little exercise leads to overweight and obesity. In England around 42% of men and 38% of women are considered overweight (BMI of 25-30 kg/m²) and an additional 25% of men and 28% of women are obese (BMI over 30 kg/m²). The British Heart Foundation (BHF) stated in their 2010 report that an estimated 7% of all disease burden in developed countries was caused by raised BMI and that around a third of CVD and ischaemic stroke and almost 60% of hypertensive disease were due to overweight (9).
Systolic and diastolic blood pressures of more than 140/90 mmHg are considered to be hypertensive. In 2010, according to the BHF, around 32% of men and 29% of women fell into this category. The INTER-HEART study estimated that 22% of heart attacks in Western Europe are due to a history of high blood pressure. This could be reduced by drug treatment, as well as lifestyle changes such as weight reduction, increased physical activity levels and a reduction in salt consumption.

Diabetes is a metabolic disease characterised by high blood sugar levels with a fasting plasma glucose value ≥ 7.0 mmol/L. According to the WHO, the risk factor of CVD is about three times higher in people with diabetes and women are at greater risk. It is estimated that approximately 15% of heart attacks in Western Europe are due to diagnosed diabetes. As prevalence for diabetes is mainly inherited, preventative measures are difficult, but the recognition of the disease is most important as blood levels can be adjusted with insulin administration.

Additionally, environmental and social factors such as the distribution of income and the level of education indirectly influence cardiovascular health. Similarly, unregulated globalisation as well as unplanned urban drift promote the risk of CVD often indirectly as they are in favour of previously mentioned determinants (3).

1.1.3 Development and progression of atherosclerosis

Atherosclerosis is a complex and multifactorial inflammatory disease and the predominant cause of most CDVs. It is characterised by accumulation of lipids in the arterial wall, which occurs in large and medium-sized muscular arteries that supply blood to vital organs such as the heart and the brain (10). The focal nature of the disease has been recognised in the early 1960s, as mainly the artery wall in predetermined sites, such as at branches or curvatures with non-laminar blood flow, is affected (11). Different stages, as summarised in Figure 1, determine the development of atherosclerosis.

In atheroprotected regions in the vasculature the healthy artery consists of three layers. The innermost tunica intima is lined by a monolayer of endothelial cells (ECs), which are in direct contact with the blood. In regions of laminar flow an alignment of the cell axis in the direction of flow can be observed (12). Vascular smooth muscle cells (VSMCs), embedded in an extracellular matrix (ECM), comprise the middle layer tunica media. The collagen fibers in the ECM give structural support to the resident cells and elastin allows the vasculature to elastically stretch during systole. The outer layer of the vessel, the adventitia, is composed of connective tissue containing mast cells, nerve endings and micro vessels (13).
In atheropron regions of disturbed flow (see section 1.2.3.2) the endothelium is dysfunctional in an inflamed and activated state, leading to the expression of pro-inflammatory molecules, including intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), the P- and E-selectins, cytokines, and chemotactic factors, such as monocyte chemotactic protein 1 (MCP-1) and macrophage colony stimulation factor (M-CSF) (14, 15). Consequently, rolling leukocytes adhere to the arterial wall and migrate into the tunica intima as seen in Figure 1 (II). After transmigration M-CSF induces the differentiation of monocytes into macrophages. Low density lipid (LDL) particles react with free radicals, which cause their oxidation (oxLDL) and results in their infiltration of the arterial wall.

The enriched cholesteryl esters and oxLDL are taken up by macrophages through scavenger receptors, which leads to their differentiation into lipid-loaded foam cells (16). These cells amplify the inflammatory process by secreting cytokines, growth factors and reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radical that damage the surrounding vascular tissue and activate more cells.

The accumulation of macrophages and dedifferentiated foam cells beneath the endothelial layer, a so-called fatty streak, may be the precursor of an atheroma (Figure 1 III). When more foam cells assemble and VSMCs migrate and proliferate, plaque development progresses into a next stage. This process is characterised by the formation of a fibrous cap above the lipid core through a layer of migrated VSMCs and deposited ECM. Within this environment the synthesis of ECM macromolecules, such as collagen, elastin and proteoglycans, contributes also to plaque expansion (17). T-cells recognise local antigens and mount Th1 responses with secretion of pro-inflammatory cytokines, contributing to an inflammatory microenvironment. In an advanced state, apoptosis of foam cells leads to the release of extracellular lipids, sometimes forming cholesterol crystals, which accumulate in the central region, the so-called necrotic core. The release of more cytoplasmic content further stimulates the pro-inflammatory plaque environment (10, 14, 18).
Advanced plaques can be categorised according to their susceptibility to rupture. The stable plaque (Figure 1 IV) is defined by a fibrous cap, which might be calcified because of the deposition of a scaffold matrix from pericyte-like cells. Its core contains only few inflammatory cells and is rich in fibrous connective tissues and VSMCs, producing a significant luminal stenosis (19).

In contrast, the unstable thin cap fibroatheroma (TCFA) shows a lack of or only speckled calcification, contributing to plaque instability. TCFAs are further characterised by a thinner fibrous cap, which weakens the mechanical resistance of the lesion, a larger underlying necrotic core, infiltration by macrophages and T-cells, and increased plaque vascularity (20). They are therefore prone to rupture, resulting in luminal thrombosis due to contact of their contents with the flowing blood, which is the initiating event in most acute coronary syndromes (21). Potential mechanisms of fibrous cap thinning include inflammation and the production of cytokines driving the expression of matrix-degrading proteases (MMPs) (10) as well as apoptosis of VSMCs (22).
Interestingly, the exposure to low endothelial shear stress has also been shown to promote the transition of a stable plaque to a TCFA through modulation of vascular inflammation, ECM and wall remodelling (23). The role of calcification is controversial as some researchers argue that calcification of the fibrous cap might actually stabilise the plaque (24), while others have demonstrated that calcification is associated with a higher risk of plaque rupture. Possibly the vessel is protected from rupture only when extensive calcification has occurred, whereas the early or intermediate stages of calcification may actually enhance plaque vulnerability due to increased stress (25).

1.1.4 Models to study atherosclerosis

1.1.4.1 Animal models

Despite the great interest in the study of plaque development, no ideal animal model has been found so far, as the complex atherogenic process in human atherosclerosis or the specific lipoprotein profile are very difficult to simulate (26). Mouse atherosclerotic models, which are used most frequently, depend on the generation of a non-high density lipoprotein (HDL)-based hypercholesterolemia. The ApoE (-/-) knock out animals lack the gene coding for apolipoprotein E (ApoE), an important factor in lipid metabolism. These mice have increased total plasma cholesterol levels and show some characteristics of lesion formation that are similar to larger animal models of atherosclerosis and humans (27). The genetic ablation of the low density lipid receptor (LDLR) causes a modest lipoprotein abnormality and on a high cholesterol diet the LDLR (-/-) animals develop large atherosclerotic plaques (28).

Although these features partially resemble the disease progression in humans, the plaque composition as well as the site in the vasculature varies. Mice usually do not develop a thick fibrous cap seen in chronic human atherosclerosis as well as TCFAs with overlying thrombosis.

At young age the site of plaque formation differs as murine models predominantly develop plaques in the aortic root (26). It has been shown that with prolonged breeding and on a high-fat feeding, mice older than 24 weeks can develop plaques also in coronary arteries (29). This was associated with myocardial infarction and produced evidence of myocardial metabolic anaerobic stress when compared with ApoE (-/-) mice on a normal diet.
With the help of a flow modifying cuff around the carotid artery of ApoE (-/-) mice, Cheng et al. could demonstrate that local hemodynamics affect plaque progression and vulnerability. In regions of low flow pattern TCFAs with a large lipid core and a thin fibrous cap developed spontaneous intra-plaque hemorrhages (14%), while more stable plaques formed in regions exposed to oscillatory flow (30).

For certain types of investigations larger animals such as pigs, rabbits and non-human primates are preferred although these studies are often associated with much higher expenses. The pig develops atherosclerotic lesions spontaneously, has a human-like lipoprotein profile and forms lesions in coronary arteries. Partially due to its size, the pig is also useful to study the heterogeneous interaction of hemodynamics and the endothelium. Streptozotocin-induced diabetes onto pigs on a high fat diet results in the formation of plaques. These resemble human coronary plaques in complexity, with a thick fibrous cap, a necrotic lipid core, calcification and hemorrhaging (31).

Rabbits are sometimes advantageous as they respond well to a high cholesterol diet, but they have very low plasma levels of hepatic lipase, lack apolipoprotein A II (ApoA-II) and the developed plaques are largely composed of macrophage-derived foam cells (26). A strain with familial hypercholesterolemia, the Watanabe hereditary hypercholesterolemic (WHHL) rabbit with a defect in the LDLR, has been identified (32). With selective breeding a substrain of WHHL was derived with males showing robust coronary atherosclerosis (33). Although genetic modifications on rabbits are by far more difficult than on mice, several transgenic strains have been bred, amongst those also the ApoE2 transgenic animal, which develops type III hyperlipidemia and atherosclerosis (34).

The study of non-human primates in atherogenesis research is limited due to the size of the animals, the associated expenses, the length of time required for them to develop atherosclerosis as well as moral objections. Models include the Old World, African Green, Rhesus, Cynomolgus monkeys and baboons and it has been demonstrated that these display humanoid lipoproteins and coronary plaques in response to high cholesterol diet (26).
**1.1.4.2 Ex vivo models**

The culture of *ex vivo* organs has the advantage that certain aspects of atherosclerosis development can be examined in a less complex environment. Important hereby is that the entire vessel, where ECs are still in their native environment, can be used.

The experimental design is fairly simple as the explanted vessel segment is cannulated at either end and connected to a perfusion circuit with controlled flow patterns of media or blood. Bardy et al. developed such an organ culture device to study the influence of flow and pressure on the endothelium using histology, histomorphometry and microscopy techniques (35).

Other groups have used a similar approach to study the effect of hemodynamic conditions in porcine thoracic arteries. These were perfused for 3 days under physiologically relevant unidirectional high shear stress ($6 \pm 0.3$ dyne/cm$^2$), low shear stress ($0.3 \pm 0.1$ dyne/cm$^2$) or oscillatory shear stress ($0.3 \pm 3$ dyne/cm$^2$). Gambillara et al. could observe that oscillatory shear stress induced endothelial dysfunction mediated through downregulation of endothelial nitric oxide synthase (eNOS) gene expression, while unidirectional shear stress preserved physiological functions (36).

**1.1.4.3 In vitro models**

*In vitro* models have been developed in an attempt to reproduce important characteristics of flow profiles for mechanistic studies on mainly ECs and VSMCs to gain inside on flow-induced atherogenesis. The experimental setting requires the isolation of cells from their native tissue or commercial acquisition of cell lines and their expansion to obtain sufficient numbers of cells, followed by cell culture under flow. Interestingly, although plaque development mainly affects arteries, the most common cell types used in flow studies are human umbilical cord vein cells (HUVECs) (37-44). Cells derived from the umbilical aorta have also been used (45). The umbilical cord has the advantage that it is large in size and often discarded postpartum. Usage of cells from other parts of the vasculature is not as common as they are difficult to get hold of. Working with human tissue, rather than different species, implies that many reagents for subsequent analysis, such as antibodies, primers and siRNA are tested and commercially available.
Mice are commonly used as *in vivo* models for atherogenesis, but cell numbers derived from murine carotid or thoracic arteries are limiting and would require pooling from several animals. Still, some research groups have successfully published their work with mouse cells (46). To overcome the problem of insufficient material, bovine and porcine tissues are also used (47-49). These animals have comparable vascular hemodynamics, geometry and vascular physiology to humans. The disadvantage is the rarely off-the-shelf reagent availability as most companies are specialised on a human-, and murine-specific product range.

Several flow devices have been designed based on the principles of parallel-plate flow chambers, cone-and-plate viscometers, orbital shakers and rectangular or tubular capillary tubes as summarised in Figure 2 (50).

![Figure 2: In vitro flow systems: parallel-plate flow chamber (A), cone-and-plate viscometer (B), orbital shaker (C) and tubular or rectangular capillary tube (D). Adapted from Chiu and Chien (2011).](image)

One of the first devices used for *in vitro* studies, the cone-and-plate flow apparatus (Figure 2B), was designed by pioneers of mechanobiology: Gimbrone, Dewey and Davies. In this apparatus shear stress is induced by rotating a cone over a stationary plate containing ECs cultured on cover slips. The cone-and-plate flow apparatus was used to study EC shape and their orientation in response to flow (51, 52). The initial system was further optimised by Franke et al., who incorporated an optical system to monitor ECs in real time (53). Also Blackman et al. further modified the speed-controlled motor so that it could produce pulsatile shear stresses in an arterial-like waveform that mimicked the flow characteristics in human arteries (54).
This model has been widely used with the advantages of being well suited for long term cultures and providing large quantities of RNA or of complementary deoxyribonucleic acid (cDNA) for further analysis.

The orbital shaker model (Figure 2C) was developed by Pearce et al. (55) and it is simply composed of a Petri dish containing cultured cells placed on an orbital shaker. Using computational fluid dynamics (CFDs) the wall shear stresses over the entire area of the bottom surface of a dish can be determined (56). The system is nevertheless very limited in the range of shear magnitudes and the shear stress is not homogenous over the entire surface.

This problem has been addressed by using a tubular or rectangular capillary tube (Figure 2D), where the cells are attached to the inner surface. Similarly to the ex vivo organ culture (see section 1.1.4.2), the capillary is connected to a flow circuit and the shear rate is only limited by the pump system used. In addition to shear, the circumferential cyclic strain, which results from the expansion of the artery during the cardiac cycle, can also be investigated (57). As a drawback, it has been reported that the tube system does not yield sufficient amounts of cells for some bioassay analysis and that the seeding procedure presents many obstacles (50).

The most commonly used device is the parallel plate flow chamber, which consists of a gasket with a rectangular cut-out to result in a uniform channel height along the path of flow generated by a perfusion or peristaltic pump (Figure 2A). Initially developed by Frangos et al., the system gained recognition in the research community and was used to document cell morphology (58), metabolic changes (59) as well as endothelial-leukocyte interaction (60). It was further modified to allow for more complex patterns, such as an asymmetric sudden expansion (61) and spatial and temporal flow gradients (62).

The modulation of flow patterns and shear stress magnitude over a wide range, the possibility of interaction studies in inflammation and wound healing, user-friendly handling as well as easy access to the culture are the main advantages of the parallel plate flow chamber. These advantages were convincing factors to choose a commercially available parallel plate flow chamber for the work presented in this thesis.
1.2 Shear stress

1.2.1 Principles of hemodynamics in the vasculature

The principles of fluid mechanics are described by the Navier-Stokes equations, a series of nonlinear partial differential equations. Flow can be classified as laminar when a solution to the Navier-Stokes equations exists, which allows for the prediction of the flow path. In contrast, the numerical solution of the Navier-Stokes equations for turbulent flow is extremely difficult. The mathematical model of steady, laminar and fully developed flow through a straight circular tube of constant cross sectional area is named Poiseuille flow. It describes shear stress ($\tau$) with the Hagen-Poiseuille equation (Equation 1) as being directly proportional to the velocity of blood flow and inversely proportional to the cube of the arterial radius ($R$), where $Q$ is the flow rate and $\mu$ is the fluid viscosity. Thus small changes in $R$ greatly influence $\tau$ and vice versa.

$$\tau = \frac{4\mu Q}{\pi R^3}$$

Equation 1: Hagen Poiseuille equation of shear stress ($N/m^2$) with $R$ the arterial radius (m), the $Q$ the flow rate ($m^3/s$) and $\mu$ the fluid viscosity ($Ns/m^2$).

Poiseuille flow rarely exists in large arteries, because these are not straight; contain branches that perturb steady flow and their cross sectional area varies. Blood is often considered as a Newtonian fluid in models, although it is non-newtonian, meaning that it does not exhibit a linear relationship between the shearing stress and the rate of deformation. Nevertheless, even with these approximations, good estimates of hemodynamic values in the vasculature can be obtained.
Shear stress (τ), measured in dyne/cm² or N/m², is the fluid mechanical force generated by blood flow on ECs at the inner lining of the aorta. The vascular shear stress of large arteries usually varies between 5-20 dyne/cm², but instantaneous values can alternate from negative measures to nearly 40 dyne/cm² during states of increased cardiac output (63).

Another important parameter in fluid dynamics is the Reynolds number (Re), which is a dimensionless ratio between inertial forces and viscous forces. It can be used to discriminate between laminar and turbulent flow (Equation 2). Where p is the fluid density (kg/m³), U is the mean velocity (m/s), d is the characteristic linear dimension (m) and μ is the viscosity of the fluid (Ns/m²).

\[
Re = \frac{pUd}{\mu}
\]

Equation 2: Reynolds number (Re) with p the fluid density (kg/m³), U the mean velocity (m/s), d the characteristic length (m) and μ the viscosity of the fluid (Ns/m²).

Reynolds numbers in the circulation vary from around several thousands in the aortic arch to <1 for the smaller arterioles. At very low Re (i.e. below 1), viscous forces dominate inertial forces, resulting in laminar flow, which follows the vessel geometry. At higher Re, when viscous and inertial forces are similar, secondary flow profiles such as vortices develop at arterial bends and bifurcations. Flow is considered to be turbulent when Re ≥ 2000 prevails.

Besides flow-generated endothelial shear stress, the cyclic strain is another local hemodynamic force. Pulsatile changes in blood pressure stretch the vessel wall circumferentially and create cyclic strain in ECs. The degree of stretch is around 9–12% in the aorta, 1–2% in carotid arteries and 6–10% in pulmonary arteries (64).

1.2.2 Mechanoreception, signal transduction and mechanosensitive gene expression

Shear stress exerts its local effects on ECs through mechanotransduction, which is the conversion of physical stress into intra-cellular biochemical signals. Understanding of the mechanisms how mechanotransducers can sense shear exerted through blood flow is currently very limited.
The following section describes some of the potentially important effectors and the subsequent signalling cascades they trigger. Figure 3 visualises schematically some of the known mechanoreceptors on a cell and their downstream signalling pathways. It is not well understood whether these are stimulated in a synchronised fashion or if they can also be activated individually. So far most publications have exclusively focussed on a single mechanoreceptor and demonstrated its responsiveness to shear stress. Detailed understanding of the mechanisms triggered to switch from a quiescent inactivated state to an activated state and the responsiveness and interplay of mechanoreceptors is currently not available. As a common signalling pathway downstream of some mechanoreceptors the mitogen-activated protein kinase (MAPK) cascade has been identified as outlined in the following section.

Microtubules, actin and intermediate filaments physically connect different regions of ECs to transmit forces from the apical domain, where shear is applied, to the basal or lateral domains, where mechanotransduction events occur (65). It has been shown that the inhibition of these by drugs or genetic intervention blocks many EC responses to flow (66). Helmke et al. could demonstrate by life cell imaging that filaments linking the luminal surface to junctions and focal adhesions were displaced upon exposure to shear (67). Further evidence has been introduced through switch-like models, where applied forces were instantaneously transmitted to load-bearing subcellular structures, inducing conformational changes in mechanosensitive proteins (68). These examples suggest that the cytoskeleton plays an important role in the shear response of ECs.

Direct signalling can also occur through ion channels, guanine nucleotide-binding protein (G–protein) activation and other mechanosensitive complexes in the luminal membrane. Ion channels have been found to be sensitive to membrane fluidity and tension and they were shown to trigger signalling in cells, leading to activation of the MAPK pathway, under flow (69). Hoger et al. have identified a shear sensitive inward rectifying potassium channel. Upon activation the ion flux has been proposed to induce vessel dilation by hyperpolarisation and thereby lowering the contraction of VSMCs (70).

G-proteins are activated by flow through ligand-dependent conformational changes on the bradykinin receptor. Studies have demonstrated that the localised molecular associations and signalling events of G–protein activation at cell–cell junctions are in conjunction with the platelet endothelial cell adhesion molecule 1 (PECAM-1).
Otte et al. have shown \textit{in vitro} and \textit{in vivo} that the flow-induced G alpha-PECAM-1 complex depends on a temporal gradient, suggesting it to be a critical mediator of hemodynamic forces (71). The cell adhesion molecule PECAM-1 is localised at the inter-endothelial cell-cell adhesion site.

In flow conditions the cell–cell junction associated proteins undergo significant dose-dependent reorganisation, whereas the integrity of the monolayer remains unaffected. PECAM-1 phosphorylation in response to mechanoforce-induced deformational changes in the molecule triggers the activation of Src family kinases.

Tzima et al. have demonstrated that vascular endothelial cadherin (VE-cadherin), a specific EC classical cadherin, is crucial for the mechanotransduction in this model, but does not require binding to cadherins on other cells. Instead, VE-cadherin seems to be an adaptor that associates with vascular endothelial growth factor receptor 2 (VEGFR2) and brings it into proximity with PECAM-1, which facilitates the transactivation of VEGFR2 by active Src. Upon activation of this trimolecular complex by the application of mechanical force, activated phosphatidylinositol 3-kinase (PI3K) is recruited, which mediates crucial downstream signals (72).

In addition, the glycocalyx (73) and caveolae (74) have been shown to be important mediators of mechanical forces, while the role of primary cilia in shear stress sensing remains controversial (75). The complex network of intra-cellular pathways, which are triggered after activation of mechanoreceptors and result in the activation of several TFs such as nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1) and KLFs, is shown in Figure 3. These can regulate gene expression and ultimately modulate cellular functions and morphology. Downstream of luminal or junctional receptors, the transduction involves generation of ROS from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, activation of protein kinase C (PKC), activation of Rho family small GTPases, release of eNOS and activation of PI3K-Akt cascade (76).
Introduction

The integrin-dependent cascade is initiated by induction of a multiple complex of non-receptor tyrosine kinases (e.g. protein tyrosine kinase 2 (PTK2), c-Src, shc-transforming protein 1 (Shc) and paxillin), adaptor protein growth factor receptor-bound protein (Grb) 2 and MAPK p38 and guanine nucleotide exchange factors. They thereby activate members of the Ras family, which hydrolyse guanosine triphosphate (GTP). GTP then mediates the remodelling of the cytoskeleton, resulting in temporary or permanent structural changes of ECs.

Active Ras plays a pivotal role in intracellular transduction of endothelial shear stress signals as it triggers various parallel downstream cascades of serine kinases; each kinase phosphorylates and hence activates the next one downstream, ultimately activating MAPKs (76). The MAPK pathway as seen in Figure 4 implicates the sequential phosphorylation and activation of the cytoplasmic protein kinases MAP3K (e.g. MEKK5), MAP2K (e.g. MEK5) and MAPK (e.g. ERK1/2). The MAP kinase cascade comprises three different pathways: the c-Jun N-terminal kinase (JNK), the p38 and the extracellular signal-regulated kinase (ERK) pathways. They are triggered in response to various stimuli, JNK and p38 pathways are preferentially activated by inflammatory cytokines and stress, whereas the ERK pathway is preferentially activated by growth factors (77).
Figure 4: MAPK signalling pathways. Four distinctly regulated groups of MAPks are known: extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38 alpha/beta/gamma/delta) and ERK5. These are activated by specific MAP2Ks: MEK1/2 for ERK1/2, MKK3/6 for the p38, MKK4/7 (JNKK1/2) for the JNKs and MEK5 for ERK5. Each MAP2K, however, can be activated by more than one MAP3K. Adapted from Jeffrey et al. (2007).

1.2.3 Role of shear stress in the development of atherosclerosis

The development of atherosclerotic plaques occurs predominantly in curved regions of the vasculature such as bends, where the blood flow rate is relatively low, and near side branches, where blood flow is non-uniform. This focal distribution has been explained by geometry-dependent differences in shear stress magnitude on the endothelium, which trigger a distinct mechanosensitive response. There is a strong correlation between EC dysfunction, areas of low mean shear stress and oscillatory flow with recirculation. In atheroprone regions, where disturbed flow dominates, pro-atherogenic genes are up-regulated, whereas atheroprotective genes are down-regulated (78) (Figure 6).
On the other hand, high laminar shear stress is considered to be atheroprotective, because it stimulates an anti-inflammatory and anti-thrombotic endothelial phenotype as shown in vivo and in vitro (Figure 5).

1.2.3.1 Atheroprotective high laminar shear stress

One of the main atheroprotective effects is the transcriptional activation of the gene NOS3 (79) and the post-transcriptional phosphorylation of its protein product eNOS by high laminar shear stress of more than 15dyne/cm² (80). eNOS catalyses the production of nitric oxide (NO) from L-arginine, which regulates many aspects of cardiovascular homeostasis. It has a relaxing effect on VSMCs, inducing vasodilation, increased blood flow as well as anti-atherogenic properties such as downregulation of leukocyte adhesion molecule expression, inhibition of platelet aggregation and suppression of VSMC proliferation (13). The anti-inflammatory phenotype is mediated by reduced induction of VCAM-1 and E-selectin in response to pro-inflammatory stimuli as demonstrated in vitro and in vivo (81, 82). High shear stress also exerts its protective function by modulating inflammatory NF-κB pathways such as p38 and JNK signalling via inhibition of apoptosis signal-regulating kinase 1 (ASK1) and thioredoxin interaction (83). This results in the inhibition of leukocyte adhesion and transmigration into the vessel wall, which presents a crucial point in the onset of atherosclerosis (section 1.1.3). The integrity of the endothelium also does not allow for the uptake of lipoproteins and their metabolites.
Coagulation stimulates the release of potent anti-thrombotic mediators from ECs. Prostaglandin 2 (PGI2), an inhibitor of platelet activation and effective vasodilator, was shown to be released from ECs on exposure to high shear stress. Also other important factors such as thrombomodulin (TM), which interacts with protein C and protein S to inactivate certain clotting factors and tissue plasminogen activator (tPA) are induced by high shear stimulus as shown in Figure 5 (84). Interestingly, some anti-inflammatory transcription factors such as nuclear factor (erythroid-derived)-like 2 (Nrf2) are also induced by high shear stress (85). Exclusive shear-induction of KLFs, which have been shown to trigger atheroprotective signalling cascades, has also been demonstrated and is discussed in more detail in section 1.3.2.

1.2.3.2 Atheroproned low or oscillatory shear stress

Low or oscillatory shear stress of less than 5 (+/-2) dyne/cm² has been shown to attenuate the NO-dependent atheroprotection, as its bioavailability is reduced by inhibition of eNOS synthesis (86). Additionally, prostacyclin, another important vasodilator, is down-regulated while the potent vasoconstrictor endothelin 1 (ET-1) is induced as demonstrated by Qiu et al. (87). Thrombogenicity is enhanced by the downregulation of NO and tPA.
Low shear stress has also been shown to trigger the inflammation process in dysfunctional endothelium (Figure 6). One of the important mediators in this cascade is the transcription factor NF-κB (76). It is highly induced by low shear stress and its nuclear translocation is enhanced through the lack of NO (88). Various NF-κB-dependent adhesion molecules and markers of inflammation are up-regulated as reviewed by Chatzizisis et al. These include VCAM-1, ICAM-1, E-selectin, chemoattractant chemokines, such as MCP-1, and pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and interferon (IFN).

The rolling and adhesion on the luminal surface of circulating monocytes is enhanced through the expression of these adhesion molecules and the transmigration process is promoted by MCP-1. As described in section 1.1.3 this is the crucial onset of the terminal differentiation process into macrophages, which sustain inflammation, oxidative stress and matrix remodelling (76).

Foam cells, lipid loaded macrophages or VSMCs, can develop as more LDL infiltrates with increasing endothelial permeability in low flow regions (15). An important aspect is also the promotion of oxidative stress through the production of ROS. Enhanced expression and activation of oxidative enzymes such as NADPH generate ROS, which then oxidise LDL (oxLDL). Furthermore, ROS also degrade NO and its cofactors and activates therefore ECs (76).

Ultimately, the shear-induced combination of all these factors also influences the VSMC layer. Overexpression of chemotactic factors stimulates VSMCs to migrate from media to tunica intima, where they deposit ECM and form the fibrous cap as detailed in section 1.1.3. During the progression of the disease low shear stress plays an important role in plaque destabilisation through induction of MMP gene expression and protein activity, which are associated with degradation of ECM (14). Additionally, the induction of the pro-angiogenic factor vascular endothelial growth factor (VEGF), thereby promoting neovascularisation, which is a key factor in plaque progression and vulnerability, has been demonstrated (20). In comparison to the anti-thrombogenic role of high shear stress, low shear stress enhances thrombogenicity as it has no effect on TM. The expression of tPA is therefore reduced and as NO and prostacyclin are also absent, thrombosis is promoted (87). These thrombogenic effects might be detrimental in the disruption of TCFA (84).
Figure 6: Low oscillatory shear stress promotes a pro-thrombotic endothelial phenotype with enhanced leukocyte adhesion and migration, which is prone to develop atherosclerosis. The abbreviations stand for tissue necrosis factor (TNF)-α, interleukin (IL)-1, reactive oxygen species (ROS), monocyte chemotactic protein-1 (MCP-1), nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1) and vascular cell adhesion protein 1 (VCAM-1). Adapted from Traub et al. (1998) and Libby et al. (2011).

1.3 Biology of Kruppel-like factors

The kruppel (German for cripple) gene was firstly identified in Drosophila as a critical embryonic patterning gene. Homozygous mutants showed segmentation defects on anterior abdominal and thoracic segments resulting in death (89). Since this discovery by Nusslein-Volhard et al. in 1980, 17 mammalian members of the KLF family have emerged. Initially, they were named according to the tissue they were derived from, but the Human Gene Nomenclature Committee (HGNC) designated a numerical identification. KLFs act as DNA-binding transcriptional regulators that play very diverse roles during differentiation and development in various tissues and organs (90). This chapter discusses structural features of highly conserved domains amongst all KLFs and the function of KLFs in the vascular endothelium, with a focus on the flow-dependent expression of KLF2 and KLF4. Also the results of a phylogenetic study on the inter-species homology are presented.
1.3.1 Zinc finger structure

Zinc finger proteins are amongst the most abundant proteins in eukaryotic genomes. Their diverse functions include DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly as well as lipid binding (91).

Zinc fingers were discovered in 1985 by Miller et al., who performed biochemical studies on the interaction of the xenopus protein transcription factor IIIA (TFIIIA) with 5S RNA (92). They discovered that each finger has a self-contained domain stabilised by a zinc (Zn) ion. This domain is ligated to a pair of cysteines (Cys) and a pair of histidines (His) with an inner structural hydrophobic core (Figure 7). Several different arrangements of the zinc coordinating residues (e.g. Cys$_2$His$_2$, Cys$_4$, and Cys$_6$) exist and they are classified based on the overall shape of the protein backbone in the folded domain. The most common classical Cys$_2$His$_2$ zinc finger motif can be linked linearly in tandem to recognise nucleic acid sequences and to bind in the major groove of DNA, typically spaced at 3-base pair (bp) intervals (93).

![Figure 7: Structure of the zinc finger motif. The zinc atom is held between two cysteines (Cys) within the β-sheet of the motif and two histidines (His) in the α-helix. The solid green lines indicate the R groups of these amino acids. ‘N’ and ‘C’ indicate the N- and C-termini of the motif. Derived from DNA-Protein Interactions by Travers et al. (1993).]
Structural homology between TFIIIA and the protein product of the KLF gene predicted from DNA sequences has been shown by Rosenberg et al. (94). All 17 members of the KLF family contain three highly conserved classical Cys$_2$His$_2$ zinc fingers at the carboxyl terminus of the protein. Zinc fingers one and two contain 23 residues, while the third finger has only 21 residues, but all enable KLFs to bind to related GC- and CACCC DNA motifs (95). The individual fingers are connected by a characteristic seven-amino acid spacer TGEKP(Y/F)X, also highly conserved among the family members.

Although the amino acid sequences in the zinc finger domains are closely related to one another, the non-DNA binding domains are highly divergent. Modular activation and repression domains mediate transcriptional regulation by KLFs, which accounts for their variable and broad biological function (96). Figure 8 shows the transactivation (red) and transrepression domains (orange) of KLF2, KLF4 and KLF6 as well as their common carboxyl terminal three zinc finger motif.

![Figure 8: Schematic drawing representing transactivation-(red) and transrepression domains (orange) and the C’terminal zinc finger domain of human KLF2, KLF4 and KLF6. Adapted from Atkins et al. (2007).](image-url)
1.3.2 Kruppel-like factors in the vascular endothelium

Within the vascular endothelium KLF2, KLF4, KLF5, KLF6, KLF7 and KLF11 are expressed. Amongst these, KLF2 and KLF4 have been so far found to coordinate the regulation of atheroprotective transcriptional programs (97).

1.3.2.1 The role of KLF2 and KLF4 in vascular development

Kuo et al. firstly detected KLF2 mRNA expression at embryonic age (E) 9.5 days in the vascular endothelium throughout murine embryos. Its expression was restricted to the cluster of differentiation (CD) 34+ endothelial lineages and absent in the tunica media, which consists mainly of VSMCs. The generation of KLF2 (−/−) mice resulted in embryonic lethality at E12.5-14.5 due to haemorrhages, which were associated with defects in blood vessel morphology (98). Tissue-specific deletion of KLF2 was shown to result in embryonic heart failure due to elevated cardiac output. Signs of anaemia or arteriovenous malformation could not be detected in these animals. Lee et al. additionally showed that the lethal embryonic phenotype could be rescued by administration of the vasorestrictive phenylephrine, which increased the vessel tone. These results suggest a crucial role of KLF2 in maintaining vascular tone and controlling hemodynamic regulations (99).

KLF4 has been shown to be highly expressed in the differentiating layers of the epidermis at E16.5. Homozygous KLF4 (−/−) mice died shortly after birth, due to loss of skin barrier function, while no vascular abnormalities could be observed. Segre et al. concluded that KLF4 is critical to and selective for establishing the skin's barrier function (100).

1.3.2.2 Regulation of KLF expression and protein activity through various stimuli

1.3.2.2.1 Shear stress

The modulation of gene expression through shear stress was firstly described in 1981 by Dewey et al. (52) and shear-dependent induction of KLF2 was later discovered by Dekker et al. This study was based on microarray analysis of HUVECs exposed to steady laminar flow (25 dyne/cm²) for 24 hours (h). Dekker et al. revealed an up-regulated gene expression of at least 5-fold for KLF2, whereas maximum levels were reached within 2h.
The expression decreased slowly after 2h and reached almost basal levels at 24h. Interestingly, compared to steady laminar flow, pulsatile flow resulted in an additional 3-fold increase in KLF2 expression. The maximum was reached after 6h and remained constant from then on. In contrast to its induction by flow, the expression of KLF2 in vitro was actually repressed by TNF-α. Nonradioactive KLF2 mRNA in situ hybridisation on the thoracic aorta demonstrated a heterogeneous expression pattern. Throughout the straight sections of the aorta KLF2 was uniformly expressed, but was absent in the areas where a branch physically disconnected from the larger aorta. The effects were explained by differences in shear stress, concluding that the TF is exclusively induced by flow (43). SenBanerjee et al. confirmed the finding and reported a 5-fold upregulation of KLF2 when exposed to laminar shear stress of 10 dyne/cm² within the first 24h. They showed that upon exposure to the inflammatory cytokine IL1β, KLF2 expression was inhibited, similarly as seen in the response to TNF-α (45).

The effect of steady flow versus (vs.) pulsatile flow on KLF2 expression in dependence of shear magnitude was investigated by Dekker et al. in 2005. They revealed a biphasic expression, where the expression of KLF2 remained similar to static when exposed to less than 5 dyne/cm². For steady flow a shear stress of 15 dyne/cm² was needed to obtain maximum expression levels of 15-fold in comparison to static, while 30 dyne/cm² of pulsatile flow could induce KLF2 36-fold. No effect was observed in response to uniaxial cyclic strain (101).

As a continuation of the in vivo studies from 2002, Dekker et al. fixed a flow restrictive collar around the carotid arteries of ApoE (-/-) mice. Using in situ hybridisation they could already after 2 days observe increased KLF2 mRNA levels of 5-, to 30-fold in the intercollar region where flow was enhanced. In the proximal-to-collar sections low KLF2 expression levels were found. These findings were in line with increased eNOS protein and therefore showed a correlation of increased eNOS with increased KLF2 expression in dependence of flow in vivo (101).

Van Thienen et al. performed time course studies of KLF2 and eNOS mRNA expression with quantitative polymerase chain reaction (qPCR) and immuno-fluorescence for protein detection. The study revealed that KLF2 mRNA levels varied within the 7 days of observation, while eNOS expression constantly increased (37).
Much earlier in 2001, McComerick et al. performed a microarray assay of HUVECs exposed to 25 dyne/cm² for 6h or 24h. They identified 32 genes, which were up-regulated by more than 2-fold. Amongst these were KLF4 (6h at 4.56 ± 0.69 and 24h at 2.78 ± 0.54) as well as argininosuccinate synthase 1 (ASS1) (6h at 2.41 ± 0.21 and 24 h 3.04 ± 0.88), while neither eNOS nor KLF2 were detected (44).

As highlighted above, the research focused mainly on KLF2 shear-dependent expression until Hamik et al. in 2007 performed functional studies on KLF4 expression in vitro as well as in vivo. qPCR analysis of HUVECs exposed for 24h to arterial shear stress of 20 dyne/cm² revealed more than 20-fold upregulation of KLF4 and approximately 10-fold upregulation in response to venous shear stress of 2 dyne/cm². The KLF2 levels for both shear regimes were significantly less, while eNOS as well as TM were highly expressed.

In summary, KLF2 and KLF4 TFs have been found to be both induced by high laminar shear stress, while their specific roles remain unexplored.

1.3.2.2.2 Myocyte enhancer factor-2

To understand the mechanisms of shear-dependent KLF2 and KLF4 expression, their promoter regions were examined and Kumar et al. discovered an A/T-rich myocyte enhancer factor-2 (MEF2) binding site on KLF2 (102). As previously described, MEF2 was identified as a downstream target of the mechanosensitive MAPK pathway and it has been implicated as a regulator of endothelial biology (103). Laminar shear stress triggers a signalling cascade through endothelial surface mechanosensors, which propagates through a MEK5-ERK5-MEF2 cascade. Parmar et al. confirmed these findings in flow studies with HUVECs. They infected these cells with a control adenovirus or a virus expressing a dominant negative MEF2A mutant (MEF2ASA). After 24h the MEF2ASA protein blocked the function of all four MEF2 family members by preventing transactivation at bound MEF2 sites. MEF2ASA, while having no effect on basal KLF2 expression, dramatically abrogated the upregulation of KLF2 observed under flow (39).

Villarreal et al. demonstrated in a microarray study the dependence of atheroprotective shear stress-induced KLF4 expression on the MEK5/MEF2 signalling pathway. They claimed ERK5-independent regulation of KLF4 by MEK5 as ERK5 siRNA did not affect its induction in their experiments (104).
This finding was only partially confirmed by Ohnesorge et al., whose studies support the observation that KLF4 mediates, at least to some degree, the gene expression in dependence of ERK5. Inhibitory RNA (RNAi) experiments were performed, showing that siRNA against MEK5 strongly decreased simvastatin-induced KLF4 mRNA expression. Consistently, short hairpin RNA (shRNA) induced depletion of ERK5 and blunted the effect of simvastatin to induce KLF4 mRNA and protein expression, demonstrating that ERK5 activation is indispensable for statin-induced KLF4 induction. They also confirmed the expression of a variety of functionally relevant genes (105).

1.3.2.2.3 Phosphatidylinositol 3-kinase

Huddleson et al. identified a 62 bp shear stress response region in the KLF2 promoter containing a 30 bp tripartite palindrome motif. They demonstrated with electrophoretic mobility supershift and chromatin immunoprecipitation assays the binding of P-300/cAMP-response element-binding protein-associated factor (PCAF) and heterogeneous nuclear ribonucleoprotein D to the KLF2 promoter in flow conditions. These are components of a shear stress-specific regulatory complex that functions in a phosphatidylinositol 3-kinase (PI3K)-dependent pathway to acetylate histones. The resulting chromatin remodelling allows then the binding of the general transcription machinery. This signalling cascade provides a molecular mechanism for the flow-dependent expression of KLF2 (106).

In vitro studies of van Thienen et al. demonstrated that flow-induced KLF2 mRNA, in comparison to statin-induced KLF2 mRNA, is dependent on signalling involving PI3K. They inhibited PI3K with the compound LY294002 and observed a long-term stabilising effect on ∼50% of KLF2 mRNA, similar to the effect observed for shear stress (37).

1.3.2.2.4 Statins

Statins are a class of drugs that can lower cholesterol levels by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), which plays a central role in the production of cholesterol in the liver. They have been shown to possess vasodilatory, immunosuppressive, anti-thrombotic and cholesterol-lowering capabilities. Studies implicate that the inhibition of isoprenoid synthesis (geranyl-geranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate (FPP)) mediates the effects of statins on GTP-binding proteins (e.g. Ras and Rho).
The induction of KLF2, even under static conditions by statins, has been shown by Kumar and co-workers. They could observe activity in HUVECs within 8h after drug treatment, which remained elevated for at least 24h. They also showed that this induction resulted from MEF2 binding to the KLF2 promoter site (107, 108).

As previously mentioned, administration of simvastatin can trigger ERK5 phosphorylation and induction of KLF4 mRNA in a concentration-dependent manner. Up to 14-fold could be observed with a dose of 10 µM. Mechanisms on how exactly statin induced ERK5 activation were not revealed. The administration of statins has hence been a widely used tool to up-regulate KLF2 and KLF4 without the need for exposure to shear stress.

1.3.2.2.5 Cytokines

The cytokine TNF-α, a major pro-inflammatory mediator, has been shown to induce activation of NFκB, JNK and p38-MAPK signalling pathways with an optional capacity to induce apoptosis (109). Administration of TNF-α leads to strong suppression of KLF2 in ECs. Kumar et al. revealed a mechanism involving a MEF2 directed inhibitory complex. They found that the subunit p65 of the NF-κB complex can cooperated with histone deacetylase-4 (HDAC 4) to associate with MEF2 on the KLF2 promoter, thereby inhibiting transcription (102). This presents an interesting finding as MEF2 alone, as stated above, is one of the main KLF2-activating factors.

Downregulation of KLF2 has also been observed in response to IL-1β, where already a 3h administration resulted in 4.7-fold decrease of KLF2 expression. For both cytokines the response stimulated activation of VCAM-1, E-selectin and ICAM-1. Functional in vitro flow assays demonstrated that cell attachment and rolling were markedly enhanced in inflamed endothelial monolayers (45, 102).

Interestingly, KLF4 has been shown to be induced in the presence of TNF-α, IL-1β and IFNγ and the effect maintained for at least 24h. Stimulation with cytokines induced expression of Tf, plasminogen activator inhibitor-1 (PAI-1) and VCAM-1, each acting directly or via recruitment of inflammatory cells to confer a pro-coagulant phenotype to ECs (97, 110).
1.3.2.2.6 Other factors

Several other factors have been described to affect KLF2 and KLF4 expression either independent or in combination with flow. For example, Huddleson et al. postulated a general role for nucleolin in gene regulation of KLF2. They reported that fluid flow-induced interaction of nucleolin with the p85 regulatory subunit of PI3K was needed to express active KLF2 (111). Cowan et al. demonstrated in protein immunoblots the dependence of Wnt signalling, which induced VE-cadherin, β-catenin and KLF4 (112).

1.3.2.3 Regulation of key mediators of vascular tone by KLFs

The vascular tone in the healthy endothelium is maintained by vasodilating key molecules such as eNOS (113) and c-type natriuretic peptide (CNP) (114). As previously mentioned, both are induced by laminar flow in physiological conditions. In pathological conditions of disturbed flow, vasoconstrictive factors such as ET-1, adrenomedullin and angiotensin-converting enzyme (ACE) are predominantly expressed. KLF2 has been shown to be the mediator of vasodilatory and vasoconstrictive molecules, hereby controlling vasoreactivity and vascular tone. The first indications derived from studies of SenBanerjee et al., which showed that KLF2 could induce expression and protein levels of eNOS. Direct interaction of KLF2 by binding to the eNOS promoter side was required. Furthermore, this activation function was mediated by KLF2 and its recruitment of the coactivator CBP/p300 to the eNOS promoter. Binding of CBP/p300, a cofactor of NF-κB, also repressed the induction of its inflammatory signalling cascade through direct competition (45).

The KLF2-mediated induction of CNP, an agonist for the B-type natriuretic receptor (NPRB) involved in VSMC relaxation, was shown by Parmar et al.; microarray and qPCR-derived data demonstrated the direct relationship for KLF2 overexpression and siRNA silencing conditions (39).

The expression of ASS1, a limiting enzyme in eNOS bioavailability (115), has been shown to be KLF2-dependent. In contrast to this, Dekker et al. demonstrated that in KLF2-silenced conditions the vasoconstrictive peptides ET-1, adrenomedullin and ACE are highly expressed (101, 116).

Similar observations have been made by Hamik et al. investigating the role of KLF4 in endothelial biology. They confirmed the induction of vasoprotective eNOS by KLF4 and transcriptional profiling revealed also ASS1 as well as ET-1 as direct targets (104, 110).
1.3.2.4 Function of KLF2 and KLF4 in the inflamed endothelium

ECs respond to inflammatory stimuli by inducing the expression of chemokines and adhesion molecules that recruit immune cells to the blood vessel wall. Overexpression of KLF2 results in severe reduction of VCAM-1 and E-selectin expression. These mediate the rolling and adhesion of lymphocytes, monocytes, eosinophils and basophils to the vascular endothelium. VCAM-1 has also been implicated to play a role in leukocyte-endothelial cell signal transduction (78). Functionality of KLF2-transduced cells was validated in an in vitro flow assay, which demonstrated that cell attachment and rolling were markedly reduced in TNFα-stimulated endothelial monolayers (45). Mechanistic studies by SenBanerjee et al. showed that these effects were caused by inhibition of the pro-inflammatory NF-κB pathway. KLF2 competes for CBP/p300, a cofactor of p65, and thereby reduces NF-κB-dependent transcriptional activity and expression of VCAM and E-selectin.

Furthermore, repression of the AP-1 related pathways by KLF2 has been published by Boon and co-workers. It was demonstrated that KLF2-induced Smad7, subsequently suppressing Smad2 phosphorylation and Smad3/4-dependent transcriptional activation. Additionally, KLF2 was shown to inhibit the transforming growth factor (TGF)-signalling cofactor (117). KLF2 overexpression studies in human ECs showed that KLF2 reduced levels of activating transcription factor (ATF) 2 by inhibiting nuclear translocation (38). The protein ATF2 is known to bind the cAMP-responsive element (CRE), which forms a homodimer or heterodimer with AP-1 and stimulates pro-inflammatory gene expression pathways. These observations suggest a potential role of KLF2 as an anti-inflammatory regulator in blood vessels.

Although KLF4 expression in the vascular endothelium was firstly described in 1998 (118), its function was explored much later. Methe et al. demonstrated that KLF4 overexpression in human ECs significantly reduced TNFα-induced E-selectin and VCAM-1 expression. Additionally, the inhibition of c-reactive protein (CRP), PAI-1 and IL-6 has been demonstrated. Shen et al. highlighted a mechanism in which KLF4 binds to kallistatin, a plasma protein that exhibits pleiotropic effects in anti-inflammation. They showed that kallistatin modulated inhibition of TNFα-induced NF-κB activation, as well as VCAM-1 and MCP-1 expression. The inhibition of these various inflammatory mediators suggests that KLF4 plays a regulating role in endothelial activation (110, 119, 120).
1.3.2.5 Role of KLFs in endothelial thrombosis

It has been shown that KLF2 can regulate key factors of endothelial coagulant gene expression and function. Lin et al. demonstrated that KLF2 induces TM, an EC surface marker that acts as a cofactor in the thrombin-induced activation of protein C. KLF2 overexpression also resulted in downregulation of genes involved in fibrinolysis. PAI-1, which functions as the principal inhibitor of tPA and inducer of fibrinolysis, was inhibited. In addition, tissue factor (Tf), which enables cells to initiate the blood coagulation cascades and functions as the high-affinity receptor for the coagulation factor VII, was reduced (47).

\(\text{siRNA-mediated silencing of KLF2 reduced anti-thrombotic gene expression, while inducing the expression of pro-coagulant factors. In a functional assay it was demonstrated that gene silencing of KLF2 significantly reduced blood clotting times and flow rates. Mechanistically, KLF2 inhibited protease-activated receptor 1 (PAR-1) expression and, as a consequence, thrombin-mediated NF-\kappa B nuclear accumulation and DNA binding (47, 121).}\)

Similar observations have been made for the anti-thrombotic function of KLF4 by Hamik et al. They discovered that the anti-thrombotic endothelial surface was maintained by KLF4-induced upregulation of TM and eNOS promoter studies confirmed direct binding of KLF4 to both promoters (via the zinc-finger domain) and transactivation or transrepression of other transcriptional regulators (via the putative protein-interaction domain). Overexpression of KLF4 also markedly inhibited the TNF-mediated upregulation of NF-\kappa B-dependent TF activity. It was also demonstrated that KLF4 essentially abrogates the adhesion of the monocytic cell line THP1 to the endothelial monolayer in an \textit{in vitro} assay (110).

1.3.2.6 Regulation of endothelial proliferation, migration and angiogenesis

Angiogenesis is the physiological process of blood vessel growth in normal development and tissue repair, but also a pathological feature in chronic inflammation and cancer. Several studies have highlighted the anti-angiogenic effects of KLF2. Overexpression of KLF2 results in reduction of angiogenic VEGF. By competing with specificity protein 1 (SP1) for binding the VEGFR2 promoter it inhibits the expression of key VEGF receptor genes. The endothelium remains inactivated, resulting in reduced cell proliferation and less Ca2+ flux (122). In standard wound assays of HUVECs growing on a fibronectin matrix, Dekker et al. could observe that wounded monolayers transduced with KLF2 migrated slower in comparison to wild type (WT). Additional gene profiling studies revealed that semaphorin-3F, a factor previously demonstrated to strongly inhibit tumour cell migration, was potently induced by KLF2 (116).
Transcriptional profiling studies with KLF4–transduced primary HUVEC revealed potentially novel KLF4-specific targets including forkhead box O1 (FOXO1), VEGF, angiopoietin 2 (Ang2) and thrombospondin 1 (THBS1). These genes play important roles in angiogenesis and cell proliferation, but were not further assessed for their functionality (104).

KLF6, in complex with SP2, has been shown to be required for farnesoid X receptor-induced endothelial cell migration. Das et al. firstly observed that KLF6 in conjunction with SP2 negatively regulates the expression of MMP9 involved in the breakdown of extracellular matrix. Upregulation of small heterodimeric partner (SHP) through activation of farnesoid X receptor disrupted the KLF6-SP2 interaction. MMP-9 was induced and led to enhanced cell chemotaxis as measured with a modified Boyden chamber. Das et al. therefore identified a mechanism for antagonising Sp/KLF protein repression function via SHP, which regulates endothelial cell motility (123).

1.3.2.7 Role of KLFs in endothelial barrier function

Thrombin and hydrogen peroxide or inflammatory processes can induce intercellular gap formation by disrupting cell-cell adhesion proteins (124) and changing the cytoskeleton (125), thus resulting in enhanced leakage formation. KLF2 as well as KLF4 have been reported to play a role in the regulation of endothelial barrier function.

In vivo studies by Lin et al. have shown that heterozygous KLF2 (+/-) mice exhibited a significantly higher degree of vascular leak in comparison to homozygous WT mice. They furthermore confirmed an endothelial protective function of KLF2 in vitro: KLF2 overexpression attenuated the thrombin-induced fluorescein isothiocyanat (FITC) leakage in endothelial monolayers, while in KLF2-silenced conditions the formation of gaps increased. The mechanism for these observations has been ascribed to the loss or gain of the tight junction protein occludin, which has been shown to be KLF2-dependent (126).

Depletion of KLF4 in mice significantly augmented both lung polymorphonuclear leukocyte sequestration and water content, which was coupled to reduction in VE-cadherin expression and endothelial barrier integrity. Cowan et al. demonstrated also in vitro the binding of KLF4 to the VE-cadherin promoter to activate protein expression required for maintaining a restrictive endothelial barrier. They further hypothesised that KLF4 makes adherent junction barriers more resistant to inflammatory stimuli and serves to prevent vascular leakage (112).
1.3.2.8 KLFs and miRNAs

MicroRNAs (miRNAs) are endogenously expressed small non-coding RNAs that regulate gene expression on the posttranscriptional level. During the last years, miRNAs have emerged as key regulators of several physiological and pathophysiological processes such as regulation of various aspects of angiogenesis, including proliferation, migration and morphogenesis of ECs (127).

In a recent study Hergenreicher et al. investigated several miRNAs induced by KLF2; miR-126, miR-143 and miR-145 have been identified amongst these. Promoter studies confirmed that direct binding of KLF2 resulted in target miRNA upregulation. Especially the expression of the miR-143/145 cluster was highly induced. Functional studies demonstrated that extracellular vesicles secreted by KLF2-transduced or shear-stress-stimulated HUVECs were enriched in miR-143/145 and could control target gene expression in co-cultured SMCs. They furthermore validated in vivo that vesicles derived from KLF2-expressing ECs also reduced atherosclerotic lesion formation in the aorta of ApoE (-/-) mice. This study therefore revealed a new KLF2-driven mechanism of communication between ECs and VSMCs (128).

In 2012 Fang et al. published a paper demonstrating the importance of miRNAs in the regulation of KLF expression. In silico studies revealed binding sites of several miRs (26a, 26b, 29a, 92a and 103) to KLF2 and miR-92a to KLF4 promoter regions. Of these, only miR-92a knock-down and knock-in caused a response of KLF4 and KLF2 expression in human arterial ECs. They confirmed the 5’GUGCAAU 3’UTR sequence to be an evolutionarily conserved miR-92a binding element on both genes, resulting in reduced expression. The functionality was confirmed in a leukocyte-endothelial interaction assay, where knock-down of endogenous miR-92a desensitised TNF-induced endothelial inflammation and leukocyte adhesion principally through KLF4 induction (129).
To summarise the information on KLF2 and KLF4 regulation and their targets the important key factors are compiled in Figure 9 and Figure 10.

Figure 9: Overview of factors that can activate and inhibit KLF2 expression and protein activity as well as its functional role as activator and repressor of several molecular pathways and their key mediators. Inhibition →, activation ←. List of abbreviations can be found on page 10.
1.3.3 Interaction between KLF2 and KLF4

The previous section provided an overview on the current data and scientific understanding of KLF2 and KLF4 signalling. Both genes are induced by atheroprotective shear stress as components of the ERK5/MEF2-dependent signalling pathway, but their response to inflammatory stimuli differs. It has also been shown that both TFs function partially redundant to control endothelial homeostasis, most importantly, their common functions as anti-inflammatory, vasodilatory and anti-thrombotic mediators. Atkins et al. showed that atherosclerotic lesions have increased by approximately 37% in a heterozygous KLF2 (-/+), mouse model crossed with an ApoE (-/-) mouse, while no difference in eNOS could be determined. They explained this effect by the significant induction of KLF4 mRNA, which might have compensated for the loss of KLF2 (130). The mechanistic and functional studies of Hamik et al. (110) then questioned the role of KLF2 as the unique key regulator of endothelial inflammation. Successive studies had to address the role of both KLFs in their scientific context so that individual targets, predominantly regulated by only one of the KLFs, could be revealed. In a genomic profiling approach, Villarreal et al. demonstrated that 42.4% of the genes regulated by KLF2 were shared transcriptional targets with KLF4.
As both KLFs have been indicated as downstream targets of the MAPK pathway, the overlap in a MEK5 dependent manner was with 59.2% even more pronounced (104). This significant degree of mechanistic and functional conservation is not surprising as a phylogenetic study revealed a close relationship of KLF2 and KLF4 and the common canonical binding site of CACCC promoter elements supports this finding (90).

In the context of signalling in other tissues, members of the KLF family have also been shown to act synergistically or as agonists. An important example in developmental biology and differentiation is the study of Jiang et al., who elucidated the role of several interacting KLFs in the self-renewal of embryonic stem (ES) cells. Systematic pairwise gene silencing of KLF2, KLF4 and KLF5 did not cause morphological changes, while simultaneous depletion of all three KLFs led to fibroblastic differentiation and consequently disruption of the self-renewal capacity. Administration of RNAi resistant cDNAs coding for KLF2, KLF4 and KLF5 could rescue the induced differentiated phenotype. Immunoprecipitation experiments revealed Nanog as the common target of the three KLFs and two binding loci upstream of the coding region were identified (131). Studies of Nanog showed that it can induce pluripotency in mouse ES cells (132). Jiang et al. demonstrated further that the Nanog enhancer is dependent on the CCCCACCC binding motif recognised by the three KLF proteins. The KLFs also share many common targets of Nanog, emphasising their functional redundancy.

In conclusion, the authors revealed that a KLF circuit was integrated into the Nanog transcriptional regulatory network in ES cells with common target genes. They also suggested that the coordinated regulation of KLFs may, at least in some parts, be mediated through autoregulatory loops (131). The data implicates that for studies in the vascular endothelium KLF2 as well as KLF4 have to be taken into consideration. They act at least to a certain degree cooperatively, but may also exert opposing effects in the regulation of the same process. As shown for the regulation of stem cell differentiation, only a close network of several KLFs was functionally significant. It could therefore be possible that such a circuit may exist also in the endothelium.

1.3.4 Porcine KLF gene family

Several studies have been performed to unravel the relationship between KLF family members. For human KLFs significant amino acid homologies beyond their DNA binding regions exist only between very closely related family members and even then it is quite low (90, 133).
As the experiments in this study were performed with porcine aortic ECs (PAECs) the structural differences to the human genome as well as phylogenetic analysis within the porcine KLF family have to be considered. Chen et al. compared the structure, mapping and evolutionary conservation between the human and porcine KLF genes and proteins. They confirmed the existence of 17 porcine KLFs and demonstrated that the proteins and their corresponding human homologues have in average 88.5% identity over the entire sequence and even 98.2% are conserved in the zinc finger domains (134). The DNA binding region is very similar to the three zing finger motif described in section 1.3.1. For KLF2, KLF4 and KLF6 the zinc finger domain is even 100% identical as shown in Table 3.

Table 3: Sequence characterisation of the porcine KLF family. Adapted from Chen et al. (2010).

<table>
<thead>
<tr>
<th>KLF</th>
<th>Synonyms</th>
<th>Chromosome location</th>
<th>Size protein (aa)</th>
<th>Identity of porcine and human orthologues (%)</th>
<th>Gene bank accession nr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLF1</td>
<td>EKLF</td>
<td>2q13 (2q21-q24)</td>
<td>367</td>
<td>78.3</td>
<td>EU669072</td>
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<tr>
<td>KLF2</td>
<td>LKLF</td>
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<td>95.5</td>
<td>EF095721</td>
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<tr>
<td>KLF3</td>
<td>BKLF</td>
<td>8p21.2 (8p12-p21)</td>
<td>347</td>
<td>95.7</td>
<td>EU669073</td>
</tr>
<tr>
<td>KLF4</td>
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<td>1q211 (1q28-q29)</td>
<td>477</td>
<td>93.5</td>
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<tr>
<td>KLF5</td>
<td>IKLF</td>
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<td>457</td>
<td>95.5</td>
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<tr>
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<td>359</td>
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<tr>
<td>KLF9</td>
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<tr>
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<td>18q13 (18q24)</td>
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<td>KLF15</td>
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<td>(6q35)</td>
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cDNA sequence analysis of KLF4 revealed that the transcript contains an alternative splicing side in the coding region. Depending on the inclusion or exclusion of intron three, two isoforms of KLF4 exist. The shorter KLF4 isoform A with 477 amino acids (aa) and the isoform B with 510 aa are known. It has also been shown that the chromosomal location of porcine KLFs is highly conserved with the human loci. When the complete aa sequences of KLFs are aligned, five related subgroups can be identified, which coincide with the human classification: the first group consisting of KLF1, 2, 3 and 17, the second of KLF5, 6 and 7, the third of KLF3, 8 and 12, the fourth only of KLF15 and the fifth of the remaining KLFs.

The classical Cys$_2$His$_2$ zinc finger domain (see section 1.3.1), as well as the linker sequence, are highly conserved between all KLFs. Specific contact with the DNA occurs most likely with the amino acids KH(A/S) within the first, RER within the second and RH(K/L) within the third zinc finger. DNA binding sites of related GC boxes and GT/CACCC sequences are largely conserved, with slight differences that also mediate affinity (134).

### 1.4 RNA interference technology

RNAi is a biological process in which the experimental introduction of double stranded RNA (dsRNA) that are homologous in sequence to the target mRNA, results in posttranscriptional gene silencing of this target. Historically, the first successful RNAi experiments have been performed with Caenorhabditis elegans and Drosophila to investigate functional genomics. Fire et al. in 1998 firstly showed that injection of siRNA caused potent and specific interference and that the effects of this interference were evident in both the injected animals and their progeny (135). Three years later, Elbashir et al. confirmed these findings also for mammalian cell lines including human embryonic kidney 293 and HeLa cells (136). Their approach using 21-nucleotide siRNA duplexes to specifically suppress expression of endogenous and heterologous genes has revolutionised the study of gene function and opened the field of gene therapy. The following paragraph describes the biological mechanism of RNAi, highlights some of its applications, describes rationales of the siRNA design and briefly discusses current limitations of the technique.

#### 1.4.1 Biological mechanism and applications

RNAi is an RNA-dependent gene silencing process initiated by short dsRNA molecules that are introduced into the cytoplasm of a cell. Several methodologies such as lipid based transfection, antibody conjugation, electroporation and viral introduction have been developed for efficient delivery. A detailed description of these methods would be beyond the scope of this thesis.
Li et al. have published an excellent review on nucleic acid delivery, which is suggested for further reading (137). The dsRNAs are firstly cleaved into 21–23 bp fragments by the enzyme dicer with two-nucleotide 3' overhangs at each end. Dicer then helps to direct the catalytically active RNase argonaute to the guide strand of the dsRNA.

One strand, which has its 5' end oriented to the less stable extremity of the duplex, is then incorporated into the RNA-induced silencing complex (RISC). The other strand is degraded. The first step in the assembly of a cleavage-competent RISC occurs with the incorporation of accessory proteins and the dissociation of dicer from the complex. With the bound single siRNA, active RISC can then recognise complementary mRNA sequences in the cytoplasm and trigger their degradation (138-140). This mechanism therefore leads to post-transcriptional suppression of target gene expression (Figure 11).

![Model of siRNA-guided post-transcriptional regulation of gene expression. Long dsRNA is introduced to a cell and the enzyme dicer cleaves the dsRNA into 21–23 bp fragments (siRNA) with 2 nucleotide 3' overhangs. Following cleavage, RNA-induced silencing complex (RISC) loads, unwinds the siRNA and binds to the complementary target mRNA. Target mRNA is subsequently cleaved and therefore made non-functional. Derived from Karpala et al. (2005).](image-url)
The RNAi technology has been widely used to inhibit protein transcription at the mRNA level leading to transcriptional silencing of proteins. Besides their function in downregulation of gene expression and protein translation, siRNAs complementary to promoter sequences can also modulate gene expression by either inhibiting and/or activating gene transcription through chromatin remodelling (141). RNAi interventions do therefore present a strong tool in studying functional genomics and a promising application for gene therapy. Therapeutic applications of RNAi are currently under investigation in clinical trials for age-related macular degeneration, hepatitis and cancer (142).

1.4.2 Sequence design of potent siRNAs

When using siRNAs in gene-silencing studies a rational sequence design is one of the most important factors contributing to their functionality. Many studies have been performed and the following rules can be considered as golden standard for siRNA sequence selection. Reynolds et al. summarise these rules as follows (143):

1. siRNA targeted sequence length should be 19-21 nt
2. regions within 50-100 bp of the start codon and the termination codon as well as introns should be avoided
3. 4 or more nucleotide repeats should be avoided
4. GC content should be >30% and <60%
5. no repeats and low complex sequence
6. single nucleotide polymorphism (SNP) sites should be avoided

Once the target sequences have been selected, a blast homology search should be performed to avoid off-target-effects (OTEs) on other sequence. It is also important that negative controls are designed by scrambling the original siRNA sequences. Hereby it is advisable to use the same length and nucleotide composition, but with 4-5 bases mismatched to the original siRNA (143). Tuschl and his co-workers have investigated the impact of chemical modifications on siRNA potency. They discovered that 3´UU overhang modification can enhance or modify the pharmacologic properties of siRNAs (144).

Several freeware tools are available and some companies have commercialised siRNA design algorithms to identify optimal sequences, integrating the above mentioned rules for potent silencing. The methodology for selection of siRNA probes used in this study is further detailed in section 2.3.1.
1.4.3 Limitations

One of the key limitations in gene silencing with siRNA is their efficient transfer into the target cell cytoplasm. Different cell types are known to vary in siRNA uptake susceptibility and it is crucial to optimise the transfection method accordingly. In this process it is important to consider the cell viability as well. For potent inhibition the transfection efficiency has to be sufficient with a high percentage of viable cells after the treatment. Several side effects have been reported, resulting from the transfection procedure or siRNA molecules. One of these aspects is the activation of a non-specific viral defense mechanism. The interferon response is known to lead to an arrest of protein synthesis and non-specific mRNA degradation in the affected cells. Tuschl et al. have shown that this mechanism is activated by dsRNA greater than 80 bp in length (145).

Another challenge in the use of siRNA for gene silencing is the occurrence of OTEs. As previously described, the introduced siRNA molecules might cause specific or non-specific side effects. The later is caused by siRNA binding to unintended sequences, thereby modulating non-target gene expression. For the off-targeting mechanism to occur, a perfect complementarity between nucleotide position 2–7 or 2–8 (seed region) of the antisense strand and the 3´UTR of the transcript is necessary. Nielsen et al. showed that the presence of adenosine opposite to siRNA base 1 and of an adenosine or uridine opposite to siRNA base 9 enhanced off-target mRNA repression (146). Non-specific side effects, which have been shown to cause an immune and toxicity related response, are due to the RNAi probe itself or the delivery method. For clinical in vivo applications of siRNA it has been shown that modification with 2´-OMe of the second ribose from the 5´-end of the siRNA reduces some of the non-specific effects (147, 148). Despite these limitations, the RNAi approach for studying functional genomics, as shown in this work, presents a very potent tool as long as the probe design and delivery method are highly optimised.

1.5 Functional genomics and microarray genome analysis

Functional genomics aims to assign biochemical, cellular, and/or physiological properties to genes and gene clusters, thereby identifying gene interactions and regulatory mechanisms. Studying these dynamic biological processes and to a greater extend the entire response of a cell and an organism helps to gain insights to a variety of questions concerning physiology and pathophysiology. The understanding how genetic information leads to a given phenotype has important implications for the prevention and treatment of diseases.
A variety of molecular biology techniques is used to study gene function and gene interaction. One of the most powerful, but also difficult to evaluate due to its large data output, is the DNA microarray genome analysis. The following section discusses the principle, briefly touches on the data analysis methodologies and concludes with an example of microarray studies involving KLFs in the context of mechanobiology.

1.5.1 Microarray principles

An important tool in studying functional genomics is the DNA microarray technology, which is growing immensely since its discovery in the early 1990s. Several types of microarrays have been developed based on the target material, which can be cDNA, mRNA, proteins, small molecules, tissues or any other material that allows quantitative analysis. In the scope of this thesis only the cDNA based technologies are reviewed, which have since the development of high throughput applications expanded the field to three major genetic explorations: the genome-wide analysis of gene expression, SNP genotyping and resequencing (149). Microarray technology has been used in many research fields such as the identification of new drug targets (150) and toxicology measures (151), discovery of biomarkers (152), genome annotations (153) and molecular profiling (154).

There are two distinct types of assays commonly used in determining the level or volume at which a certain gene is expressed. These are cDNA microarrays and oligonucleotide microarrays. They differ in the technique applied for spotting the DNA sequences on a solid surface, the surface material as well as the type and the length of oligonucleotides used (150).

In microarrays, cDNA from fully sequenced clones, collections of partially sequenced cDNAs known as expressed sequence tags, or randomly chosen cDNAs from any library of interest are printed on a glass surface using computer controlled high-speed robotics (155).

Oligonucleotide arrays have been developed by Fodor et al., who could synthesise a chain of 25–mers, using light-directed, spatially addressable parallel chemical synthesis, which was immobilised on a surface (156). The concept was commercialised and nowadays the most commonly used Affymetrix GeneChip technology allows high-density in situ oligonucleotide synthesis of over 6.5 million features (half are negative controls) on a 5 µm scale.
Other companies such as Agilent, CodeLink, Combimatrix 18, Illumina and Nimblegen have developed different synthesis techniques, most with fewer features per array. The manufacturing process used for each array determines the number and quality of features achieved, influences the flexibility of the platform and controls the cost (157).

In principle, five basic steps are involved in each array: RNA extraction, sample preparation and labelling, hybridisation, scanning and data analysis by computational methods. As the genome analysis of porcine ECs in this work has been conducted using Affymetrix GeneChips, the workflow of standard steps is described on the basis of this example.

At first, the total RNA of the target samples has to be isolated, containing mRNA that ideally represents a quantitative copy of genes expressed at the time of sample collection (e.g. experimental sample and reference sample). The overall success of any gene-profile microarray experiment depends on the quality of the RNA and as described in section 2.5.2 all RNA samples used in this study had an integrity number of 10 or higher. mRNA extracted from the sample material is then converted into cDNA, using a reverse transcriptase enzyme, purified, fragmented by DNase, labelled with biotin and used for hybridisations. The commercially manufactured GeneChips are modified with a stable surface layer of hydroxyalkyl groups. Linker molecules with photolabile protecting groups are then attached covalently to this layer to form a surface that may be spatially activated by light. A photolithographic mask, which is aligned with the quartz wafer, can then spatially and temporarily activate the terminal hydroxyl groups. To initiate the oligonucleotide synthesis, a solution containing deoxynucleoside phosphoramidite monomers with a light-sensitive protecting group is flushed over the surface and the nucleosides attach to the activated linkers (149). Deprotection and coupling steps are repeated until all of the full-length probe sequences, usually 25-mers, are completed.

These so called features are only 5 µm apart so that 6.5 million different ones can fit on the same array. For a genome-wide analysis as conducted in this work, total genomic DNA is digested and ligated to a common primer followed by PCR amplification. The amplicons are then fragmented and labeled as described previously for other DNA targets. The Affymetrix porcine genome array provides comprehensive coverage of the sus scrofa transcriptome as it contains 23,937 probe sets that interrogate approximately 23,256 transcripts from 20,201 sus scrofa genes. The sequence information for this array has been selected from public data sources including UniGene GenBank mRNAs, porcine mitochondrial sequences and rRNA sequences.
The third step, hybridisation of the cDNA fragments to the array features, takes place over 16h in a hybridisation oven with 40-50°C. Afterwards the unhybridised sequences are removed by repetitive washes and the bound target molecules are marked with a fluorescent streptavidin–phycoerythrin conjugate. The fluorescence emission is captured by a laser scanner, which can resolve more than 65,000 different fluorescence intensities. These are then converted to a numerical value and stored as pixel values that comprise the image data file (.dat file).

1.5.2 Microarray bioinformatics for data analysis

To robustly assign pixels to features a process called feature extraction is done and the intensity of each feature is calculated and stored in a .CEL file. Analysis tools can then be used to detect sequence variations or to differentiate gene expression levels of transcripts from these files. For gene expression different algorithms have been employed to summarise multiple probe intensities into an aggregate signal estimate that is correlated to the relative abundance of the transcript in the experimental sample (149).

Further analysis of microarray gene expression data typically involves three different steps of preprocessing: differential gene expression analysis (DGEA), clustering and functional annotation as summarised in Figure 12.

1.5.2.1 Normalisation, background correction, differential gene expression and functional analysis

For an unbiased and true measurement the background of the intensity measures has to be corrected. The raw probe intensity for a perfect match (PM) or a mismatch (MM) is the sum of a true hybridisation signal, specific cross-hybridisation signal, non-specific binding signal, and some system noise. Background consists of everything but the true signal and subtraction of the MM probe intensity is considered as an accurate method. There are several software packages either provided by the array manufacturer or available as open source, such as The Bioconductor suite, which perform background correction, normalisation and further analysis. Affymetrix provides a Robust Multi-array analysis (RMA) in Expression Console software for these steps. It ignores MM altogether and relies solely on correcting PM values based on stochastic models for the global distribution of probe intensities, resulting in corrected gene expression values. Furthermore, preprocessing can also include the detection and removal of outliers and filtering. For example, filtering genes without annotation or genes that show the least amount of variation across all samples reduces the number of statistical tests to be used (158).
The second stage in microarray data analysis is to determine the genes which are significantly over-, or under-expressed when compared against control data. This involves the annotation of genes, which is at least for the human genome, a software-guided process. For mapping of non-human genomes, the annotation has partially been published by research groups, like for the porcine genome by Tsai et al. (159). Nevertheless, it is almost inevitable to build a custom annotation base combining annotation data from different sources. After annotation several statistical testing procedures, such as LIMMA, significance analysis of microarrays (SAM) or analysis of variance (ANOVA) are employed to quantify differential gene expression. For further information there are several reviews available, which in detail discuss the methodology of strategies (160, 161). The false discovery rate (FDR) and adjustments for multiple testing by the Benjamini-Hochberg method, which is a less conservative procedure for comparison than the Bonferroni correction, are usually applied to additionally control the number of false positives (162).

Using the per comparison error rate (q value) might be a complimentary approach for biological data, because it assumes dependency between genes as they form signalling pathways in tight interaction (163).

As stated above, functional genomics aims to understand functional relationships amongst different genes. By clustering genes according to certain criteria such as their gene expression levels in different biological conditions, genes in the same cluster are more similar to each other than to those in other clusters. This computational tool can help to unravel insight on gene interaction, pathway analysis and even network compositions.
There are many different clustering algorithms that compress high-dimensional array data to simplify data management without losing important information. The most widely used techniques in analysis of gene expression data are hierarchical clustering (164), K-means clustering (165) and self-organised maps (SOMs) (166). SOMs address an important aspect of visualisation with the objective to represent all data points in the source space by points in a colour-coded target mosaic map, where the distance and proximity relationships are preserved.

The detection and clustering of differentially expressed genes in certain biological conditions still does not reveal any information of their putative function and network relationships. Functional annotations are needed to better interpret the obtained results and a widely used database is Gene Ontology. It can relate newly acquired gene expression level data to known functional and partial pathway information in three distinct categories of molecular function, biological process and cellular component (167). The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a compilation of seventeen databases and associated software that combines current knowledge on molecular interaction networks in biological processes, genes and their proteins, as well as chemical compounds (168). These databases function as reference knowledge bases for biological interpretation of genomes and high-throughput molecular datasets through the process of KEGG mapping. The Database for Annotation, Visualization and Integrated Discovery (DAVID) consists of a knowledge base and five integrated, web-based functional annotation tool suites. It has been recently expanded by analytic features that provide investigators with additional means to explore and extract biological meaning from large gene lists. Biological information coverage is expanded by the integration of many public databases. Furthermore, the development of new algorithms can highlight the enriched and redundant relationships among many-genes-to-many-terms and dynamic visualisation within the most relevant KEGG and BioCarta pathways can be done (169).

Within the scope of this thesis, only a few methods for microarray data analysis, functional interpretation and visualisation have been covered, by far not a full representation of the software, statistical tools and web resources available (170-172). The degree of complexity and variety requires a detailed understanding of the data and the distinct features of analysis options. A key in exploring biological networks is to combine as many data and analyses as possible to compare different outcomes to truly rely on the data and the resulting biological implications.
Introduction

1.5.2.2 Limitations, noise and reproducibility

Even if the most suitable analysis method has been employed, microarrays have limitations, which can alter the outcome and it is crucial that investigators are aware of these. For example, the reproducibility of microarrays from in vitro experiments, as in this study, can differ due to the biological variability of the cell samples such as cell cycle stage, susceptibility to mechanosensation, different dynamics in mechanotransduction, cell passage and the unknown donor history. This inter-chip noise also includes variations in the batch reagents and the protocol such as background hybridisation levels (including cross-hybridisation), differential probe hybridisation properties, the laser system and dye binding variances (162). These errors can be reduced by repetition and replication of the measurements to obtain a number of samples, which can be analysed using standard statistical means. Nevertheless, there is a financial limitation to how many replicates can be assessed for one experiment. Although the advancements in technology and more competition have lowered the total price, the technique is still expensive (157).

Not to underestimate is also the role of the operator processing the samples. Changes in the obtained data can result from this variability, although the application of standard operating procedures (SOPs) should limit the influence of an individual. Intra-chip noise refers to possible deviations from the standardisation of the manufacturing process such as errors in the oligonucleotide synthesis or feature assembly for genome-wide assays. The intra-chip derivations cannot be ruled out by repetitions, but are kept to a minimum by quality control assurance.

Another limitation of the microarray technique is the availability of sequence information. Complementary binding of the probe to the target sequence is crucial, though small variations due to both polymorphism and error can be tolerated by adapting the hybridisation and washing protocols. In addition to the intra-platform variability as discussed above, the inter-platform variability also presents an important aspect as there are currently no accepted guidelines on standard techniques in data processing and it remains questionable if experiments can be compared across platforms. Dupuy et al. have addressed the issue of transparency in their recent review on microarray data from over 90 publications and suggest guidelines on statistical analysis and reporting. Some of these include that a clear objective of the study, which also has an influence on the sample characteristics, has to be stated. Furthermore, the acquisition of data should be detailed and the raw dataset made publicly available (173).
1.5.3 Microarray studies of endothelial cells

Reviewing last decade’s literature, the microarray technology has been used by many investigators to explore genomic changes of ECs in response to mechanical stimuli and the overexpression of genes involved in the downstream signalling cascade of the mechanoresponse. *In vitro* experiments were carried out with cells exposed to different shear stress regimes (39-41, 43, 44, 174-177), co-cultured with VSMCs (178) and/or, as mentioned above, virally transduced to over-express mechanosensitive genes (39, 116, 179, 180) and/or exposed to inflammatory cytokines (39, 176). In most of these studies venous derived cells from the umbilical cord were used. Just in two exceptions human aortic ECs (178) and human dermal microvasculature ECs (179) were considered. It is debatable if ECs from the vein reflect the same phenotype as arterial cells, especially in the context of investigating endothelial inflammation triggering the development of exclusively arterial plaque formation. The difficulty of receiving human arteries, in contrast to the fairly easy availability of umbilical cords, may be a valid argument for the selection. For this study nevertheless aortic ECs were preferred.

In addition to cell culture experiments, some groups have also studied changes of the endothelial transcriptome *in vivo* (82, 181-184). These investigations were mainly conducted in pigs, as these animals have similar hemodynamic features in comparison with humans. A common strategy to this approach is firstly the assessment of shear patterns in different geometrical parts of the main arteries. Through CFD flow patterns can be modelled and shear stresses extracted from images obtained through magnetic resonance imaging (MRI) or ultrasound. Secondly, after explantation of the tissue, patches from the endothelium of very distinct regions in the vasculature are scraped off. Through this combined approach it can be determined which shear regime the obtained cells have been exposed to *in vivo*.

As in this study the genomic changes of PAECs in response to repression of KLF2 and KLF4 in combination with high shear stress exposure were investigated, the following section reviews only briefly the literature findings involving differential expression on KLF2 and/or KLF4.

In 2001, McCormick et al. were the first group, who addressed the question how gene expression in HUVECs changes when exposed to laminar shear stress (25 dyne/cm²) for 6h or 24h in comparison to static culture. The microarray used contained only a restricted number of features, as the entire human genome was not deciphered until 2003.
Nevertheless, 52 genes were found to be differentially expressed, of which 32 genes increased, whereas the expression of 20 genes decreased upon exposure to shear stress. The increasing genes such as cytochrome P450, ASS1 and human pGL transporter are known as markers of atheroprotection. Interestingly, as mentioned in section 1.3.2.2, KLF4 was firstly shown to be shear responsive at both time points (at 6h 4.56 ± 0.69 and at 24h 2.78 ± 0.54). Genes that play a key role in the initiation of atherosclerosis were consistently down-regulated such as connective tissue growth factor, which mirrors some of the effects of TGFβ, ET-1 and MCP-1 (44).

Dekker et al. followed a different experimental approach by shearing HUVECs with either steady flow (25 dyne/cm²) or pulsatile flow (12 ± 7 dyne/cm²) for 24h and up to 7 days. 12 of 18,000 genes were identified with significantly altered expression (> 5-fold), amongst these also up-regulated eNOS, TM, cytochrome P450, Diaphorase 4, PECAM-1 and regulators of G-protein expression. KLF4 expression could not be detected, but the shear-dependent upregulation of KLF2 (4.9 fold ± 1.74) was shown for the first time. In line with the shear susceptibility theory, inflammatory and thrombogenic markers such as VCAM-1 and Tf were down-regulated (43).

Chiu et al. expanded the experimental conditions by additional administration of TNFα after exposure to static, high level shear stress (HSS, 20 dyne/cm²) or low level of shear stress (LSS, 0.5 dyne/cm²) for 24h. They concluded that pre-shearing of ECs decreased TNFα responsiveness of many pro-inflammatory, pro-coagulant, proliferative and pro-apoptotic genes, whereas it increased the responsiveness of some anti-oxidant, anti-coagulant and anti-apoptotic genes. LSS showed less regulatory effects than HSS on EC gene expression in response to TNFα. Superoxide dismutase 2-mitochondrial, Tf, inflammatory markers such as ICAM and E-selectin as well as KLF4 (2.08 ± 0.07) were up-regulated, while cytochrome P450, TM and bone morphogenic protein (BMP) 4 had decreased expression levels.

Comparing HSS exposure to TNFα, antioxidants such as eNOS, cytochrome P450, hemeoxygenase (HO) 1, TM and KLF4 (3.87 ± 0.50) were up-regulated, while as expected many inflammatory markers such as E-selectin, VCAM-1, TGFβ, Tf and cyclins were down-regulated. For the ratio of LSS to TNFα a generally reduced response could be observed, while KLF4 (2.85 ± 0.07) still showed significant upregulation (176).
In 2006, Dekker et al. wanted to further investigate the role of KLF2 and used a lentiviral system to constitutively over-express KLF2, without exposure to shear. They could determine KLF2-dependent overexpression of atheroprotected genes such as eNOS, TM, MAPK9 and Von Willebrand factor (VWF), but also NF-κB, while BMP4, IL8 and SERPINE1 were down-regulated. Interestingly, 42 endothelial signature genes including VEGF-receptor1/2, Tie-like receptor tyrosine kinase 1/2 (Tie1/2), platelet-derived growth factor (PDGF) β receptor, their ligands and VE-cadherin were not significantly affected by KLF2 (116).

Parmar et al. added complexity to the shear regimes by modelling an atheroprotective and atheroprone shear stress waveform to which HUVECs were exposed for 24h. They also over-expressed KLF2 with an adenovirus and investigated the response to IL-1β. KLF2 was highly up-regulated in response to shear stress with the atheroprotective waveform. Genes that were down-regulated by KLF2, but not modulated by IL-1β, included Ang-2 and ET-1, while nuclear factor of activated T-cells cytoplasmic-dependent (NFATc3), eNOS and ASS1 were down-regulated. Genes that were up-regulated by IL-1β and antagonised by KLF2 included IL-6, VCAM-1 and Tf. Synergistically up-regulated by both stimuli were PI3K, prostaglandin E synthase (PTGDS) 1 and VEGF. The group further demonstrated with other experiments that KLF2 expression is involved in the activation of a MEK5/ERK5/MEF2 pathway (185).

Convey et al. compared the transcriptional profile of HUVECs after 24h exposure to one of the three shear regimes: reversing shear stress (RVSS) (1 dyne/cm² ± 11 dyne/cm²), HSS (15 dyne/cm²) and LSS (1 dyne/cm²). They found that in comparison to static conditions the RVSS and LSS exhibited a similar pattern. Up-regulated genes were metallothionein 1 (MT1) and cell cycle genes such as cyclin B3, cytochrome P450 and syndecan 1, while KLF2 and natriuretic peptide receptor A (NPR1) were down-regulated. In the high shear scheme KLF2 expression was not enhanced, while KLF4, MEF2, and TGFα were significantly up-regulated (40).

Clark et al. over-expressed MEK5 in cultured human dermal microvascular ECs without exposure to shear stress. In comparison to WT cells, many atheroprotective genes such as TM, signal transducer and activator of transcription (STAT) 6, cytochrome P450, ASS1 and ICAM1 were up-regulated. A surprising finding was that KLF4 was extremely up-regulated (15.5-fold) and KLF2 (5.6-fold) only moderately. In comparison to other studies (44, 116) in the investigations from Clark et al. KLF4 was more responsive. As expected, amongst the down-regulated genes, cyclooxygenase (COX) 2, Tf, and ANG2 could be found (179).
In 2010, Villarreal et al. addressed for the first time the issue of partially overlapping roles of KLF2 and KLF4 in response to shear stress or an upstream stimulus of constitutively expressed MEK5. They combined overexpression studies of KLF2 and KLF4 and discovered that 59.2% of genes regulated by the activation of MEK5 were similarly controlled by KLF2 or KLF4. These genes were either coordinated in a combinatorial approach or individually in dependence of MEK5. MEK5/KLF2-dependent up-regulated genes were CD59, 2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase) and PTGDS, while BMP4 was down-regulated. Cathepsin b (CTSB), sex determining region Y-box 7 (Sox7) and VEGF were over-expressed in dependence of MEK5/KLF4, while INFγ-inducible protein 16 was down-regulated. A synergistic upregulation could be observed for TIE-2, ASS1, nephroblastoma over-expressed (NOV), while MCP1 and IL8 were down-regulated (104).

The last in vitro study discussed in this context was conducted by White et al. They investigated the expression profile of HUVECs exposed to extreme shear conditions of 75 dyne/cm², as it can be experienced in stenosed areas of the aorta, in comparison to average physiological shear stress of 15 dyne/cm². Comparing the two conditions, they observed an upregulation of 145 genes. Amongst the up-regulated ones were also two members of the KLF family; KLF4 (3.6-fold) and for the first time KLF6 (2.2-fold). Other up-regulated genes were integrin β 8 (ITGB8), ATF3, dual specificity phosphatases (DUSPS), prostacyclin and IL8. Approximately 158 genes, including GTPase IMAP family member (GIMAP) 4/7, ET-1 and IL6, were down-regulated. White et al. concluded that the elevated shear modified gene expression, reduced ROS levels, altered MAP kinase signalling and decreased cAMP levels. Elevated shear appeared to combine an amplification of the atheroprotective effects of laminar shear with unique responses to this stimulus (41).

The only in vivo study, which reports changes in KLF levels, was conducted by Zhang et al. in 2008. They investigated the transcriptomal differences between porcine coronary ECs (CECs), which derived from a more atheroprone vessel, and iliac artery derived cells (IECs). Comparing the gene expression patterns of CECs versus IECs, many inflammatory and thrombogenic genes such as DUSP1, ICAM-1, activated leukocyte cell adhesion molecule (ALCAM), PECAM-1, E-selectin, NF-κB and AP-1 were up-regulated. Cytoprotective genes such as members of the homeobox family HOXA10, HOXA9, HOXD3 and superoxide dismutase 2 (SOD2) as well as KLF2 and KLF4 were down-regulated (183).
These examples of *in vitro* and *in vivo* studies should provide an overview of the recent experimental designs and findings centred around the question which transcriptional patterns ECs activate when exposed to different shear stress regimes. In conjunction with details provided in section 1.1.3, these microarray studies provide enormous insight on the potential function of KLF2 and KLF4 in the mediation of an atheroprotective phenotype.

### 1.6 Aim of the study

The aim of this study was to investigate the expression profiles and regulatory roles of KLF2, KLF4, KLF6 and eNOS in dependence of time, shear stress magnitude and interference with siRNA. It was hypothesised that an interacting network of KLFs, rather than just a single regulator as currently postulated in the literature, contributes to the atheroprotective transcriptional program. All KLFs have been shown to be induced by shear stress, but only little effort has been made to clearly distinguish the roles of KLF2 and KLF4. KLF6 has only recently been found to show expression modulation in dependence of shear, but its function in this context remained unknown. Three main objectives were defined to investigate the working hypothesis.

1. Develop a reliable flow system, which allows to culture primary porcine endothelial cells in atheroprone low shear stress of 5 dyne/cm$^2$ and atheroprotected high shear stress of 20 dyne/cm$^2$ for up to 48h. The cell material has to be sufficient for subsequent molecular analysis and cell phenotypes have to correspond to the *in vivo* morphology in different areas in the vasculature.

2. Design and optimise experiments for quantitative gene expression analysis of PAECs after exposure to shear stress or static culture. Quantify KLF2, KLF4, KLF6 and eNOS gene expression levels and protein in time course experiments ranging from 2−48h in normal and siRNA-interfered conditions. Study a potential relationships and dependencies of KLFs, which might contribute to a KLF network modulating eNOS and mediating atheroprotection. Mathematical modelling should be employed in these investigations.

3. Conduct a genome-wide microarray analysis to determine global endothelial gene expression patterns in response to high atheroprotective shear and compare these to conditions in which KLF2 and KLF4 were silenced. Identify genes that might play a role in the development and progression of shear-induced endothelial inflammation. Investigations on a genome scale will enable to differentiate the specific roles of KLF2, KLF4 and KLF6.
2 CHAPTER MATERIALS AND METHODS

2.1 Design of flow apparatuses

Design and optimisation of the flow set up was an iterative process. This section describes the selection and optimisation of the main components such as the microfluidic chamber, tubing material, media chamber and three pump prototypes that were tested. Chapter 3 outlines the evaluation of the components and provides a rationale for selection of the final flow circuit.

2.1.1 Microfluidic chamber

Cell culture under shear was conducted in μ-Slides VI 0.4 flow chambers (ibidi) (Figure 13). The chambers have the advantage that they provide excellent optical qualities, a sterile culture environment and compatibility to flow circuit connection. Additionally, the small surface areas suited the experimental design as cells were limited and reagents used rather costly.

As the uncoated μ-Slides were manufactured from hydrophobic plastic to which cells do not easily attach, the channels were coated for 1h prior to cell culture experiments with various adhesive coatings such as gelatine, fibronectin or collagen IV. Table 4 summarises the specifications of the chambers.

Figure 13: Schematic drawing of a flow chamber μ-Slide VI 0.4 (ibidi) with six flow channels. Figure adapted from online catalogue at http://www.ibidi.com.
Table 4: Dimensions of the flow chamber µ–Slide VI 0.4.

<table>
<thead>
<tr>
<th>Dimensions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of channels</td>
<td>6</td>
</tr>
<tr>
<td>Channel volume</td>
<td>30 µL</td>
</tr>
<tr>
<td>Channel length</td>
<td>17 mm</td>
</tr>
<tr>
<td>Channel width</td>
<td>3.8 mm</td>
</tr>
<tr>
<td>Channel height</td>
<td>0.4 mm</td>
</tr>
<tr>
<td>Adapters</td>
<td>Female luer</td>
</tr>
<tr>
<td>Volume per reservoir</td>
<td>60 µL</td>
</tr>
<tr>
<td>Growth area</td>
<td>0.6 cm² per channel</td>
</tr>
<tr>
<td>Coating area using 30 µL</td>
<td>1.2 cm² per channel</td>
</tr>
<tr>
<td>Bottom matches coverslip</td>
<td>No 1.5</td>
</tr>
</tbody>
</table>

Shear stress was calculated according to Equation 3 as specified in the user manual Application Note 11 (ibidi) (186). Further information on the mathematical background and derivation of this equation are also detailed in this reference.

Equation 3: Formula to convert shear stress $\tau$ (dyne/cm²) to flow rate $\Phi$ (mL/min).

$$\tau \left( \frac{\text{dyne}}{\text{cm}^2} \right) = 1.761 \Phi \left( \frac{\text{mL}}{\text{min}} \right)$$

Each pump system was calibrated individually before initiation of a flow experiment according to the conversion rates in Table 5. The dyne is a unit of force from the centimeter-gram-second system of units and is equal to 10 µN ($=10^{-5}$ kg·m/s²) (see section 1.2.1).
Materials and Methods

Table 5: Conversion shear stress $\tau$ to flow rate $\Phi$.

<table>
<thead>
<tr>
<th>$\tau$ (dyne/cm$^2$)</th>
<th>$\Phi$ (mL/min)</th>
<th>$\tau$ (dyne/cm$^2$)</th>
<th>$\Phi$ (mL/min)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0.57</td>
<td>13</td>
<td>7.38</td>
</tr>
<tr>
<td>2</td>
<td>1.14</td>
<td>14</td>
<td>7.95</td>
</tr>
<tr>
<td>3</td>
<td>1.70</td>
<td>15</td>
<td>8.52</td>
</tr>
<tr>
<td>4</td>
<td>2.27</td>
<td>16</td>
<td>9.09</td>
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<tr>
<td>5</td>
<td>2.84</td>
<td>17</td>
<td>9.65</td>
</tr>
<tr>
<td>6</td>
<td>3.41</td>
<td>18</td>
<td>10.22</td>
</tr>
<tr>
<td>7</td>
<td>3.98</td>
<td>19</td>
<td>10.79</td>
</tr>
<tr>
<td>8</td>
<td>4.54</td>
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</tr>
<tr>
<td>9</td>
<td>5.11</td>
<td>21</td>
<td>11.93</td>
</tr>
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<td>10</td>
<td>5.68</td>
<td>22</td>
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</tr>
<tr>
<td>11</td>
<td>6.25</td>
<td>23</td>
<td>13.06</td>
</tr>
<tr>
<td>12</td>
<td>6.81</td>
<td>24</td>
<td>13.63</td>
</tr>
</tbody>
</table>

2.1.2 Tubing and connectors

Different tubing materials, as listed in Table 6, were tested to evaluate the material’s compatibility with cell culture under flow. The tubing was cut in 7 cm long pieces and connected in series to the flow channel inlets using polypropylene male luers (The Westgroup) with a homogenous inner diameter (ID) of 3.2 mm or elbow luer connectors (ibidi) of varying ID with 0.8 mm in the smallest section. A platinum-cured silicon tube with 3.4 mm ID (Cole Parmer) was used in the pump head.

For time course experiments, several flow chambers were connected in series by longer pieces of tubing without trapping of air bubbles. The remaining media was disposed after usage and the tubing cleaned with soapy hot water. It was rinsed twice with deionised water and left to dry. Tubes were then sealed in autoclaving bags and sterilised. After 3-5 times usage, tubes were disposed off.

Table 6: Different tubing materials used in flow apparatus set up.

<table>
<thead>
<tr>
<th>Material</th>
<th>ID (mm)</th>
<th>Manufacturer</th>
<th>Order nr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear flex tubing (non gas permeable)</td>
<td>2.4</td>
<td>Cole Parmer</td>
<td>RZ-06422-03</td>
</tr>
<tr>
<td>Platinum cured silicon</td>
<td>2.4</td>
<td>Cole Parmer</td>
<td>RZ-95802-03</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>2.0</td>
<td>Pharmed</td>
<td>F1825103</td>
</tr>
<tr>
<td>Silicon</td>
<td>2.0</td>
<td>ibidi</td>
<td>10962</td>
</tr>
<tr>
<td>Platinum cured silicon</td>
<td>3.4</td>
<td>Cole Parmer</td>
<td>EW-96410-34</td>
</tr>
</tbody>
</table>
2.1.3 Media reservoirs

In the course of flow circuit optimisation, two different media reservoirs were tested. Firstly, a round glass chamber of 150 mL volume, having the loosely fitted lid modified with a media inlet and outlet. The second one was a self-designed modified 500 mL glass bottle (Schott). The screw-on polypropylene lid was perforated to fit three tubes functioning as media inlet, media outlet and gas exchange, fitted with a 0.22 µm hydrophobic filter. Both reservoirs were made of inert material with a high temperature resistance. Before usage, they were washed with warm soapy water and rinsed twice with deionised water and sterilised for 20 min at 131°C by autoclaving. The media reservoirs were either filled with 30 mL or 300 mL complete media Dulbecco’s Modified Eagle Medium (CM-DMEM) 12h prior to initiation of a flow experiment and left in the incubator at 37°C and 5% CO₂. This procedure of degassing proved to be beneficial as it reduced bubble formation during a flow experiment.

2.1.4 Peristaltic system prototype I

The first prototype, a Minipuls 3 peristaltic pump (Gilson) as seen in Error! Reference source not found., combined microprocessor speed control with a high-torque stepper motor. The pump head was equipped with ten stainless steel rollers, which were supposed to produce smooth, low-pulse flow and reproducible flow rates at higher pressures. Due to overheating problems, the pump had to be placed outside of the incubator. Tubing running outside the incubator was enclosed in rubber as a thermal insulator. Within the circuit, the pump was positioned between the microfluidic chambers and the media reservoir.

Figure 14: Top view onto prototype I Minipuls 3 pump head (Gilson) equipped with ten stainless steel rollers. Due to technical limitations, this pump could not operate inside the incubator.
2.1.5 Perfusion system

The perfusion system, as depicted in Figure 15, was composed of two main components: a control unit and a fluidic unit (ibidi). The control unit consisted of a computer-controlled air pump, which generated pressure ranging from ± 100 mbar with ± 1 mbar accuracy. The user-friendly software presented a flexible tool to control the pressure, which was automatically converted into shear rates. The fluidic unit contained two reservoirs for the culture media, a slide holder and all required tubes for connecting the syringe reservoirs with the microfluidic slides. 12h prior to initiation of a flow experiment, 12 mL CM-DMEM were filled into the syringe reservoirs and left over night to degas.

A switch valve generated unidirectional flow and the rate was monitored and recorded during the time course of an experiment. Unexpected changes in pressure initiated an alarm signal so that the user could investigate the source of error. The control unit and the fluidic unit were connected via a thin, low-voltage electric cable and a tube for transducing the air pressure to drive the flow. The air tube and the electric cable were mounted into the incubator through the front door seal. This system had the advantage that the entire flow circuit could be placed inside the incubator, being exposed to a homogenous temperature and gas environment.

Figure 15: Front view onto the fluidic unit of the perfusion system (ibidi) showing the two pressurised syringe reservoirs connected with C-flex tubing to a microfluidic chamber with the individual channels connected in series. The switch valve guaranteed unidirectional flow.
2.1.6 Peristaltic system prototype II

The second peristaltic pump prototype II (Atto) was equipped with six plastic rollers as seen in Figure 16 and produced a constant flow rate within the desired shear range. It suited the experimental set up better than prototype I as it could be placed inside the incubator without causing an increase of the overall temperature.

The tubing in the pump head was fixed with adjustable side clamps and kept in place by mounting a plastic cover tightly on top. The position of the pump within the circuit was similar to prototype I between the microfluidic chamber and the media reservoir.

![Figure 16: Top view onto prototype II pump head of Perista SJ1220 peristaltic pump (Atto) equipped with six plastic rollers.](image)

2.2 Cell culture

Specifications for media components, reagents, chemicals, solutions and equipment can be found in the Appendix section 7.3. All static cell culture experiments and preparation of flow experiments were carried out under sterile conditions using aseptic techniques in a 1.5 Topsafe (Bioair) cell culture hood. Shear stress experiments and static cell culture were conducted in an Incusafe incubator (Sanyo) at 5% CO₂ and 37°C. Growing cells were monitored on a daily basis and mycoplasma tests were frequently conducted to minimise the risk of undetected contaminations.
2.2.1 Isolation of porcine aortic endothelial cells

Descending thoracic aortas of Landrace Cross pigs, aged 4–6 months and weighing 80 kg, were obtained immediately after slaughter (Fresh Tissue Supplies) and stored for 24h in Hank's Balanced Salt Solution (HBSS) containing penicillin (200 U/mL), streptomycin (200 µg/mL), amphotericin (5 µg/mL) and gentamycin (100 µg/mL).

Using collagenase digestion, PAECs were isolated from the aortas according to the method of Bogle et al (187). Briefly, in a sterile environment, vessels were trimmed of fat and connective tissue and intercostal branches were ligated. The proximal end of the aortic segment was cannulated and the vessel was flushed with PBS to remove blood cells. The distal end of the vessel was then clamped and the aorta was filled with 0.2 mg/mL type II collagenase. After incubation at 37°C for 15 min, the aorta was gently massaged to loosen PAECs and the collagenase solution was collected and centrifuged at 1,200 rpm for 5 min.

The cell pellet was resuspended in 5 mL DMEM supplemented with 10% (vol/vol) FBS, 5 mM L-glutamine, 5 µg/mL endothelial cell growth factor (ECGF), 100 U/mL penicillin, 100 U/mL streptomycin, 50 µg/ml gentamycin, 50 µg/ml amphotericin and 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES). The media composition (CM-DMEM for PAECs) derived from an original standard protocol from the lab in which initially 10% neonatal calf serum (NCS) (vol/vol) was used. The rational for culturing in only 10% total serum is described as part of the results in section 2.2.1. Cells were seeded in T-25 flasks coated with 1% gelatine and they were maintained at 37°C in CM-DMEM under a humidified atmosphere containing 5% CO₂. Media was changed after 24h to remove non-adherent cells and loose debris. Subsequently, media was changed regularly every 2–3 days. Cells were monitored on a daily basis and discarded in the case of contamination with VSMCs that could have been accidentally loosened and collected during the collagenase digestion isolation procedure.

2.2.2 Maintenance of porcine aortic endothelial cells

PAECs were isolated from thoracic arteries and subcultured as described in 2.2.1 until 80% confluence. For passaging the medium was removed and the cells were washed once with 2–5 mL phosphate buffered saline (PBS). Then 2 mL of 2.5% trypsin-ethylenediamine-tetraacetic acid (EDTA) solution was administered to the cell layer and cells were incubated at 37°C for 3-5 min to detach.
Materials and Methods

The remaining trypsin was inactivated by resuspending the cells in CM-DMEM and subculture was carried out at a ratio of 1:3 and grown to 80% confluence. Cells were only subcultured and used for flow experiments until passage 3 (P3). The culture of PAECs under flow is described in Chapter 2.2.5.

2.2.3 Cryopreservation and thawing of adherent cells

Cells to be cryopreserved were washed once with PBS and then trypsinised for 5 min at 37°C with 2.5% trypsin-EDTA solution. Afterwards they were collected, centrifuged, resuspended in CM-DMEM and the cell concentration was then evaluated. Subsequently, they were centrifuged once more before being placed on ice. The supernatant was removed and the pellets were resuspended in freezing media I (FM I) containing 10% more FBS than complete media, making 50% of the total end volume. FM II, containing 10% more FBS than complete media and 20% dimethylsulfoxide (DMSO), was added drop wise (50% of the total end-volume), while gently mixing. The cell suspension was then distributed in cryovials (1 mL/vial with 0.5–5.0 Mio cells per tube). Cryovials were placed in a freezing box, which was kept at -80°C for 24 h prior to storage in a cryotank filled with liquid nitrogen.

Frozen PAECs were quickly thawed by placing the cryovial in a 37°C warm water bath. To remove toxic DMSO, cells were resuspended in 45 mL CM-DMEM, centrifuged and resuspended. Culture media was exchanged once the cells had adhered.

2.2.4 Determination of cell numbers

A 50 µL sample of the cell suspension to be counted was transferred to a microcentrifuge tube and 50 µL of trypan blue were added and mixed. The solution was allowed to stand at room temperature for 5 min. Then 10 µL of the mixture were loaded into both counting chambers of an improved Neubauer Haemocytometer. The slide was placed on the stage of microscope and the cells (unstained: live cells, stained: dead cells) in 4-9 large squares (1 mm²-areas) of each chamber were counted using a 10x objective.

The mean number of viable cells per mL was calculated as follows:

\[ C_v = \bar{X}_v \times D \times 10,000 \]

\( C_v \) = the viable cell concentration (cells/mL)

\( \bar{X}_v \) = the average number of viable cells/1 mm x 1 mm square

\( D \) = the dilution factor (for 25 µL cell suspension in 25 µL trypan blue, \( D = 2 \))

Equation 4: Cell number evaluation using a Neubauer Haemocytometer.
2.2.5 Porcine aortic endothelial cell culture under flow

For shear stress experiments, PAECs (P1-3) were cultured in microfluidic chambers coated with 1% gelatine. Gene silencing experiments required transfection beforehand as described in section 0. Briefly, cells were washed once with PBS and were then trypsised for 5-6 min at 37°C with a 2.5% trypsin-EDTA solution. Afterwards they were collected, centrifuged, resuspended in CM-DMEM and the cell concentration was evaluated (see section 2.2.4). Cells were then seeded into flow chambers with a concentration of 30,000 cells per channel and a maximum volume of 35 µL. During the optimisation phase, the cell concentration initially varied between 9,000-35,000 cells until the optimal density was found. Static culture of at least 12h was conducted at 37°C in CM-DMEM under a humidified atmosphere containing 5% CO₂. Media was exchanged before connecting the flow chambers to the pump system. A preconditioning phase of 1 dyne/cm² was performed before the shear stress was set to 5 dyne/cm² or 20 dyne/cm².

2.3 Gene silencing with RNAi approach

Gene silencing of KLF2 and KLF4 required the design of functional siRNAs and scrambled mock probes. Cell transfection using electroporation was optimised with FACS and confocal microscopy and the gene expression was quantified by qPCR.

2.3.1 Design of siRNA probes

For the KLF2 and KLF4 mRNA targets three different siRNA sequences (termed siRNA A, B and C) were designed as well as a mock sequence (M) mismatch siRNA with GC exchange and one AT pair exchanged with GC according to the golden standard rules in section 1.4.2 (Table 7). In silico analysis was used to blast the designed siRNA sequences against the pig genome with Blastn program of the National Centre for Biotechnology Information (NCBI, database name: gp/9823.10718/ssc_refm) with sus scrofa RefSeq RNA. Sequences were designed using the online tool siRNA Design centre from Dharmacon with the gene sequences as FASTA files and the respective accession numbers (NM format of NCBI). Binding in an open reading frame region as well as a min G/C of 30% and max G/C of 64% were criteria for the selection. All of the chosen siRNAs were 19-mers with 3´UU overhang modification and they were desalted and duplexed for ready use.
Table 7: Overview of siRNA sequences (A-C) targeting KLF2 mRNA, KLF4 mRNA and their respective mock sequences (M). Letters highlighted in bold present the amino acids for the mock sequence where GC was changed into AT and one CG exchanged from the original sequence ID A.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>ID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLF2</td>
<td>A</td>
<td>GCGGCAAGACCTACACAAA</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>ACAAAGAGCTGCACCTCA</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>TGGAGCTACTAGGGCCCA</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>GCATAACAGCAGGAGAAA</td>
</tr>
<tr>
<td>KLF4</td>
<td>A</td>
<td>GGTCACTCAGGGTACGAAA</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>CAGAGGAGCAAAGCCAAA</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>GGGAGACGGAGGAGTCAA</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>CCTGCGGACTCTTAAATAAA</td>
</tr>
</tbody>
</table>

2.3.2 Transfection using electroporation

Transfection of cells with plasmid DNA or siRNA was done using microporator technology (MP-100 from Digital Bio). Electroporation efficiencies are known to vary greatly between different cell types and the electroporation parameters had to be adjusted accordingly. As the availability of primary cells was limited, an initial optimisation of the microporation parameters was conducted with HeLa, following the manufacturer’s 24 well version for 10 µL tip quick protocol (Digital Bio). Briefly, subconfluent HeLa were harvested and washed with PBS. Approximately 3×10⁶ cells were resuspended in 275 µL suspension buffer R and incubated with 15 µg of a standard green fluorescent protein (GFP) plasmid provided by the manufacturer to yield a concentration of 0.5 µg per well.

Microporation was performed at room temperature using 24 different programs varying pulse voltage (850–1700 V), time (10-40 ms) and pulse numbers (1-3). As a control, a setting suggested by the manufacturer was used (1005 V, 35 ms and 2 pulses). After electroporation, cells were distributed into 24 wells, containing 500 µL of DMEM with 10% FBS (without antibiotics and supplements) and cultured at 37°C in a 5% CO₂-humidified atmosphere. Media was changed to CM-DMEM approximately 6h after the electroporation. Cells were harvested after 24h and washed twice with PBS before visualisation of GFP using confocal microscopy to qualitatively assess electroporation efficiency. For cell culture under flow, cells were directly seeded with optimised density into the individual channels of the pre-coated flow camber (ibidi).
The transfection method using primary PAECs was optimised with a smaller number of parameters, taking the optimal transfection setting for HeLa as guidance. Briefly, subconfluent PAECs were harvested and washed with PBS. Approximately $2 \times 10^5$ cells were resuspended in 12 µL suspension buffer R and incubated with different concentrations of All stars transfection negative siRNA control (Qiagen) according to Table 8. The electroporation protocol as detailed above was followed and cells were subsequently assessed via flow cytometry as described in Chapter 2.7.

Table 8: Range of electroporation parameters (A-C) and siRNA concentrations from 0-150 nM (1-5) used to optimise electroporation settings for PAECs.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Pulse (V)</th>
<th>Pulse width (ms)</th>
<th>Pulse nr.</th>
<th>Conc. siRNA (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1500</td>
<td>50</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>A2</td>
<td>1500</td>
<td>50</td>
<td>1</td>
<td>100</td>
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<tr>
<td>A3</td>
<td>1500</td>
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<td>A4</td>
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<td>150</td>
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</tbody>
</table>

To assess transfection efficiency also qualitatively, siGLO Red Transfection Indicator (Thermos Scientific) was used in combination with functional siRNA against KLF2 and KLF4. PAECs were electroporated and transfected with 300 nM total siGLO and KLF2 ABC or KLF4 BC (1:1), 300 nM KLF2 ABC or KLF BC or none and seeded into flow chambers. The cells were exposed to 2h high flow following the protocols outlined in section 2.2.5. They were then washed, stained with Draq5 (Biostatus Limited), a nuclear dye for live cell imaging, and imaged as detailed in section 2.4.2.
2.3.3 Optimisation of siRNA cocktail composition and concentration

The functionality of the designed siRNA sequences had to be validated, fine-tuning siRNA composition and concentration.

Three different siRNAs (Table 7) against KLF2 and KLF4 were designed with the support of the siRNA Design centre from Dharmacon. To test their functionality, PAECs were electroporated with siRNA sequence A, B and C individually, combinatorial ABC as well as a scrambled mock sequence (M) at a total concentration of 500 nM, using the optimised electroporation setting (1200V, 20 ms pulse width and 2 pulses). Cells resuspended in antibiotic-free DMEM were seeded into flow chambers and media was exchanged with CM-DMEM 6h after electroporation. After 12h of static culture, chambers were connected to the flow circuit and cells exposed to 20 dyne/cm² for 2h. The protocol for qPCR in section 2.5.4 was followed to quantitatively assess changes in target gene expression.

Once the optimal composition had been determined, titration experiments were conducted for the functional siRNA cocktail as well as the unspecific mock siRNAs with concentrations ranging from 50-1500 nM total. The same procedure as described in the paragraph above was followed. qPCR was used to determine the optimal siRNA concentration for the probes with the most efficient gene-silencing effect. All further gene silencing experiments targeting KLF2 and KLF4 mRNA were carried out with the siRNAs optimised in composition and concentration.

2.4 Imaging

2.4.1 Bright field microscopy

Cells in culture were monitored frequently with an inverted light microscope (Leica DM IL LED). Magnifications of 4x, 20x and 40x were used to observe cell distribution and density, possible contamination of bacteria, fungus and other cell types such as VSMCs and the cell shape. Especially for flow experiments and in the initial optimisation phase, cell phenotypes were frequently analysed as the alignment of cells in high shear conditions presented a visual quality control. Bright field images were taken with a digital microscope camera (Leica DFC290) and processed in jpg format.
2.4.2 Confocal microscopy

Confocal microscopy was used to qualitatively assess the uptake of siRNA in PAECs after electroporation and exposure to high shear stress for 2h. The flow chambers were uncoupled from the circuit and the individual chambers were rinsed twice with PBS before staining with 5 mM Draq5 (Biostatus Limited) as nuclear stain. Alive cells were covered in sufficient solution of Draq5 (1:500 PBS + 1% bovine serum albumin (BSA)) for 10 min at room temperature followed by rinsing 3x with PBS. The samples were kept in PBS 1% BSA for confocal analysis.

Confocal microscopy (Leica model TCS SP5 X) was conducted to image the distribution of fluorescent siRNA. This RISC-independent control was chemically modified and used as a qualitative indicator of delivery. siGLO siRNA (Thermos Scientific) was detected with the 561 nm laser (absorbance at 557 nm and emission max at 570 nm, imaged green). The nuclear stain Draq5 with the 633 nm laser (absorbance 646 nm and emission max at 700 nm, imaged blue).

2.5 Gene expression studies

The mRNA levels of KLF2, KLF4, KLF6 and eNOS in PAECs were quantified by qPCR in response to shear stress exposure in comparison to static control conditions. After flow experiments, cells were first harvested by trypsinisation before RNA was purified and reverse transcribed to cDNA.

Hypoxanthine-guanine phosphoribosyltransferase (HPRT1), which plays a central role in generation of purine nucleotides, was chosen as a reference gene to normalise for variations of cDNA in the samples. Nygard et al. have investigated genome-wide expression profiles with SYBR green qPCR methodology and confirmed HPRT1 as a suitable housekeeping gene in porcine endothelial tissue as its expression levels did not change in dependence of various treatments (188).

2.5.1 Preparation of samples

After exposure to shear stress, cell chambers were uncoupled from the circuit, placed in Petri dishes and the remaining media was aspirated. The individual channels were rinsed 3x with ice-cold PBS and images of the cell layer were taken with an inverted light microscope. During the optimisation phase, cells were detached by trypsinisation and the pellet was collected in a 1.5 mL Eppendorf tube. 500 µL of RNA later were added and the samples were kept at 4°C until further processing.
This procedure was later changed to direct lysis as it resulted in severe cell loss. For all experiments described in this work, cells were lysed directly in each flow chamber channel with 350 µL cell lysis buffer containing 1% β-mercaptoethanol. The lysate was collected in a 1.5 mL Eppendorf tube and stored at -20°C until further processing.

2.5.2 RNA extraction

The RNA extraction was performed once all samples for an individual time course experiment were collected to reduce technical variability. A dedicated RNA isolation workspace, cleaned with RNase AWAY (Sigma), was used to minimise the risk of RNase contamination. Briefly, cell lysates were thawed to room temperature and processed using column extraction with the RNeasy Mini Kit (Qiagen). For homogenisation 350 µL of 70% ethanol were added and the samples transferred to columns. These were centrifuged for 15 sec (8000 x g for all steps unless otherwise stated) and the flow-through was discarded. 700 µL of buffer RW1 were added followed by a spin and disposal of flow-through. Two washes with 500 µL RPE were performed, the last one with an increased centrifugation time of 2 min. The columns were then placed in new 2 mL tubes and centrifuged at 12,000 x g for 1 min. Subsequently, they were placed in new 1.5 mL tubes and the RNA was eluted with 32 µL of nuclease free water and the samples were kept on ice. The RNA concentration, purity and integrity were determined with a UV-Vis Spectrophotometer (NanoDrop 2000c). Samples were only further processed, if the ratio of the absorbance at 260 and 280 nm was between 1.8 and 2.1 and without any contamination. This quality check ensured that only high quality RNA was used in qPCR analysis.

2.5.3 cDNA reverse transcription

The conversion to cDNA was performed straight after RNA isolation to avoid freeze-thaw cycles that could potentially damage the RNA integrity. The High Capacity RNA to cDNA kit (Applied Biosciences) protocol was followed in a 20 µL reaction. Briefly, a master mix of 2x RT buffer and 20x RT enzyme was prepared and pipetted into a 96-well plate. 9 µL RNA per sample were added and the plate sealed with an adhesive foil. The reverse transcription reaction was carried out with a thermo cycler (Multigene Labnet, Appleton Woods) at 37°C for 60 min, followed by 95°C for 5 min and an infinite cooling at 4°C. The cDNA samples were aliquotted into small PCR tubes and stored at -20°C.
2.5.4 Quantitative polymerase chain reaction

qPCR was carried out using SYBR Green PCR Master Mix (Applied Biosciences) in 20 µL reactions. Primers for each individual gene were added to the master mix to yield a final concentration of 300 nM. 15 µL of master mix were transferred into dedicated wells of a 96-well plate and 5 µL of cDNA sample (1:4 working solution) were added. All reactions were performed in triplicates and negative controls containing no primer (NPC) as well as no template (NTC) were added to each experiment. The plate was sealed with an adhesive foil and briefly centrifuged to spin down the content and to eliminate any air bubbles. For the real-time PCR reaction a thermo cycler (realplex, Eppendorf) was used. Reactions were incubated for 45 sec at 95°C before thermal cycling at 95°C for 15 sec, followed by 58°C (or 61°C for certain genes as specified in Table 14) for 30 sec. After 45 amplification steps a gradient ranging from 65°C to 95°C over a time period of 20 min was performed. The resulting melting curve was used as a quality control to monitor specificity of the amplification reaction.

2.5.5 Primer design

All parameters described in the following section were taken into consideration for the design of qPCR primers. Briefly, the amplicon length for qPCR is meant to be around 100-200 bp with a product position close to the 3’ end. Primer annealing temperatures (Tₘ) were optimised as too high Tₘ will produce insufficient primer-template hybridisation, resulting in low PCR product yield. Melting temperatures (Tₘ) in the range of 52-58°C are supposed to generally produce the best results and the primers were chosen accordingly. The GC content, which should be 40-60%, presents another important parameter. Repeats and runs in the primary structure as well as hairpins, self assembly and cross hybridisation in the secondary structure were avoided as much as possible. The freeware programs Beacon designer and NetPrimer were used to address these criteria. Primer specificity was checked in silico using the NCBI primer blast against the pig genome, which helped to avoid cross homology to other sequences (189).

Primers that fulfilled all criteria were purchased from Invitrogen, reconstituted to a stock solution (SS) of 100 µM (100 pmol/µL) and aliquotted to a working solution (WS) of 5 µM. They were stored at -20°C and freezing and thawing cycles were avoided. Primer sequences are listed in Table 9 and primer specifications are summarised in Table 10.
Table 9: Primer pair sequences.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sense (forward)</th>
<th>Antisense (reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLF2</td>
<td>AGACCACGATCCTCCTTGAC</td>
<td>AAGCCTCGATCCTCTAGTGAG</td>
</tr>
<tr>
<td>KLF4</td>
<td>CCGTCCTCTCCACCTCCTTG</td>
<td>AGCACCGGGGGAAAATCTTTGC</td>
</tr>
<tr>
<td>KLF6</td>
<td>CATCCTGGCGCCGGAGCATA</td>
<td>ACTTCGCCGTGGGGTCGGA</td>
</tr>
<tr>
<td>eNOS</td>
<td>GGCCGTCAGTCCTCCTCGAA</td>
<td>AGACCCAGGGGAGGAAACAGG</td>
</tr>
<tr>
<td>HPRT1</td>
<td>CCCAGCGTGCGATTAGTGATG</td>
<td>CATCACATCTCGAGCAAGCCGT</td>
</tr>
</tbody>
</table>

Table 10: Primer pair specifications.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>GC% Sense</th>
<th>GC% Antisense</th>
<th>Amplicon (bp)</th>
<th>Sequence binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLF2</td>
<td>55.00</td>
<td>50.00</td>
<td>105</td>
<td>1,367-1,431</td>
</tr>
<tr>
<td>KLF4</td>
<td>66.67</td>
<td>61.90</td>
<td>125</td>
<td>25-129</td>
</tr>
<tr>
<td>KLF6</td>
<td>65.00</td>
<td>65.00</td>
<td>198</td>
<td>408-586</td>
</tr>
<tr>
<td>eNOS</td>
<td>65.00</td>
<td>59.09</td>
<td>168</td>
<td>3,662-3,808</td>
</tr>
<tr>
<td>HPRT1</td>
<td>54.17</td>
<td>54.55</td>
<td>148</td>
<td>111-237</td>
</tr>
</tbody>
</table>

2.5.6 Dynamic range of primers

Absolute quantification strategies for PCR utilise calibration curves to obtain the copy number or concentration of a sample. A 5x dilution series of a processed cell sample exposed to 2h of high shear stress was prepared, following the procedure described in section 2.5.1. The samples were used to generate standard curves with a linear relationship between the cycle threshold (Ct) values and the dilution ratio on log scale. A logarithmic trendline determined the slope of the curve (m), the y intercept (b) and the coefficient of determination $R^2$ at a threshold Ct value, which was individually determined for each primer. For accurate and reliable calculations it was ensured that each standard curve encompassed the entire concentration scale of unknown samples and covered the dynamic range of the reagents. Standard curves were not performed for each experiment, but on a regular basis to check that they were consistent. All reagents and providers were kept to guarantee no inter-experimental differences in all performed PCR experiments due to variability in reagents.
2.5.7 Calculation of the amount of unknown sample

The slope (m) of the standard curve was used to determine the relative concentration of unknown samples (x), which was calculated from an average of 3 reactions, as shown in Equation 5. The qPCR measurements were repeated if \( \Delta Ct \geq 0.5 \) for all three measures in a single reaction.

\[
    x = e^{\frac{y-m}{b}}
\]

*Equation 5: Calculation of relative sample concentration x derived from a standard curve with slope (m), the y-axis intercept (b) and the Ct value (y).*

2.5.8 Calculation of the amplification efficiency

The efficiency of a PCR assay can also be calculated from the above described standard curve as a plot of cycle numbers versus the logarithm of the standards’ concentrations. The PCR efficiency for a graph with Ct plotted on the y-axis and dilution ratio (log) on the x-axis was calculated as shown in Equation 6.

\[
    PCR \ efficiency = \left( \left( 10^{-\frac{1}{m}} \right) - 1 \right) \times 100\%
\]

*Equation 6: Calculation of the amplification efficiency of a qPCR assay with the slope of the standard curve (m).*

2.5.9 Statistical analysis

In statistics, a standard score (z-score) indicates how many standard deviations an observation is above or below the mean. The acquired qPCR data, already normalised to the housekeeping gene, was initially processed by identification of outliers using the z-score analysis (Equation 7). All values above the selected threshold value of 1.4 were excluded. For the time series evaluation, the data was also normalised to 1 for the static control condition at 0h.

\[
    z = \frac{x - \mu}{\sigma}
\]

*Equation 7: Z-score equation used to identify and exclude outliers above a threshold of 1.4.*
All normalised qPCR gene expression data are presented as fold difference and the error bars represent the standard deviation (± SD).

\[
\sigma = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2}
\]

Equation 8: Calculation of the standard deviation.

For the statistical analysis and graphical representation of the results SPSS and GraphPad Prism were used. A power analysis to evaluate an ideal sample size was not conducted prior to the experimental phase, but all data shown is at least with a sample number n ≥ 3, unless otherwise stated. Normal distribution of qPCR data was confirmed and statistical significance of normalised data was determined using Student’s t-test, one-way or two-way ANOVA followed by Bonferroni’s post-hoc test when appropriate. Differences were considered significant at \( p < 0.05 \).

2.6 Protein detection using immunoblotting

Preliminary results have been obtained in the effort to quantify KLF2 and KLF4 proteins using immunoblotting.

2.6.1 Sample preparation

Cell pellets were lysed in RIPA buffer (SIGMA) containing proteinase inhibitors. The cells were then homogenised with an ultrasonic homogenizer (Model 3000, BioLogics) by repeating the following program four times for a total of 4 min: interval on for 3 sec, interval off for 5 sec. During this procedure the samples were placed on ice. After sonication, cell lysates were combined with the equal quantity of 2x sample buffer and final 5% β-mercaptoethanol and then boiled for 5 min. The protein immunoblot samples were aliquotted in PCR tubes and stored at -20°C.

2.6.2 SDS-PAGE gel electrophoresis

Mini gel cassettes (Invitrogen) with 1 mm widths and 12 well combs were used to prepare gels for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Running gels and stacking gels were prepared according to the compositions in Table 11 and Table 12.
Table 11: Recipe for 12% SDS-PAGE running gel (15 mL total for 2 gels).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>4.9</td>
</tr>
<tr>
<td>30% AA-bisAA mix</td>
<td>6.0</td>
</tr>
<tr>
<td>1.5 M Tris</td>
<td>3.8</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.15</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.15</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Table 12: Recipe for SDS-PAGE stacking gel (6 mL total for 2 gels).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>4.1</td>
</tr>
<tr>
<td>30% AA-bisAA mix</td>
<td>1.0</td>
</tr>
<tr>
<td>1 M Tris</td>
<td>0.75</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.06</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.06</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Briefly, the cassettes, filled with 6 mL of 12% running gel, were left in a vertical position covered with 800 µL water. After 1h settling, 2 mL of the stacking gel were poured into the cassette and a comb was inserted. Once this gel settled, the comb was removed and the gels were wrapped in wet blue towel to avoid drying up and then stored at 4°C or used immediately. Frozen samples were thawed and 10-15 µL were loaded on the SDS gel, which was placed into the transfer tank filled with 700 mL ice-cold running buffer. Gel electrophoresis was conducted at 200V at constant voltage for 50-75 min.

2.6.3 Transfer

The gel was removed from the cassette after electrophoresis. Two paper sheets (Whatman) and one polyvinylidene fluoride (PVDF) membrane (Millipore) were cut out in the size of the gel. The membrane was soaked for 1 min in 100% methanol before sandwiching the transfer casts in the following order: red plastic cover-sponge-paper-membrane-gel-paper-sponge-black plastic cover. All air bubbles were removed and the firmly sealed sandwich was placed in the transfer tank filled with 1.4 L cold transfer buffer.
The transfer tank was covered in ice and electrophoresis for vertical transfer of the proteins from the gel to the membrane was conducted for 60 min at 100V. The membrane was then removed from the cast and sealed in 5 mL blocking solution for overnight blocking at 4°C.

2.6.4 Antibody incubation

For detection of KLF4 protein the membrane was incubated on a shaker for 1h with the primary antibody (SC20691 anti-KLF4, Santa Cruz) 1/100 dilution or for detection of the His-tag with the primary antibody (70796-3 His-tag, Millipore EMD).

It was then washed twice for 10 min with 0.1% Tween20-PBS (-). Secondary antibody incubation for KLF4 (SC2030 anti-rabbit) and Anti-His (386445 anti-mouse) 1/5000 dilution in cold blocking buffer was conducted for 1h on a shaker before washing four times for 5 min with 0.1% Tween20-PBS (-). Finally, the membrane was washed for 3 min with MilliQ water.

2.6.5 Protein detection

The ECL Prime Protein immunoblotting reagent (GE) was used to detect protein bands by chemiluminescence. 1 mL of prime solution and 1 mL of peroxide solution were mixed on cling foil. The washed membrane was fully immersed in the solution without bubbles and incubated for 1 min. Excess detection reagent was drained off by touching the edges against a tissue. With the protein side up, the membrane was placed on a tray in the UV chamber and luminescence was detected by automated exposure for 120 sec. Images were converted to jpg and annotated in Power Point.

2.7 Flow cytometry to quantitatively assess electroporation efficiency

Flow cytometry was used to assess the ideal electroporation parameters for the transfection of primary PAECs with All stars transfection negative siRNA control (Qiagen). Cells were electroporated as described in section 0. Samples electroporated without siRNA (sample ID 4) and unaffected cells, being exposed to 150 nM control siRNA (sample ID 5), were used as negative controls for each of the three different electroporation settings.

After electroporation, cells were resuspended in 100 µL fluorescence-activated cell sorting (FACS) buffer, containing TO-PRO-3 iodide (Invitrogen) staining (1:10000) for viability assessment. The tubes were kept on ice and in the dark. 10,000 cells were acquired per condition using a dual laser 4 colour FACSCalibur (Becton Dickinson) analyser. A standard protocol was followed using the programs Cellquest and FlowJo for data analysis and visual presentation (190).
2.8 Porcine microarray

Commercially available Porcine Genome Arrays (Affymetrix) containing 23,937 probe sets that interrogate approximately 23,256 transcripts from 20,201 Sus scrofa genes were used as gene chips for the transcriptional profiling. The sequence information of the spotted features was selected from public data sources including UniGene GenBank mRNAs, porcine mitochondrial sequences and rRNA sequences.

2.8.1 Endothelial cell samples

PAECs were transfected with KLF2 ABC siRNA and KLF4 BC siRNA as described in section 2.3.3, while the controls remained non-impaired, before seeding into microfluidic chambers. After 12h of static culture, the microfluidic chambers were connected to the designed flow circuit (see section 2.1.6) and PAECs were exposed to high laminar shear stress of 20 dyne/cm² for 48h. Cell lysis and RNA isolation were performed as detailed in section 2.5.1 and 2.5.2 and the RNA integrity number (RIN) was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). The RIN had to be greater than 10 as a quality control measure of sample RNA integrity.

In total 9 samples (3x control sheared without transfection, 3x sheared and transfected with KLF2 ABC and 3x sheared and transfected with KLF4 BC) were collected and 100 ng of total RNA from each sample were shipped to the Central Biotechnology Service in the Institute for Translation, Innovation, Methodologies and Engagement at Cardiff University School of Medicine. Some samples were generated with the support of Nataly Maimari, a collaborating PhD student also supervised by Prof. Rob Krams. According to the manufacturer’s 3’ IVT expression kit manual (191), the RNA samples were reverse transcribed to cDNA, further processed and fragmented. Hybridisation was performed at 45°C for 16h, prior to washing and scanning the gene chip according to the GeneChip expression wash, stain and scan manual (192).

2.8.2 Data analysis

CEL files obtained from the microarray facility were firstly analysed in-house by Nataly Maimari as her project involved the generation of a computational model from this genome array dataset. Normalisation was done using RMA in Expression Console, which comprises background subtraction, quantile normalisation and intensity summarisation.
Furthermore, outliers were excluded based on principle component analysis (PCA), hierarchical clustering and RLE plots, which are the log-scale expression values relative to the median expression value computed by probe set basis.

Filtering of probe sets that had either no associated gene symbol, expression levels in the lower 5% percentile or which showed little variation across all samples, were removed to improve the power of statistical tests. The Combat tool was subsequently used to correct for the batch effect. Statistical analysis of the data, which was grouped in gene lists, was then performed considering unpaired correlation. Using the statistics tool LIMMA estimates of the variance were obtained and FDR correction was performed based on q-value.

The pre-processed data obtained from Nataly Maimari was then analysed without further contribution using DAVID and KEGG databases. Functional annotation of the data was done with DAVID, which determined clusters according to an enrichment score (EScr). Firstly, genes were ranked by their expression difference and then the cumulative sum over the ranked genes was computed. The magnitude of the increment depended on the correlation of the gene with the phenotype. The maximum deviation from zero was recorded as EScr. Additionally, the biological meaning and functionality of the differentially expressed genes and their protein products were further assessed by pathway analysis based on KEGG.
3 CHAPTER DEVELOPMENT OF A FLOW SYSTEM

3.1 Introduction

ECs line the inner walls of arteries and veins, where they are constantly exposed to the blood flow, which exerts shear stress, a fluid mechanical friction force, onto the cells. The ex vivo study of ECs under flow, in comparison to static culture, takes this physiological flow environment into account. Low shear stress has been shown to be a plaque-modulating factor and therefore it plays a key role in the development of atherosclerosis (30). It is of interest to study shear stress-dependent gene expression, but in vivo studies of exclusively shear-related cellular responses are difficult to conduct. In vitro cultures enable the investigation of direct shear stress effects on cells, without the complexity of an in vivo system. Hereby parameters such as sterility, reproducible and defined flow rates, broad range of shear regimes, real time monitoring of cells, sufficient material for further molecular biology analysis as well as user-friendliness, have to be considered. These factors presented the main criteria for the selection of a suitable flow system.

Several flow apparatuses as discussed in section 1.1.4.3 have been developed and some of them even commercialised. Independent of their design, cell culture media is commonly used to mimic the friction force onto the cells, which is exerted by blood in vivo. From this the cells can withdraw all needed nutrients and secrete waste products, but frequent media changes are indispensable.

The orbital shaker model has been widely used to culture cells in vitro under flow. Cells are plated on a standard Petri dish, which is filled with media and placed on an orbital shaker (see section 1.1.4.3). The large area allows the culture of many cells at the same time, but these are not exposed to homogenous flow. Berson et al. have employed a CFD model to describe fluid behaviour in a cylindrical cell culture dish resulting from motion imparted by an orbital shaker apparatus (56). They concluded that the oscillatory flow varies between 0–5 dyne/cm² depending on the position of the cell in the dish.
The study described in this thesis investigated two distinct shear stresses. The orbital shaker design could not be employed, because the resulting shear rates were too low and not homogenous. Several other flow systems with parallel flow chambers have been developed to address the issue of non-homogenous flow exposure. One of these, the commercially available Bioflux system, is based on computer controlled perfusion and can achieve shear rates of up to 200 dyne/cm$^2$. The 96-well system subjects cells plated in separate wells to the same shear stress. It has been successfully used in drug screening, platelet adhesion and thrombosis assays on endothelial monolayers (193, 194). While the set up can be advantageous for high throughput applications, the incompatibility with standard microscopy slides did not suit the experimental design in this study as monitoring of the cells was necessary.

Cellflex designed a peristaltic flow system that can generate a shear stress range of 0.1-35 dyne/cm$^2$ using cells cultured on cover slips. Wang et al. studied the proteome of vascular ECs in response to laminar shear stress with this set up (195). The cells were cultured on a coated microscopy slide that had to be mounted upside down so that an upright microscope had to be used for visualisation. Such a microscope was not available and therefore use of the Cellflex system was not feasible.

Glycotech produces flow chambers casted in acrylic, which are compatible with standard microscopy slides. The acrylic cast is not autoclavable and the producer recommends using a new flow chamber for each experiment, which is not economical. Various gaskets shapes and sizes are available to adapt to the user needs, which is advantageous in comparison to the other systems described. Wiese et al. have nicely demonstrated the applicability of the Glycotech apparatus in leukocyte adhesion assays on the micro vascular endothelium (196). The system could unfortunately not be modified for time course experiments, which required the frequent collection of cell samples.

3.2 Design of a bespoke flow system

None of the above described shear stress apparatuses was either affordable or suited the experimental needs in this study. Therefore, the development of a robust, precise and easy to handle flow system for the culture of ECs in vitro under flow was aimed firstly. The only standard protocol in the lab was for the isolation of PAEC from the aorta and cell culture in transwells (187).
The establishment of a flow apparatus presented a novel undertaking in iteratively testing and improving relevant parameters. Not all of these parameters were defined from the beginning and some emerged only during the testing phase. The time-consuming process required a step by step approach as only a single parameter was changed at once to maintain traceability. The goal was to establish a system, which could be used to shear intact cell monolayers \textit{in vitro} at different magnitudes for prolonged times of up to 48h, preferably under sterile conditions and in a humidified incubator at 37°C and 5% CO$_2$. Experiments resulting in apoptotic phenotypes and cell loss were not regarded as successful, underlining the need for further optimisation.

It is known from literature that cells \textit{in vitro} have the ability to align to the direction of high flow when exposed to more than 15 dyne/cm$^2$ for 12h (197) as observed \textit{in vivo} at atheroprotected sides in the vasculature (198). The alignment was taken as a quality control read out for a successful experiment as it partially resembled the \textit{in vivo} morphology.

\section*{3.3 Results and discussion}

Assessment of flow experiments was mainly qualitative by monitoring the set up and observing the cell morphology. After several trial experiments, parameters influencing the experimental results could be summarised as follows: cell donor and passage, flow channel geometry and coating, cell seeding density and confluence before onset of shear, tubing material, media reservoir design, direction of flow and the pump system to maintain steady flow rates. The next paragraph addresses these issues in the aforementioned order. As stated before, the optimisation process was of reiterative nature rather than chronological as it is described here.

\subsection*{3.3.1 Optimisation of endothelial cell culture conditions}

The initial standard cell culture protocol for PAECs required a 20% serum concentration in CM-DMEM composed of 10% FBS (vol/vol) and 10% NCS (vol/vol) for static conditions. Phenotype studies showed that the cell morphology varied from the typical cobble-stone endothelial cell type (199). Large round cells, sometimes with almost dendritic elongations, could be observed (Figure 17 A). A media composition study was conducted varying several media components and their concentrations. It was found that the cell morphology changed greatly in different serum compositions.
Culturing in 10% FBS only (Figure 17 B) resulted in a homogeneous cobblestone endothelial-like monolayer, while culturing in 10% NCS only (Figure 17 C) resulted in a non-confluent, elongated, flat dendritic cell morphology that did not show similarity to ECs cultured in 10% FBS. This media optimisation study suggested that cell morphology could be greatly improved by excluding NCS from the media composition and that 10% total FBS seemed sufficient to maintain an endothelial phenotype. All further studies were conducted with 10% FBS only.

![Figure 17: Morphological study of PAEC (P1), derived from the same passage, seeded at the same density and cultured statically for 5 days either in 10% FBS and 10% NCS (A), 10% FBS (B) or 10% NCS (C). Bright field microscopy images at 10x (scale bar =75 µm).](image)

3.3.2 Cell donor and culture passage

During the development phase, inter-experimental variability could be noticed, which seemed to be independent from flow apparatus design. Although the same set up was used, some cells appeared more fragile in response to flow than others. This variability can be seen as an inter-donor variability. The in-house cell isolation procedure was standardised, but there was no control during the pig breeding period and slaughter procedure. Hence the observed variability could result from different treatments of the donor pigs from which the cells were isolated.

Another important parameter was the passage of cells. It has been shown that prolonged 2D culture of ECs in vitro leads to dedifferentiation The process of cellular dedifferentiation causes endothelial dysfunction, accompanied by downregulation of endothelial specific transcription factors (200). To avoid this phenomenon, only P1 and P2 cells were used in the present study.
3.3.3 Optimisation of the flow chamber

Several ECM protein coatings of the individual channels and different cell seeding densities were evaluated.

3.3.3.1 Chamber coating

The microfluidic chambers were coated with several *in vivo* ECM components that act as surface adhesives to investigate cell attachment and proliferation affinity in static and flow conditions. Briefly, chambers were coated for 1h with 1% gelatine, a hydrolysed form of collagen, 1% fibronectin, or 0.1% collagen IV as these reagents are mostly used for 2D culture of endothelial cells. After rinsing with PBS, 12,000 cells of the same passage were seeded into each channel. Cell distribution and density were evaluated 24h after seeding. Coating with gelatine and fibronectin (Figure 18 A and B respectively), resulted in homogenous cell distribution and approximately 80% confluence in static conditions. PAECs seemed to have less affinity for the collagen IV coated cell surface as they only attached, but did not proliferate (Figure 18 C). Hence testing of collagen IV as a chamber coating in sheared conditions was not further pursued.

![Figure 18: PAEC distribution and density 24h after seeding into flow channels coated with gelatine (A), fibronectin (B) and collagen IV (C). Bright field microscopy images at 10x (scale bar =75 µm).](image)

The cell seeding experiments on gelatine-, and fibronectin-coated surfaces were repeated followed by exposure to flow. Firstly, a flow regime of only 2h at 20 dyne/cm² after a 2h priming phase of 1 dyne/cm² was tested. Cell morphology changed to less elongated, but no cell detachment could be observed for either of the conditions (Figure 19). To assess the long term effects at reduced shear stress, a flow regime of 5 dyne/cm² for 24h was tested.
The morphological evaluation of the cells revealed cell loss and possibly apoptotic phenotypes for the fibronectin-coated condition (Figure 20 B), while cells seeded on gelatine seemed to be of cobblestone-like morphology and with an intact monolayer (Figure 20 A). This experimental series concluded that all flow chambers were to be coated with gelatine prior to cell seeding, because cell affinity and adaptation in static and sheared conditions were superior in comparison to fibronectin or collagen IV.

![Figure 19: PAEC distribution and density 12h after seeding followed by exposure to 20 dyne/cm² flow in horizontal direction for 2h in flow channels coated with gelatine (A) and fibronectin (B). Bright field microscopy images at 10x (scale bar = 75 µm).](image1)

![Figure 20: PAEC distribution and density 12h after seeding followed by exposure to 5 dyne/cm² flow in horizontal direction for 24h in flow channels coated with gelatine (A) and fibronectin (B). Bright field microscopy images at 10x (scale bar = 75 µm).](image2)

3.3.3.2 Cell seeding concentration

In addition to an adhesive coating, the initial cell seeding concentration presented an important parameter for successful flow experiments. The chamber manufacturer ibidi recommended a seeding density of 9,000 HUVECs per channel per 0.6 cm². This estimate also required a static culture prior to the onset of flow for 2-4 days.
Results and Discussion

A shorter culture period was preferred for this study and the seeding density had to be optimised accordingly. To investigate the proliferation capacity of PAECs, P1 cells were seeded at different concentrations on gelatine-coated channels, ranging from 9,000–65,000 cells per channel and monitored 12h, 24h and 36h (not shown) post seeding. Figure 21 provides an overview of the cell morphology and distribution for the varying conditions. Rows present the seeding densities of PAECs per channel and columns display the time when a representative image of the condition was taken. The first two columns display the morphology and cell density 12h after seeding at 10x and 40x magnification and the third column after 24h at 10x magnification.

A seeding density of < 30,000 cells/channel resulted in a non-confluent cell monolayer 12h post seeding. The 40x magnification clearly shows that cells were sparsely distributed without cell-cell contact. Especially in flow experiments, the formation of cell junctions is important for cell communication and endothelial homeostasis. In vivo junctions are required to maintain the integrity of the vessel wall. Modification of the molecular organisation and intracellular signalling of junction proteins might have complex effects on vascular homeostasis (201). Without these cells are much likely to detach as they cannot withstand the physical force. This could be demonstrated in an example experiment, where cells were seeded on gelatine at a density of 12,000 cells/channel. After 12h of static culture, cells were subjected to 5 dyne/cm² flow for 12h. Figure 22 shows images of the static control in comparison to the sheared condition. It can be noticed that even at a low shear rate cells lost cell-cell contact, they shrank in size and the light deflections are a sign of cell detachment. Hence, a seeding density of < 30,000 cells/channel was regarded as non-ideal. After 24h, giving cells the time to proliferate, the previously seen gaps have decreased and a seeding density of 24,000 cells resulted in a confluent monolayer.

For seeding densities > 30,000 cells, a dense over-confluent monolayer could be seen at 12h. Dome-like structures appeared in the condition of 65,000 cells/channel (Figure 21). Proliferation was inhibited and the cells grew as tightly together as possible (24h) and eventually detached. For a density of 30,000 cells/channel, an ideal monolayer at 12h after seeding could be achieved. Homogenous cell-cell contact was evident, which was regarded as a prerequisite for cell exposure to flow. Even 24h post seeding the monolayer was approximately 90% confluent, which would still be feasible for flow studies. Slight inter-experimental differences in attachment affinity and proliferation capacity are possible, but this small study determined an optimal seeding density of 30,000 cells/channel. This seeding density was used in all further experiments, unless stated differently.
Figure 21: PAEC distribution and density 12h and 24h after seeding in gelatine-coated microfluidic chambers with 0.6 cm² area (columns) at different concentrations ranging from 9,000–65,000 cells/channel (rows). Representative bright field microscopy images at 10x (scale bar =100 µm) and at 40x (scale bar =25 µm).
Results and Discussion

3.3.4 Validation of tube material and connectors

One major issue that had to be overcome was the trapping of gas bubbles in the flow circuit. As described in the section 3.3.6, the formation of small bubbles travelling through the system caused damage to the cells and led often to cell detachment over time as seen in Figure 23. Approximately 4h after initiation of flow the first phenotypical changes could be observed. Cells lost their cobblestone morphology changing into a more elongated state. Cell junctions were disrupted and the monolayer disintegrated over time (Figure 24). It can be assumed that the continuous scratching of bubbles activated pathways leading to apoptosis. This has also been reported by O Lane et al., who could observe similar effects using a comparable flow circuit design (202).

Figure 22: PAEC distribution and density in gelatine-coated flow channels after seeding at low concentration of 12,000 cells/channel followed by 12h static culture and exposure to 5 dyne/cm² flow in horizontal direction for 12h (shear) in comparison to static condition (ctr). Representative bright field microscopy images at 10x (scale bar =75 µm).

Figure 23: PAEC distribution and density in gelatine-coated flow channels after seeding 30,000 cells/channel followed by 12h static culture and exposure to 20 dyne/cm² flow in horizontal direction for 6h and 12h in comparison to static condition (ctr). The flow circuit was equipped with gas permeable silicon tubing and ibidi luers. Representative bright field microscopy images at 10x (scale bar =75 µm).
Results and Discussion

Figure 24: PAEC morphology in gelatine-coated flow channels after seeding 30,000 cells/channel followed by 12h static culture and exposure to 20 dyne/cm² flow in horizontal direction for 6h and 12h. The flow circuit was equipped with gas permeable platinum cured silicon tubing and ibidi luers. Representative bright field microscopy images at 20x (scale bar =35 µm).

To investigate the cause of cell detachment and death the individual components of the flow circuit were analysed. Several tubings as listed in Table 6 were tested to determine if the material of the tubing could play a role. All used materials were Food and Drug Administration (FDA) approved and according to the International Organization for Standardization (ISO) regulations suitable for cell culture experiments, because they were inert, chemically stable and autoclavable. As it is difficult to quantify bubble formation, the results described are of rather subjective nature. A great improvement could be achieved by using the gas impermeable C-flex material. In comparison to polypropylene, platinum cured silicon and pure silicon tubes, less bubbles formed and were trapped in the system. The cells did not experience gas insufficiency as the bottom of the channel was manufactured of gas permeable material. Positioning of the chambers in a small angle guaranteed that gas exchange at these surfaces was not hindered. In addition to the material choice it seemed important that the media was completely degassed prior to flow initiation and that the entire flow system was kept inside the incubator.

Figure 25: Polypropylene male luers fitting to female luer adapters of the channel inlets. Luer A (ibidi) with a narrowing outlet of 0.8 mm and luer B (The Westgroup) with a 1/3 longer luer adapter and uniform diameter of 3.2 mm.
Initially, the elbow luers manufactured by ibidi were used (Figure 25 A). They did not hinder bubbles from travelling through the individual channels. Once bubbles were introduced into the circuit, they almost certainly moved through the entire set up, which was not desirable as they scratched the monolayer and eventually caused cell detachment. In addition, the geometry of the elbow luer produced a pressure increase. The outlet of the elbow luer reduced 4-fold from 3.2 mm to 0.8 mm ID. As a consequence, this “funnel effect” led to increased flow and pressure at the narrowing, which affected the flow profile.

The device was changed to a polypropylene elbow luer of uniform diameter with an ID of 3.2 mm from The Westgroup (Figure 25 B). Hence it was not flow constrictive as the design from ibidi. Also, the side coupled to the channel was 1/3 longer, which proved to be beneficial. In case bubbles formed, they collectively stayed in this part of the luer, without travelling through to the next channel. Dynamic up and down bouncing could be observed when using a peristaltic pump, but to the degree that the channel surface was not reached. It can be concluded that the selection of non-gas permeable tubing material C-flex and a different elbow luer design greatly improved the problem of gas bubble travel through the circuit. Bubble formation could not be completely avoided, but limited to the extent that contact with cells did not occur and that these could therefore not be scratched of.

3.3.5 Media reservoir design

Two different media reservoir designs (see section 2.1.3) were tested in the flow set up in combination with prototype II of the peristaltic pumps. Both worked well over time periods < 12h. The self-designed glass chamber provided enough closure to keep the media sterile, but did allow for sufficient gas exchange. This was an important parameter as pressure increase due to degassing in the reservoir has been observed over prolonged times. The Schott bottle had to be modified with a hydrophobic filter, which also allowed for gas exchange. For time periods > 12h, the Schott bottle seemed advantageous as it provided a larger media capacity (300 mL) in comparison to the glass chamber (50 mL). Due to the larger volume, media did not have to be exchanged even for culture periods of up to 48h. Another aspect was that, although all system components were of optimised configuration, cell damage could be noticed when the glass chamber was used. It can be hypothesised that cell debris circulating in the media could have mechanically damaged the cells, possibly leading to detachment and enrichment of even more debris.
In the small volume of the glass chamber these were not diluted sufficiently, while the larger volume could compensate the effect. This is a rather subjective evaluation, but it provides an explanation for the observations. As a result, the Schott bottle filled with approximately 300 mL of culture media was used for flow experiments of prolonged time.

3.3.6 Pumps and experimental set up

In addition to the components mentioned above, a pump system had to be selected for the flow system. The prerequisites were that the pump would generate reproducible low-pulse flow rates over a period of 48h under the load of an experimental set-up. The system had to be compatible with cell culture requirements, for example facilitate fast sterile uncoupling of a channel. Additionally, easy handling was of advantage as the time line experiments required quick processing. Figure 26 provides a schematic drawing of the circuits’ main components.

![Figure 26: Schematic drawing of the flow circuit with a pump, a media reservoir and the microfluidic chamber with 6 flow channels coupled in series.]

3.3.6.1 Peristaltic system prototype I

The first pump system integrated into the flow circuit was a Minipuls 3 pump (Gilson). The pump head consisted of 10 stainless steel rollers and the tubing was fixed with adjustable clamps. In order to stay in place for a prolonged period of time, the clamps had to be tightened quite strongly, leading to over-compression of the tubing in the pump head. This also resulted in a pulsatile flow profile at higher flow rates.

Initially, the entire flow circuit as seen in Figure 26 was placed into the incubator, which maintained a humidified environment of 37°C and 5% CO₂. While operating, the pump unfortunately generated access heat causing the temperature to increase to > 39°C.
This temperature rise resulted in cell death, not only of the sheared, but also of the static conditions. To adapt to this problem, the pump was placed outside the incubator. The colder room temperature favoured uptake of gas, which then formed bubbles when entering the warmer incubator environment. These then enriched the media in the reservoir with gas and caused the circulation of bubbles. As previously described, bubbles could tear cells off the surface, which resulted in severe cell loss. Besides cell detachment, changes in cell morphology could be observed (Figure 27). This was not in conjunction with cell loss, which could usually be observed already at 10x magnification.

![Figure 27](image)

*Figure 27: PAEC distribution and density in gelatine-coated flow channels after seeding 30,000 cells/channel followed by 12h static culture and exposure to 20 dyne/cm² flow in horizontal direction for 6h in comparison to static condition (ctr). The flow circuit was equipped with non-gas permeable C-flex tubing and optimised elbow luers. Representative bright field microscopy images at 10x (scale bar = 75 µm).*

Only at higher magnifications the formation of vacuoles could be noticed (Figure 28). The individual cells were still in contact with neighbouring cells, but their shape changed from cobblestone, as in static culture, to very round and compact in sheared conditions. The effect occurred between 2-4h of high flow exposure and worsened with time. It was hypothesised that the distinct phenotype changes were in response to the irregular pulsatile flow. As mentioned previously, the tube clamps could not be fine tuned and in order to stay in place, they had to be tightened strongly. This over-compressed the tubing and the 10 rollers did not synchronise the flow rate. At high shear stress of 20 dyne/cm² the cells were exposed to pulse frequencies many folds higher than the *in vivo* rate (203). This could have triggered an abnormal apoptotic response.
In summary, the Minipuls 3 pump did not fulfil the criteria required for the flow circuit in this study. Although the problem of over-heating was compensated, it resulted in gas enrichment of the media, which caused bubble formation and led to cell loss. In addition, high pulse frequencies might have initiated an apoptotic cellular response as cells appeared round with vacuoles located in the cytoplasm.

### 3.3.6.2 Perfusion system

The commercially available perfusion system (ibidi) had the advantage that the fluidic unit (Figure 15) could be placed inside the incubator, while the pump was located outside. As the flow was driven by filtered air, the mechanical stress onto cells through pulsation was very low. The generated flow could be precisely tuned with ± 1 mbar accuracy. After a couple of validation experiments, which confirmed good reproducibility and user friendly handling, the system was used for time course evaluations. As described in section 4.3.2, sampling after 2h, 4h, 6h, 12h and 24h was performed to investigate gene expression changes over time and in dependence of flow magnitude.

The gene expression results are presented and discussed in Chapter 4, but the phenotypical changes are discussed below. Criteria for validation of a functional pump system were the maintenance of endothelial morphology for sheared PAECs and the alignment to the direction of flow when exposed to high shear over prolonged times (Figure 32).

Images of sheared cells were taken before further processing for real-time PCR applications and these are shown for a representative time course experiment in low flow at 5 dyne/cm² (Figure 29) and high flow at 20 dyne/cm² (Figure 31).
In the time course of 24h exposure to 5 dyne/cm² no detachment of cells could be observed (Figure 29). The endothelial monolayer was intact and the cells did not show an abnormal morphology, such as enhanced elongation or vesicle formation, as observed with the peristaltic pump. Their phenotype was comparable to the non-sheared control condition, which can be best seen at a higher magnification (Figure 30). The cells were in tight contact with each other and had cobblestone, polygonal morphology. Horizontal alignment in the direction of flow could not be observed at any time for the exposure to low flow.

**Figure 29:** PAEC distribution and density in gelatine-coated flow channels with a seeding density of 30,000 cells/channel followed by 12h static culture and exposure to 5 dyne/cm² in horizontal direction for 2h, 4h, 6h, 12h and 24h respectively in comparison to static condition (ctr). The flow system was composed of a perfusion pump using C-flex tubing and elbow luers. Representative bright field microscopy images at 10x (scale bar =75 µm).

**Figure 30:** PAEC morphology in gelatine-coated flow channels with a seeding density of 30,000 cells/channel followed by 12h static culture and exposure to 5 dyne/cm² in horizontal direction for 24h in comparison to static condition (ctr). The flow system was composed of a perfusion pump using C-flex tubing and elbow luers. Representative bright field microscopy images at 20x (scale bar =35 µm).
Also exposure to 20 dyne/cm² for prolonged time of up to 24h did not cause cell detachment (Figure 31). The monolayer remained intact throughout the entire channel with possibly tight junction formation between individual cells as observed by microscopy. None of the previously described apoptotic phenotypes could be noticed. Still, high shear stress caused a morphological alteration from polygonal cobblestone to spindle-shaped with the major axis aligned horizontally in the direction of flow. This response could be observed from 12h after initiation of flow and it increased with time (Figure 32).

The observed effect has been described by several other groups (12) and it validates the functionality and applicability of the perfusion system for flow experiments. In can therefore be concluded that the perfusion system set up as described in Chapter 2.1.5 presented an ideal experimental design as it could operate in low and high shear stress regimes, triggering a functional response in the sheared cells.

Figure 31: PAEC distribution and density in gelatine-coated flow channels with a seeding density of 30,000 cells/channel followed by 12h static culture and exposure to 20 dyne/cm² in horizontal direction for 2h, 4h, 6h, 12h and 24h respectively in comparison to static condition (ctr). The flow system was composed of a perfusion pump using C-flex tubing and elbow luers. Representative bright field microscopy images at 10x (scale bar =75 μm).
3.3.6.3 Peristaltic system prototype II

The availability of the perfusion system was unfortunately limited. Different pumps were investigated and the peristaltic prototype II (Atto) presented a suitable alternative (Figure 16). The advantage over prototype I (Gilson) was that it could be placed inside the incubator without causing an increase of the overall temperature. Still, the motor has been proven strong enough to generate the rotation speeds required for shear regimes of up to 35 dyne/cm². Six plastic rollers could produce a constant flow rate and the tubing in the pump head was fixed with adjustable side clamps and kept in place by mounting a plastic cover tightly on top. This set up reduced over-compression of the tube and resulted in an almost non-pulsatile flow profile. Readjustments of the pump head tubing had to be made after prolonged flow as the tube slightly stretched with time and could get jammed between the rollers. As a result, the flow was reduced and in a worst case scenario the tubing would eventually break, causing spillage and complete cease of flow.

Different flow directions, “pushing” or “pulling” mode, were also investigated, but the obtained results were similar. The final configuration as shown in Figure 26 was by withdrawing of the media from the reservoir through the flow chamber, rather than “pushing” it through.

The design of prototype II addressed basically all weaknesses that were identified for the peristaltic prototype I. In comparison to the perfusion pump, reproducibility was similar and the same results could be achieved. Cells did not change their morphology in comparison to the static condition when exposed to low flow. The monolayers remained intact, showing no sign of cell loss for both shear regimes. In high shear conditions, cell alignment in the direction of flow could be observed after approximately 12h of shear stress exposure. The pump was used for all gene silencing studies (see section 4.3.3) and examples of cell morphologies are given in Chapter 4.
3.4 Conclusion

This chapter covered the design and optimisation of all components required for the flow circuit, resulting in the development of a reliable and reproducible system for culturing PAECs under flow. The reiterative evaluation addressed issues such as cell detachment and abnormal morphological changes, which occurred during the exposure to flow in non-optimised conditions. Firstly, the media composition used for static and flow culture was reduced to 10% total FBS serum. It could be shown that the addition of 10% NCS, which was so far the standard procedure, led to dedifferentiation of PAECs into macrophage-like cells. These qualitative observations were based on cell morphology.

As cell culture devices for flow experiments, the commercially available µ-Slides VI 0.4 were chosen. In comparison to other devices such as the Bioflux perfusion system, sufficient numbers of cells for further applications such as real-time PCR and protein immunoblotting could be retrieved. The sterility was guaranteed as the chambers were for single use only. In comparison to other disposable systems such as Glycotech chambers, the µ-Slides VI 0.4 were still affordable. The standardised microfluidic chamber geometry was very well defined and mathematically assessed for accurate flow calculation.

The channel surface had to be coated with adhesive proteins as PAECs did not show high affinity to the tissue culture-treated surface. Coating with 1% gelatine resulted in better cell attachment and proliferation as in comparison to collagen IV and fibronectin, especially in flow conditions. The determined optimal seeding density was 30,000 cells/channel. Cells grew in 80% confluent monolayers when cultured statically for 12h before the onset of flow. Tight cell junctions, formed during this time period, gave enough support to withstand the physical stress of flow exposure. Nevertheless, the cell density still allowed for dynamic cell movement so that alignment in the direction of flow at high shear stress occurred.
Several tubing materials were investigated and non-gas permeable C-flex was the most suitable for the application. Especially in the set up where not all circuit components were placed in the incubator. Gas exchange was still possible through the bottom surface of the flow chamber as well as the media reservoir.

The formation of bubbles and their travelling through the system presented a major obstacle as cells were damaged, often resulting in severe cell detachment. Changing the initially used luer adapters to a type with a longer neck solved the problem partially. Bubbles that formed were trapped, moving up and down the elbow luer, but they did not reach the cell surface nor did they travel through the tubing. Hence, this modification could reduce the harmful effects caused by bubbles, scratching over the cell monolayer.

Both media reservoirs tested were suitable for flow applications as they kept the equilibrated media sterile and they could be easily coupled to the tubing. A large volume seemed more advantageous over longer culture periods as media changes were not needed and small debris circulating in the system were better diluted. Therefore, the converted 300 mL Schott bottle was used for shear stress experiment lasting more than 12h.

Three different pumps were tested for their suitability to drive the liquid flow through the circuit. The peristaltic prototype I did not perform well as the temperature gradient within the circuit, due to the fact that the pump could not be accommodated in the incubator, resulted in gas uptake outside and release of gas bubbles inside the system. The configuration of the pump head also required extreme compression of the tubing, so that the resulting flow profile was pulsatile. The perfusion pump and the peristaltic prototype II suited the experimental design in this study. They were compatible with all cell culture requirements, produced reliably the desired flow rates over prolonged time and their handling was user friendly. To summarise, the developed flow circuit fulfilled all aimed requirements and the application of cell culture under flow is further described in the following Chapter 4.
4 CHAPTER GENE EXPRESSION STUDIES

4.1 Introduction

The focal nature of plaque development in curvatures and bifurcations of the arterial tree is caused by differences in blood flow along the endothelium. In regions of laminar blood flow, high shear stress results in an atheroprotected endothelium (198, 204, 205), while low shear stress is associated with the occurrence and progression of plaques (30, 204).

Mechanistic studies have shown that shear stress can profoundly influence the endothelial phenotype by modulating vascular gene expression (52, 206). Dekker et al. first described the flow-dependent expression of KLF2 TF, which mediates an atheroprotective phenotype generated by shear stress (43). Several studies provide in vivo evidence for the importance of KLF2. Firstly, the generation of KLF2 null mice resulted in embryonic lethality at E12.5−14.5 due to haemorrhages, which was associated with specific defects in blood vessel morphology (98). In the developing chick embryo, KLF2 is expressed in regions of highest shear stress, while ET-1 and eNOS expression, at least in the later stages, are related to shear stress (207). Parmar et al. have investigated a silent heart zebrafish model, which did not show KLF2 expression in animals with a non-contractile heart in comparison to wild type fish with a functional heart. They demonstrated the clear expression dependence on blood flow and hypothesised that the mode of regulation may be evolutionary conserved (39). Several groups also showed the same dependence in mature tissue from human, pig or mouse arteries (116, 129).

In vitro studies of cultured ECs gave further insight on the regulation and function of KLF2. Direct binding of KLF2 to the promoter region of eNOS and the induction of total enzymatic activity has been observed by SenBanerjee et al. (45). They further proved evidence that KLF2 overexpression potently inhibited the induction of VCAM-1 and endothelial adhesion molecule E-selectin in response to various pro-inflammatory cytokines. In vitro flow assays demonstrated that cell attachment and rolling were markedly attenuated in KLF2-transduced endothelial monolayers. Also recruitment of the transcriptional co-activator cyclic AMP response element-binding protein (CBP/p300) by KLF2, as a unifying mechanism for these various effects, has been shown.
Results and Discussion

CBP/p300 is a cofactor for the activation of the inflammatory NF-κB response and by competing against this factor, KLF2 negatively regulates p65, a subunit of NF-κB and AP-1 (208). Additionally, an anti-thrombotic effect has been observed by Lin et al., who found that KLF2 strongly induced TM and reduced PAI-1 expression (47). In summary, these findings support the role of KLF2 as an atheroprotective, anti-inflammatory, anti-oxidant and anti-coagulant regulator.

Upstream regulators of KLF2 have also been identified. Shear stress-induced activation of the mechanosensitive MAPK pathway has been shown to activate KLF2 expression by MEF2 binding (72). Kinase family members PI3K and 5′ adenosine monophosphate-activated protein kinase (AMPK) have also been implicated to positively stimulated KLF2 expression (37, 209). Treatment with simvastatin and resveratrol led to the upregulation of KLF2 levels (45, 210), which were comparable to expression levels induced by high shear stress. The latest publications have also implied that single stranded non-coding miRNAs, in particular miR-92a, can up-regulate KLF2 (128). Pro-inflammatory stimuli and cytokines, such as TNFα and IL1β, suppress KLF2 through inhibition of MEF2 by NF-κB and HDACs (47, 102).

Another member of the KLF family, KLF4, emerged in the last decade as a novel shear-induced vasoprotective transcriptional regulator. Microarray results published in 2001 by McCormick et al. (44) showed a 5-fold upregulation of KLF4 in sheared HUVECs in comparison to static conditions. Later on a study revealed that KLF4 could be induced by pro-inflammatory stimuli and shear stress. Overexpression led to an increase of multiple anti-inflammatory and anti-thrombotic factors including eNOS and TM, whereas gene silencing of KLF4 caused enhancement of TNFα-induced VCAM-1 and Tf expression (110). Functionality of KLF4 was demonstrated by decreased inflammatory cell adhesion to the endothelial surface and prolonged clotting time under an inflammatory state. Villarreal et al. further investigated the signalling mechanisms of KLF4 by transcriptional profiling and confirmed its anti-inflammatory, vasodilatory and anti-thrombotic effects. These roles are functionally similar to what has previously been observed for KLF2. A comparison of global gene regulation demonstrated that 552 (42.4%) of the genes regulated by KLF2 were also controlled by KLF4 (104), suggesting a significant degree of transcriptional redundancy between these transcription factors. Indeed, it has been observed that both share the CACCC motif as a common canonical transcriptional binding site (211).
Phylogenetic studies indicate that KLF2 and KLF4 have the closest relationship amongst all members of the KLF family (90) as described in section 1.3. It has been shown that both play similar functional roles as they can induce key endothelial vasoprotective genes such as eNOS, TM and CNP (110). They differ in their response to pro-inflammatory stimulation by TNFα and IL1-β. While KLF2 is repressed, KLF4 is positively induced. It remains unknown if their transcriptional regulatory functions have compensatory effects.

Although KLF5, KLF7 and KLF11 are also expressed in ECs, the shear-dependent expression of any other member of the KLF family has not yet been confirmed. KLF6 was shown to be up-regulated during vascular injury and that it could induce expression of genes important in response to injury such as tPA and TGFβ (212). Das et. al have demonstrated that MMP9 was suppressed by KLF6 in conjunction with SP1 (123). White et al. have reported an upregulation of KLF6 comparing expression levels after 24h exposure to elevated shear stress of 75 dyne/cm² or 15 dyne/cm² (41).

### 4.2 Hypothesis and Aim

This study aimed to investigate the gene expression levels of KLF2, KLF4, KLF6, as they have previously been shown to be shear-dependent, and eNOS in response to different shear stresses in a time-dependent manner. Recent publications indicated a possible compensatory mechanism between KLF2 and KLF4. The work in this thesis further examined a potential relationship.

The hypothesis was that core circuitry of KLF2, KLF4 and KLF6 exists, similar to what has been observed for the regulation of ES cell self-renewal (131), which synchronises the atheroprotective transcriptional program. eNOS was chosen as the common target gene for all KLFs. The hypothesis also included a compensatory mechanism for loss of function of a single KLF by the remaining KLFs. Flow experiments were conducted with primary PAECs and their gene expression was analysed by qPCR. This technique, once optimised, can be very precise and robust. Therefore, it presented an ideal method for the quantification of even small gene expression differences (213). As outlined in section 4.1, the expression of KLF2, KLF4 and KLF6 is known to be shear responsive. In this study the temporal effects of shear stress were examined in more detail by conducting time course experiments using the developed flow system as described in Chapter 3.
It was of interest to investigate first the response of KLF2, KLF4, KLF6 and eNOS to an atheroprone shear stress of 5 dyne/cm² and an atheroprotected shear stress of 20 dyne/cm². To further understand a possible interaction between the KLFs and eNOS, gene silencing using siRNA was performed, individually targeting KLF2, KLF4 or both simultaneously. The resulting data was then used as a foundation to hypothesise a KLF network that modulates the atheroprotective phenotype of ECs.

4.3 Results and discussion of qPCR optimisation and time-dependent gene expression analysis in response to low and high shear stress

4.3.1 Optimisation of qPCR methodology

The rational of primer selection and verification of sequence specificity in silico has been described in section 2.5.5. In order to ensure specificity and selectivity of the primers to their target sequence an initial optimisation of reaction parameters was required. The process is described in the following chapter with an example for each step. A series of qPCR assays was performed using the optimised reaction parameters. The expression level analysis for KLF2, KLF4, KLF6 and eNOS at different shear regimes over a time course of 24h is presented and discussed in section 4.3.2. The chapter concludes with a comparison of gene expression patterns observed for the different shear regimes.

4.3.1.1 Specificity of primers

The specificity of primers to their target sequence is very important and has to be tested as amplification of false sequences can lead to incorrect data acquisition. All PCR products for a particular primer pair should have the same melting temperature (T_m) unless contamination, mispriming or primer-dimer artefacts occur. Determination of the T_m and validation of the amplicon length for each primer pair have been used as quality control checks.

For each qPCR reaction an additional temperature gradient has been added after the amplification cycle. The temperature was always raised by a fraction of a degree and the change in fluorescence (dl) was measured. At T_m the two strands of DNA dissociated and the fluorescence rapidly decreased. The melting curve was generated by taking the first negative derivative of the plot, which results in a peak at the T_m. A representative example for KLF4 is shown in Figure 33, in which only a single sharp peak at T_m of 83.75°C can be detected. As no other peaks were present, contamination of the reaction, unspecific amplification or primer dimerisation can be excluded. Primer-dimer artefacts would have resulted in a second peak with a lower T_m.
Results and Discussion

Figure 33: Melting curve for KLF4 (A) and the graph of the corresponding negative first derivative from the temperature gradient probe dissociation curve at 50% (B).

For each qPCR experiment shown in this thesis it has been confirmed that all individual primer pairs had always nearly the same T<sub>m</sub>. A representative validation of the T<sub>m</sub> for one experiment is shown in Table 13.

Table 13: Average of melting temperatures ± standard deviation (SD) of primer pairs in qPCR reactions of representative experiments (n=60).

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Melting Temperature (T&lt;sub&gt;m&lt;/sub&gt;) °C</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLF2</td>
<td>77.22</td>
<td>± 0.25</td>
</tr>
<tr>
<td>KLF4</td>
<td>83.75</td>
<td>± 0.22</td>
</tr>
<tr>
<td>KLF6</td>
<td>84.00</td>
<td>± 0.29</td>
</tr>
<tr>
<td>eNOS</td>
<td>83.73</td>
<td>± 0.28</td>
</tr>
<tr>
<td>HPRT1</td>
<td>81.61</td>
<td>± 0.32</td>
</tr>
</tbody>
</table>
As a second visual quality control check SDS gel electrophoresis with qPCR product samples and their NTC containing no DNA was performed. Lanes loaded with samples derived from amplification reactions with KLF2, KLF4, KLF6, eNOS and HPRT1 primers showed only a single band at the expected base pair size of their amplicon (Figure 34) as detailed in Table 10. This quality control check excluded the possibility of unspecific template amplification during qPCR and validated the specificity of the primer pairs for their target gene. Only the NTCs for KLF4 and HPRT1 resulted in very weak bands of low molecular weight (MW) of around 50 bp. These are likely to be products of primer dimerisation, rather than reaction contaminations. Further primer concentration optimisation could have helped to eliminate dimerisation. As the amplification efficiencies were already more than 90% for both genes and the dissociation curves for both did not show primer dimer peaks at low melting temperatures, further optimisation was neglected.

![Figure 34: SDS gel to verify the amplicon length and primer specificity for KLF2, KLF4, KLF6, eNOS and HPRT1. The qPCR product (+) and the NTC (-) were separated via electrophoresis.](image)

### 4.3.1.2 Optimisation of primer concentration and melting temperature for qPCR

For initial optimisation experiments the primer concentrations were fixed at 300 nM to optimise the $T_m$. PCR experiments with a 12-step temperature gradient, ranging from 52°C to 72°C horizontally across a PCR plate, were conducted. From comparison of plots displaying fluorescence intensity versus number of cycles, the ideal amplification temperature was determined. The selection criteria were based on threshold Ct in the dynamic range and shape of the amplification curve.
Figure 35 shows representative graphs from the obtained results for KLF4 as an example for too low amplification temperature (A), ideal amplification temperature (B) and too high amplification temperature (C). In a range from 52-54.5°C the fluorescence signals reached threshold levels only at very late cycle numbers and the amplification curves had no linear range (A). With increasing temperature the Ct value decreased and at around 60.7°C to 61.4°C ideal sigmoidal amplification curves could be obtained (B). Quantification was performed during the exponential phase, when the target DNA sequence starts to double in concentration with each cycle and for this temperature range the efficiency was optimal (see section 4.3.1.3). Hence, an ideal amplification temperature of 61°C was determined for KLF4. Temperatures of more than 63.5°C did not change the Ct value drastically, but resulted in decreased slope steepness, which reduced the efficiency (C).

Figure 35: Amplification curves of KLF generated at different temperatures as examples for too low amplification temperature (A), ideal amplification temperature (B) and too high amplification temperature (C).
4.3.1.3 Dynamic range of qPCR standard curves

The ideal temperature determined through the gradient amplification in section 4.3.1.2 was chosen to test the dynamic range of primers. Serially diluted samples were used to generate amplification curves, which ideally showed a linear amplification relationship over a broad dynamic range (Figure 36).

Standard curves for each primer were generated between the Ct values (y-axis) and the dilution ratio on a log scale (x-axis). With a linear regression analysis of the standard plot the relative concentration of unknown samples and the slope of the equation, which signifies the efficiency of the PCR reaction, could be determined.

![KLF4 dynamic range](image)

*Figure 36: Amplification curves for KLF4 from a 5x serially diluted sample at optimal Tm. The red line represents the fluorescent threshold at which the Ct value is determined.*

Figure 37 represents an example of a standard curve showing the linear dynamic range of the KLF4 primers. The coefficient of determination $R^2=0.999$ for this standard curve means that the regression line almost perfectly fits the data.
Results and Discussion

The efficiency for all qPCR assays varied between 90-99% and the range of Ct values, for which a linear relationship could be assumed, was very broad covering the entire concentration scale of unknown samples within the dynamic range of the reagents. The coefficient of determination $R^2$ ranged from 0.980 to 0.999 indicating that the regression line approximated the real data points very well. Absolute quantification assumes that all amplification efficiencies are approximately the same for all samples. The parameters for all primer pairs are listed in Table 14.

Table 14: Overview of the standard curve parameters obtained for the primer pairs used in qPCR assays.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>$T_m$ (°C)</th>
<th>Efficiency (%)</th>
<th>Threshold fluorescence</th>
<th>Slope</th>
<th>$Y$ - intercept</th>
<th>$R^2$</th>
<th>Ct range</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLF2</td>
<td>58</td>
<td>90</td>
<td>492</td>
<td>-1.54 ln</td>
<td>21.07</td>
<td>0.998</td>
<td>21-33</td>
</tr>
<tr>
<td>KLF4</td>
<td>61</td>
<td>91</td>
<td>873</td>
<td>-1.54 ln</td>
<td>19.70</td>
<td>0.999</td>
<td>19-32</td>
</tr>
<tr>
<td>KLF6</td>
<td>61</td>
<td>95</td>
<td>705</td>
<td>-1.50 ln</td>
<td>21.24</td>
<td>0.998</td>
<td>21-33</td>
</tr>
<tr>
<td>eNOS</td>
<td>58</td>
<td>90</td>
<td>524</td>
<td>-1.56 ln</td>
<td>21.08</td>
<td>0.980</td>
<td>21-34</td>
</tr>
<tr>
<td>HPRT1</td>
<td>61</td>
<td>99</td>
<td>800</td>
<td>-1.45 ln</td>
<td>20.40</td>
<td>0.996</td>
<td>20-31</td>
</tr>
</tbody>
</table>

4.3.2 Results and discussion for shear-dependent gene expression of KLF2, KLF4, KLF6 and eNOS

4.3.2.1 Time course 2-24h exposure to low or high shear stress

As detailed in the introduction, the anti-inflammatory and anti-thrombotic endothelial phenotype in atheroprotected regions of high shear stress differs greatly from the phenotype in regions prone to atherosclerosis, where shear stress is low.
This section presents the results on gene expression patterns of PAECs over time when exposed to high shear stress of 20 dyne/cm$^2$ or low shear stress of 5 dyne/cm$^2$. For each measurement at 2h, 4h, 6h, 12h and 24h one microfluidic chamber was attached in series to the flow circuit. At the respective time point, the flow was stopped briefly to uncouple an individual chamber for further processing. It was of interest to quantify the gene expression and changes of KLF2, KLF4, KLF6 and eNOS over time using PCR methodology.

For each time point a total of 5 to 16 samples (n=5-16) has been analysed after exclusion of outliers using z-score analysis with threshold of 1.4 as detailed in section 2.5.9. The difference in the number of samples for each condition is due to the fact that not all time points were collected similarly for each experiment and that some data points were excluded based on z-score analysis. This methodology was chosen for the identification of outliers based on statistics and not arbitrary selection. A score between 1.3 and 1.6 is a commonly selected as the exclusion criteria. The distribution of z-scores for all time points per gene in high and low shear is shown in the scatter plots with the selected threshold z-score of 1.4 for the exclusion of outliers indicated by a line (Figure 38 and Figure 39).

![Figure 38: Scatter plot showing the distribution of z-scores from expression data for all time points (0-48h) for KLF2, KLF4, KLF6 and eNOS. A line at z-score 1.4 indicates the threshold level above which outliers were removed. Samples were sheared at 20 dyne/cm$^2$. Data was assessed by qPCR and normalised to HPRT1.](image-url)
Quantitative gene expression data from qPCR is presented as a function of average relative gene expression levels with SD normalised to HPRT1 and set to unity for the static condition at 0h. Data points were connected with trendlines, dotted for high shear stress and dashed for low shear stress. The sampling was not continuous, but display of a trend line was chosen to better visualise changes in expression over time. Comparison of individual experiments revealed that the absolute gene expression values differed in magnitude, but showed similar expression patterns in dependence of flow. This can either be due to large experimental errors in the procedure that propagated or due to a true biological inter-donor variability. Computational modelling efforts addressed this interesting observation in more detail, as briefly described in section 7.1.2.

KLF2 expression in response to high shear stress was highly significant (p< 0.001) at all time points in comparison to static (Figure 40). A very dynamic response after the onset of shear could be observed with relative expression level increase to 12.55 (± 2.76) at 2h. The level oscillated between 6.61 (± 1.86) at 4h, 9.12 (± 2.15) at 6h and 6.6 (± 1.94) at 12h from where on it decreased. All neighbouring timepoints were significantly different from each other with 2h and 4h p< 0.001, 4h and 6h p< 0.01 and the others with p< 0.05.
One possible interpretation of this observation could be that the mechanosensitive signalling pathways, leading to KLF2 activation, respond very quickly when exposed to high shear stress, resulting in a significant increase of KLF2 mRNA already after 2h. The oscillation could result from a negative feedback loop and the biological meaning of this implication is further discussed in section 5.3.3. In low shear stress conditions, KLF2 was only significantly expressed after 2h exposure (2.77 ± 0.92). For the other time points, the expression levels were decreasing with no significant difference to the static control. These experiments revealed that indeed KLF2 response is highly significant at atheroprotective high shear stress, while the expression in response to low shear stress is not significant.

Figure 40: Relative KLF2 expression normalised to HPRT1 for PAECs sheared at high shear stress of 20 dyne/cm² for 0-24h is represented by a dotted line. Significant differences of p< 0.001 (***') between static 0h (n=15) and shear were observed for all time points 2h (n=10), 4h (n=8), 6h (n=8), 12h (n=6) and 24h (n=6). KLF2 expression normalised to HPRT1 for PAECs sheared at low shear stress of 5 dyne/cm² for 0-24h is represented by a dashed line. Significant differences of p< 0.05 (†) between static 0h (n=5) and shear were observed for time point 2h (n=4). The expression pattern comparing low and high shear stress differed significantly with p< 0.001 (###) at all time points. Statistical analysis based on one-way and two-way ANOVA with post tests. Error bars represent standard deviation (SD).
For high shear stress, the pattern of KLF4 expression over time was similar to the oscillating response seen for KLF2 (Figure 41). In respect to 0h, time point 2h (3.22 ± 1.57) and 6h (2.55 ± 1.33) formed two peaks, but with less magnitude than observed for KLF2. Significantly different expression levels were detected at 2h (p< 0.001), 6h (p< 0.05) and 24h (2.9 ± 1.16) (p< 0.05). The neighbouring timepoints 2h and 4h, as well as 4h and 6h, were significantly different from each other with p< 0.01. In response to low shear stress, a slight induction of KLF4 could be observed after 2h, but without significance as for the other time points. Also KLF4 expression was shown to be highly dynamic and significantly different than static, while low shear stress did not induce KLF4.

Figure 41: Relative KLF4 expression normalised to HPRT1 for PAECs sheared at high shear stress of 20 dyne/cm² for 0-24h is represented by a dotted line. Significant differences of p< 0.001 (*** between static 0h (n=15) and shear were observed at time points 2h (n=10) and with p< 0.05 (*) at 6h (n=8) and 24h (n=8). KLF4 expression normalised to HPRT1 for PAECs sheared at low shear stress of 5 dyne/cm² for 0-24h is represented by a dashed line. No significant differences can be observed. The expression pattern comparing low and high shear stress differed significantly with p< 0.05 (#) at 2h, 6h and 24h. Statistical analysis based on one-way and two-way ANOVA with post tests. Error bars represent standard deviation (SD).
KLF6 levels did not significantly change during the investigated time course (Figure 42). The relative expression levels remained similar to static levels at 0h for high shear stress and in response to low stress KLF6 expression decreased by 60% in the first 6h to 0.33 (± 0.04). Despite recent literature reports (41), it can be concluded that for the experimental set up in this study, KLF6 was not shown to be induced by shear stress. It remains to be investigated whether KLF6 is differentially expressed in comparison to static when KLF2 or/and KLF4 mRNAs are silenced.

Figure 42: Relative KLF6 expression normalised to HPRT1 for PAECs sheared at high shear stress of 20 dyne/cm² for 0-24h, represented by a dotted line, is not significant. KLF6 expression normalised to HPRT1 for PAECs sheared at low shear stress of 5 dyne/cm² for 0-24h, represented by a dashed line, is also not significant. The expression pattern comparing low and high shear stress differed significantly with p< 0.05 (#) at 6h. Statistical analysis based on one-way and two-way ANOVA with post test. Error bars represent standard deviation (SD).
The expression levels of eNOS increased with time in response to high shear stress (Figure 43). Significance with p< 0.05 in comparison to 0h was reached already after 4h with 1.61 (± 0.78) and continuously increased (p< 0.01) at 6h (1.73 ± 0.41) and 12h (1.84 ± 0.64) until reaching 2.08 (± 0.40) at 24h. It is an interesting observation that the atheroprotective KLF2 and KLF4 showed an oscillating behaviour, while eNOS increased steadily. The time delay of significant expression induction could result from a delay needed to transcribe active KLF2 and KLF4 protein. Exposure to shear at 5 dyne/cm² did not affect eNOS levels significantly, showing that low shear stress does not induce the dilatory factor as also seen in atheroprone regions in the vasculature.

Figure 43: Relative eNOS expression normalised to HPRT1 for PAECs sheared at high shear stress of 20 dyne/cm² for 0-24h is represented by a dotted line. Significant differences of p< 0.01 (**) to static 0h (n=12) are observed at time points 6h (n=8), 12h (n=5), 24h (n=5) and with p< 0.05 (*) at 2h (n=11). eNOS expression normalised to HPRT1 for PAECs sheared at low shear stress of 5 dyne/cm² for 0-24h is represented by a dashed line. No significant difference could be observed. The expression pattern comparing low and high shear stress differed significantly with p< 0.01 (##) at 4h, 12h and 24h and with p< 0.001 (###) at 6h. Statistical analysis based on one-way and two-way ANOVA with post test. Error bars represent standard deviation (SD).

**4.3.2.2 Comparison of expression patterns in response to low and high shear stress**

For some genes very distinct expression profiles, comparing high shear and low shear conditions, could be observed. Two-way ANOVA analysis was used to evaluate differences between expression levels for a single time point and the significances (p-value) are displayed in Table 15.
Table 15: Results from two-way ANOVA analysis of significance (p-value), comparing relative expression levels for KLF2, KLF4, KLF6 and eNOS at 5 dyne/cm² (low shear) to 20 dyne/cm² (high shear) at time points 2-24h.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Gene expression low versus high shear</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KLF2</td>
</tr>
<tr>
<td>2</td>
<td>p&lt; 0.001</td>
</tr>
<tr>
<td>4</td>
<td>p&lt; 0.001</td>
</tr>
<tr>
<td>6</td>
<td>p&lt; 0.001</td>
</tr>
<tr>
<td>12</td>
<td>p&lt; 0.001</td>
</tr>
<tr>
<td>24</td>
<td>p&lt; 0.001</td>
</tr>
</tbody>
</table>

Comparison of the graphs, displaying the expression level series of KLF2 in low and high shear environment, shows that the response to high shear stress was much more dynamic in magnitude and in expression pattern. An oscillatory behaviour could be observed in high shear conditions, while exposure to low shear stress resulted in a single peak. The relative expression levels varied significantly (p< 0.001) at all time points. It can be concluded that shear of 5 dyne/cm² might be too low to activate shear stress-stimulated pathways. Dekker et al. observed that shear stress lower than 5 dyne/cm² did not provoke an upregulation of KLF2 (116). It was initially hypothesised that the low shear condition might, to a certain extent, capture the flow environment at atheroprone sides of the vasculature. It is therefore possible that the decreasing levels of KLF2 could actually lead to an atherosclerotic phenotype. The functional studies needed to proof this hypothesis were not conducted, but even prolonged exposure to low shear stress did not result in alignment of the cells in the direction of flow. In comparison, cells exposed to high shear stress rearranged after approximately 12h and their longitudinal axis was in line with the local velocity direction, as seen in the comparison of PAEC morphology in Figure 28 and Figure 31.

For KLF4 the differences between the results obtained for high and low shear stress exposure were significant at 2h, 6h and 24h (p< 0.05). As the response to high shear stress was oscillating, the difference between constant expression at low shear was when the expression level reached a maximum. These explicit differences in expression pattern in dependence of time have not been observed so far.

The expression of KLF6 decreased in response to low shear stress and reached an absolute minimum at 6h, which differed significantly (p< 0.05) from the almost constant expression level detected in high shear stress conditions.
Conditions of high and low shear stress also triggered a different response in eNOS expression levels, which was significant after 4h of exposure (p< 0.05). An increasing trend could be observed for high shear stress, while low shear was similar to static, which enhanced the significance of the expression after 6h (p< 0.01). These results support the current understanding that eNOS expression is shear-sensitive. It highlights that the experimental conditions of high and low shear stress could stimulate a distinct response, which might, to a certain extent, resemble in vivo conditions at atheroprotected and atheroprone regions of the vasculature.

4.3.3 Results and discussion of gene silencing studies

4.3.3.1 Optimisation of primary cell transfection using electroporation

The transfection parameters for microporation were initially optimised in the scope of another project by testing 24 different voltage pulsing programs on HeLa cells with GFP plasmid. GFP expression was visualised 24h after transfection by fluorescence microscopy. It could be determined that a setting of 1200V, 20 ms pulse width and 2 pulses was optimal, compromising viability and efficiency (data not shown). For the transfection optimisation of PAECs All stars siRNA Alexa 488-conjugates were selected as a transfection control. Different sets of varying electroporation parameters (A-C), similarly to the optimal setting for HeLas, in combination with a concentration range of 25-150 nM (1-3), were tested and evaluated quantitatively by FACS as summarised in Table 16. The electroporation efficiency resulting from the optimal setting was additionally confirmed by visualisation of the fluorescent conjugate with confocal microscopy.

4.3.3.2 Quantitative FACS analysis

The siRNA transfected PAECs were stained with TO-PRO-3 iodide and analysed by flow cytometry. Firstly, the cell viability was assessed using side scatter (SSC) and forward scatter (FSC) dot plot representation. Healthy viable cells were gated (M1 first column, Figure 44) based on experience and in the fraction of viable cells a comparison between transfected and non-transfected cells was made. The histograms in Figure 44 present in the first column TO-PRO-3 iodide (viability) stain and in the second for the gated M1 population the control siRNA (efficiency) for the different electroporation parameters A-C.
The transfected cells were gated with the marker R1. The concentrations of siRNA corresponding to the sample ID 1-5 were colour-coded with pink (25 nM siRNA), blue (100 nM siRNA), orange (150 nM siRNA), purple (unaffected cells 150 nM) and green (0 nM siRNA). The viability for most settings was more than 80%.

In comparison to a lipid based transfection method using Lipofectamin (Invitrogen), which yielded only 30-50% survival rate (data not shown), the obtained results were encouraging. It could be concluded that for PAECs the method of electroporation did not result in severe cell loss, which presented an important parameter as the availability of primary cells was limited. Transfection efficiency as the other important parameter varied broadly between the differently concentrated samples.

The lowest voltage of 1200V with more than 100 nM siRNA (sample C2 blue and sample C3 orange) showed the best result without compromising on viability or efficiency, both being more than 90%. Validation of the microporation results was done qualitatively by confocal microscopy. For functional siRNA experiments, a final probe concentration of 300 nM was selected as it resulted in most potent gene silencing for KLF2 and KLF4 as discussed in section 4.3.3.4.
Results and Discussion

Figure 44: FACS analysis for quantitative electroporation efficiency assessment. The transfection parameters for PAECs with microporator technology were optimised by transfection with an Alexa-488-labelled All stars siRNA using flow cytometry. The concentrations of siRNA are colour coded with pink (25 nM siRNA), blue (100 nM siRNA), orange (150 nM siRNA), purple (unaffected cells 150 nM) and green (0 nM siRNA). TO-PRO-3 iodide (1:10000) was used as a viability marker. A total of 10,000 cells were assessed. The first column presents viable cell fraction gated M1 for TO-PRO-3 iodide detected by PMT sensor FL-4. The second column presents the viable and transfected cells gated R1 for Alexa-488 detected by PMT sensor FL-1.
All electroporation settings with varying voltage, pulse width, pulse number and All stars siRNA concentration as well as the results in percentage for viability and efficiency are summarised in Table 16.

Table 16: Viability and efficiency in percent (%) of the different electroporation parameters (A-C) and siRNA concentrations ranging from 0-150 nM (1-5).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Pulse (V)</th>
<th>Pulse width (ms)</th>
<th>Pulse nr.</th>
<th>conc. siRNA (nM)</th>
<th>Viability % M1</th>
<th>Efficiency % R1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1500</td>
<td>50</td>
<td>1</td>
<td>25</td>
<td>76</td>
<td>48</td>
</tr>
<tr>
<td>A2</td>
<td>1500</td>
<td>50</td>
<td>1</td>
<td>100</td>
<td>81</td>
<td>71</td>
</tr>
<tr>
<td>A3</td>
<td>1500</td>
<td>50</td>
<td>1</td>
<td>150</td>
<td>97</td>
<td>35</td>
</tr>
<tr>
<td>A4</td>
<td>1500</td>
<td>50</td>
<td>1</td>
<td>-</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>A5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>150</td>
<td>96</td>
<td>1</td>
</tr>
<tr>
<td>B1</td>
<td>1300</td>
<td>30</td>
<td>2</td>
<td>25</td>
<td>91</td>
<td>5</td>
</tr>
<tr>
<td>B2</td>
<td>1300</td>
<td>30</td>
<td>2</td>
<td>100</td>
<td>93</td>
<td>8</td>
</tr>
<tr>
<td>B3</td>
<td>1300</td>
<td>30</td>
<td>2</td>
<td>150</td>
<td>92</td>
<td>68</td>
</tr>
<tr>
<td>B4</td>
<td>1300</td>
<td>30</td>
<td>2</td>
<td>-</td>
<td>79</td>
<td>3</td>
</tr>
<tr>
<td>B5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>150</td>
<td>Na</td>
<td>na</td>
</tr>
<tr>
<td>C1</td>
<td>1200</td>
<td>30</td>
<td>2</td>
<td>25</td>
<td>88</td>
<td>21</td>
</tr>
<tr>
<td>C2</td>
<td>1200</td>
<td>30</td>
<td>2</td>
<td>100</td>
<td>85</td>
<td>92</td>
</tr>
<tr>
<td>C3</td>
<td>1200</td>
<td>30</td>
<td>2</td>
<td>150</td>
<td>91</td>
<td>96</td>
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<tr>
<td>C4</td>
<td>1200</td>
<td>30</td>
<td>2</td>
<td>-</td>
<td>88</td>
<td>1</td>
</tr>
<tr>
<td>C5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>150</td>
<td>92</td>
<td>1</td>
</tr>
</tbody>
</table>

4.3.3.3 Qualitative confocal microscopy analysis

PAECs were transfected with KLF2 ABC (300 nM) in combination with a RISC-independent siGLO Red (DY-547) siRNA (ratio 1:1, 300 nM) and sheared for 2h at 20 dyne/cm². Alive cells were analysed with confocal microscopy after nuclear stain using Draq5 as a qualitative control. Figure 45 displays representative images of KLF2 ABC transfected cells (negative CTR) and KLF2 ABC transfected cells in combination with siGLO in a 40x magnification. siGLO localised in the cytoplasm and partially to the nucleus in almost all cells. The fluorescent intensity differed amongst the cells, which could mean that the amount of siGLO uptake per cell varied. Further magnification using 4x zoom revealed that the majority of siGLO probes was distributed in small vesicles throughout the cytoplasm, but did not translocate into the nucleus (Figure 46).
These qualitative results show that PAECs can be efficiently transfected, but not uniformly, with siRNA using the optimised microporation settings. This may explain the inter-experimental variations of transfection efficiency as not all cells might take up the same amount of siRNA. In cells with low siRNA uptake mRNA processing may not be substantially impaired. This might contribute to the fact that the gene silencing efficiencies with KLF2 ABC and KLF4 BC never reached 100%.

**Figure 45:** Confocal microscopy images for qualitative electroporation efficiency assessment. siGLO transfection indicator (300 nM) was transfected into PAECs (green) using optimised microporation parameters (1200V, 2 pulses, 30 ms). Cells were sheared for 2h at 20 dyne/cm², washed with PBS, stained with Draq5 (blue) and imaged with a 40x objective. Non uniform distribution of siGLO in the cytoplasm of PAECs can be observed (scale bar =75 μm).
Results and Discussion

4.3.3.4 Composition of siRNA gene silencing cocktail

The assessment and optimisation of effective and specific siRNA is most efficient when performed as a matrix, using different concentrations and varying siRNA sequences. As resources were limited, initial experiments with individual and combinatorial siRNAs were conducted at a defined concentration. The most promising sequences were then titrated and used at their lowest effective concentration. This assessment was important as non-specific silencing effects may be seen when siRNAs are transfected into cells at too high concentrations and gene silencing effectiveness might be impaired at too low concentrations.

A cocktail of several siRNAs to the same target is sometimes used to mediate gene silencing, but it may theoretically increase the chances of off-target effects (OTEs). Three different siRNAs (short form A, B and C) have been designed and the results shown here are from the optimisation phase of testing each siRNA for its functionality.

Figure 46: Confocal microscopy images for qualitative electroporation efficiency assessment. siGLO transfection indicator (300 nM) was transfected into PAECs (green) using optimised microporation parameters (1200V, 2 pulses, 30 ms). Cells were sheared for 2h at 20 dyne/cm², washed with PBS, stained with Draq5 (blue) and imaged with a 40x objective and zoomed 4x. siGLO appears to localise in vesicle mainly in the cytoplasm (scale bar =25 μm).
Results and Discussion

PAECs were electroporated with a fixed concentration (500 nM) of A, B and C as well as a combination of all sequences together (ABC) and a scrambled mock sequence (M). In addition, transfection without any siRNA was conducted to evaluate the effects of electroporation. Seeded in flow chambers, the cells were then subjected to 2h high shear stress and the gene expression levels of the targets were assessed by qPCR as previously described.

The results for KLF2 and KLF4 are shown as relative gene expression, with 1 being equal to the untreated control (not electroporated), but exposed to shear. In comparison to this, the static condition was 87% lower as expected since KLF2 is highly shear regulated. Electroporation caused a slight decrease of gene expression by 7%, but this was considered as an acceptable variation. As pictured in Figure 47, the treatment with siRNAs resulted consistently in at least 47% downregulation (A) with KLF2 ABC, the combination of all three probes, being the most efficient with 65%. In compliance with the data shown in the next paragraph, the KLF2 M (mock siRNA) did not alter expression levels significantly. As KLF2 ABC successfully silenced its target gene with a high efficiency, all further experiments were conducted using the cocktail.

The same methodology was followed for the functionality assessment of KLF4 targeting siRNAs. Similar to the findings for KLF2, the electroporated sample without any siRNA as well as the condition electroporated with mock KLF2 M did not significantly differ from the non-impaired control. The siRNAs treatment resulted consistently in at least 84% downregulation (A) and did even further increase to 92% (ABC), 94% (B) and 95% (C) (see Figure 48). These gene silencing efficiencies demonstrate the functionality of the in-house designed siRNA probes. In all future experiments, a mix of KLF4 B and KLF4 C was used as the addition of KLF4 A slightly decreased the efficiency.

The assessment for KLF2 and KLF4 also revealed that the optimised methodology for siRNA transfection of PAECs by electroporation did not alter target gene expression significantly. This was a very important validation as biological variability due to the treatment could be nearly neglected.
Results and Discussion

Figure 47: Relative KLF2 expression levels to test the functionality of KLF2 A, KLF2 B, KLF2 C and KLF2 ABC siRNA at a concentration of 500 nM. Additionally, the relative expressions for the static condition and electroporated with 0 nM siRNA are shown. After electroporation, PAECs were subjected to shear at 20 dyne/cm$^2$ for 2h. Data was assessed by qPCR and normalised to HPRT1 and to a non-impaired control condition, which was electroporated without siRNA (set to 1.0) ($n=1$).

Figure 48: Relative KLF4 expression levels to test the functionality of KLF4 A, KLF4 B, KLF4 C and KLF4 ABC siRNA at a concentration of 500 nM. Additionally the relative expressions for the static condition and electroporated with 0 nM siRNA are shown. After electroporation, PAECs were subjected to shear at 20 dyne/cm$^2$ for 2h. Data was assessed by qPCR and normalised to HPRT1 and to a non-impaired control condition, which was electroporated without siRNA (set to 1.0) ($n=1$).
Results and Discussion

Titration experiments were conducted for KLF2 with siRNA concentrations ranging from 50–1500 nM to assess if downregulation of the target gene could be further increased as this is also concentration-dependent. The best, most efficient silencing was achieved with a concentration of 250-500 nM. Initially, increasing concentration of siRNA lowered absolute KLF2 expression levels, but the trend was not preserved for concentrations higher than 500 nM. The expression levels of the KLF2 M siRNA condition did not differ from the untreated condition besides at 1000 nM. This was probably due to biological variability, but has not been further evaluated. The condition electroporated with 0 nM siRNA did also not differ significantly from the non-impaired shear condition. It can therefore be concluded, as highlighted in the previous dataset, that electroporation effects were non-specific. For all future experiments, a concentration of 300 nM from the cocktail KLF2 ABC was used for gene silencing of KLF2. For gene silencing of KLF4 with KLF BC the same concentration has been shown to result in good gene silencing efficiencies (Figure 49).

Figure 49: Absolute expression levels of KLF2 in PAECs sheared at 20 dyne/cm² for 2h. Cells were electroporated with varying concentrations (0-1500 nM) of KLF2 ABC and KLF2 M or remained untreated. Gene silencing efficiency was evaluated comparing to non-impaired shear condition (sheared). Data was assessed by qPCR and normalised to HPRT1. Only where indicated error bars represent the standard deviation (n=2). The other conditions were tested only once.
4.3.3.5 Assessing the effects of electroporation and transfection with mock siRNA

RNAi experiments require experimental controls to discount any changes to the gene expression profile that may have resulted from the siRNA delivery method. As described in chapter 4.3.3.5, scrambled versions of targeting siRNA, referred to as M for mock, have been designed to assess these. Here, an overview of three representative experiments is shown, comparing four different experimental conditions of PAEC treatment followed by exposure to shear for 2-48h: no electroporation, electroporation but without siRNA, electroporation with 300 nM KLF2 M and electroporation with 300 nM KLF4 M. The effects on KLF2 and KLF4 expression have been assessed by cell morphology and qPCR. Figure 50 summarises the results over time for absolute KLF2 expression. The KLF2 and KLF4 scrambled siRNA (KLF2 M and KLF4 M) showed at no time point significant absolute expression levels in comparison to the non-electroporated condition. They even followed the oscillatory pattern at early time points. The electroporated condition without any siRNA varied from the non-electroporated condition only with significance (p< 0.01) at 4h, where an upregulation could be observed. This can partially be explained by large expression level variation for those particular samples, possibly due to a technical error.

![Graph showing absolute KLF2 expression over time for different conditions](image)

**Figure 50:** Absolute KLF2 expression normalised to HPRT1 over time for four different conditions. PAECs were electroporated with 0 nM siRNA, 300 nM KLF2 M, 300 nM KLF4 M or remained untreated before exposure to shear at 20 dyne/cm² for 0-48h. Statistical analysis by two-way ANOVA revealed significant differences of p< 0.01 (**) comparing conditions electroporated with no siRNA the control condition with no electroporation at 4h. Error bars represent the standard deviation (n=3).
It can be concluded that KLF2 expression was not impaired by electroporation with the only exception being at 4h and that the scrambled siRNA versions can serve as a positive control for RNAi experiments, as they did not alter KLF2 expression. Similar results were obtained from the assessment of KLF4 expression (Figure 51). None of the conditions varied significantly at any time point from the non-electroporated condition. These results validate the use of KLF2 M and KLF4 M as suitable transfection controls, because no changes in gene expression caused by siRNA delivery were detected.

Figure 51: Absolute KLF4 expression normalised to HPRT1 over time for four different conditions. PAECs were electroporated with 0 nM siRNA, 300 nM KLF2 M, 300 nM KLF4 M or remained untreated before exposure to shear at 20 dyne/cm² for 0-48h. Error bars represent the standard deviation (n=3).

4.3.3.6 Time course 0-6h high shear with KLF2 or KLF4 gene silencing

Previous time course experiments revealed a dynamic oscillatory pattern for KLF2 and KLF4 at early time points when exposed to 20 dyne/cm². Transfection experiments with the optimised electroporation settings and siRNA probes (KLF2 ABC and KLF4 BC) were conducted, followed by exposure to shear to assess changes in expression patterns resulting from mRNA silencing of KLF2 and KLF4. The individual siRNA probes were not tested in this experimental setting as a combination of several siRNAs was shown to cause optimal gene downregulation.
Figures 52-55 show the relative gene expression for KLF2, KLF4, KLF6 and eNOS respectively at 2h, 4h and 6h. The figures display quantitative expression levels in the condition of gene silencing with KLF2 ABC as a blue dotted graph and silencing of KLF4 with KLF4 BC as a grey graph. These conditions are compared with the previously shown WT expression pattern for the individual genes, shown as a black dotted graph, in response to high shear stress. All data is presented as average values with SD, normalised to HPRT1 and 0h (static) set to 1.

For siRNA silencing with KLF2 ABC, the KLF2 expression as expected was significantly reduced (p< 0.001) to 2.41 (± 1.84) at 2h, 2.82 (± 2.08) at 6h and slightly less significant (p< 0.01) at 4h with 2.92 (± 2.13) compared to the WT condition.

When transfected with KLF4 BC, relative KLF2 expression was significantly down-regulated (p< 0.001) for 2h (2.52 ± 0.78) and 6h (2.56 ± 0.99), while p< 0.05 at 4h (3.29 ± 1.95). This is a very interesting observation, which could mean that KLF4 acts as a direct activator of KLF2, though no detailed hypothesis on the interaction can be made.

**Figure 52: Relative expression levels of KLF2 in PAECs sheared at 20 dyne/cm² for 2h, 4h and 6h in response to gene silencing with KLF2 ABC siRNA (300 nM) represented as blue dotted line and KLF4 BC siRNA (300 nM), represented as grey straight line in comparison to non-impaired wild type (WT) control, represented as black dotted line. Data was assessed by qPCR and normalised to HPRT1 with 0h=1. Significant differences for KLF2 ABC are shown as stars (p< 0.05=*, p< 0.01=** and p< 0.001=***). Error bars represent the standard deviation (n=4).**
The possibility of unspecific binding of KLF4 BC to a homologues KLF2 mRNA region could also explain the observed downregulation. This is a feasible option even though the exclusion of OTEs was assessed in silico in the siRNA design phase. Various blast tools, as described in section 2.3.1, were used to evaluate the siRNA specificity and did not result in a positive hit. It can therefore be concluded that KLF2 was silenced possibly either because of the lack of KLF4 protein or due to OTEs.

KLF4 expression was only slightly impaired at 2h (1.2 ± 0.64) by intervention with KLF2 siRNA (p< 0.05). In contrast, KLF4 BC was very potent and resulted in significant downregulation (p< 0.001) at 2h (0.21 ± 0.15) and 4h (0.13 ± 0.06) and (p< 0.01) at 6h (0.14 ± 0.08). As only a slight expression difference in absence of KLF2 at 2h could be detected, a mutual interaction, at least at early time points, seems unlikely.

![Figure 53](image-url)

**Figure 53:** Relative expression levels of KLF4 in PAECs sheared at 20 dyne/cm² for 2h, 4h and 6h in response to gene silencing with KLF2 ABC siRNA (300 nM), represented as blue dotted line and KLF4 BC siRNA (300 nM), represented as grey straight line, in comparison to non-impaired wild type (WT) control represented as black dotted line. Data was assessed by qPCR and normalised to HPRT1 with 0h=1. Significant differences for KLF2 ABC are shown as stars (p< 0.05=*, p< 0.01=** and p< 0.001=***)) and KLF4 BC as crosses (p< 0.05=†, p< 0.01= †† and p< 0.001= †††)). Error bars represent the standard deviation (n=4).
KLF6 has been shown to be present in ECs (212), but this study, for the first time, assessed its expression in dependence of shear as well as in the presence and absence of KLF2 and KLF4 mRNA. Figure 54 shows that KLF6 expression did not differ from the WT control in KLF2-silenced condition 2h (1.06 ± 0.61), 4h (0.76 ± 0.47) and 6h (1.06 ± 0.55) as well as in KLF4-silenced conditions at 2h (0.81 ± 0.18), 4h (0.88 ± 0.48) and 6h (1.11 ± 0.45). Hence a role of KLF6 in a direct functional context of network interaction is rather unlikely.

Figure 54: Relative expression levels of KLF6 in PAECs sheared at 20 dyne/cm² for 2h, 4h and 6h in response to gene silencing with KLF2 ABC siRNA (300 nM), represented as blue dotted line and KLF4 BC siRNA (300 nM), represented as grey straight line, in comparison to non-impaired wild type (WT) control represented as black dotted line. Data was assessed by qPCR and normalised to HPRT1 with 0h=1. Significant differences for KLF2 ABC are shown as stars (p < 0.05 = *, p < 0.01 = ** and p < 0.001 = ***)) and KLF4 BC as crosses (p < 0.05 = †, p < 0.01 = †† and p < 0.001 = †††). Error bars represent the standard deviation (n=4).
eNOS has been introduced as a target gene of KLF2 and KLF4 (110) and it was of interest to study its dependence on KLF2 and KLF4 at very short time scales. For KLF2-silenced conditions with KLF2 ABC, only the 2h time point (0.60 ± 0.18) showed a significantly different decrease in the expression level (p< 0.05). The effect was more severe in the KLF4−silenced condition, where relative expression levels of eNOS decreased significantly at 2h (0.37 ± 0.04) and (0.61 ± 0.19) at 6h (p< 0.05) and even more at 4h (0.05 ± 0.23) (p< 0.01). These results show that eNOS is directly affected by gene silencing of KLF2 and KLF4 thereby confirming its role as direct target gene for the network modelling.

Figure 55: Relative expression levels of eNOS in PAECs sheared at 20 dyne/cm² for 2h, 4h and 6h in response to gene silencing with KLF2 ABC siRNA (300 nM), represented as blue dotted line and KLF4 BC siRNA (300 nM), represented as grey straight line, in comparison to non-impaired wild type (WT) control represented as black dotted line. Data was assessed by qPCR and normalised to HPRT1 with 0h=1. Significant differences for KLF2 ABC are shown as stars (p< 0.05=*, p< 0.01=** and p< 0.001=***') and KLF4 BC as crosses (p< 0.05= †, p< 0.01= †† and p< 0.001= †††). Error bars represent the standard deviation (n=4).
4.3.3.7 Time course 2-24h high shear with KLF2 and KLF4 mutual gene silencing

Double gene silencing experiments with KLF2 ABC and KLF4 BC (total of 300 nM) were conducted in addition to the single RNAi approach to better investigate the relationship between the genes of interest. This set of experiments also aimed to investigate the effects on relative gene expression at a wider time scale, adding measurements at 12h and 24h.

As seen in Figure 56, KLF2 expression levels were consistently down-regulated at all time points with significance levels of \( p<0.001 \), besides at 24h (2.03 ± 1.82) where the difference was less significant \( (p<0.05) \). This was due to the fact that the expression levels for WT condition decreased.

![Graph showing relative KLF2 expression levels](image)

Figure 56: Relative expression level of KLF2 in PAECs sheared at 20 dyne/cm\(^2\) for 2h, 4h, 6h, 12h and 24h in response to gene silencing with a combination of KLF2 ABC siRNA and KLF4 BC siRNA (total 300 nM), represented as a grey straight line, in comparison to non-impaired wild type (WT) control represented as black dotted line. Data was assessed by qPCR and normalised to HPRT1. Significant differences between WT and KLF2 ABC/KLF4 BC as analysed by two-way ANOVA are shown as triangles \( (p<0.05=\Delta, p<0.01=\Delta\Delta \) and \( p<0.001=\Delta\Delta\Delta) \). Error bars represent the standard deviation \( (n=3) \).
For KLF4 significant reduction of the relative expression levels could be observed at time points where the oscillation of WT expression levels reached a maximum (Figure 57). These were significantly different at 2h (0.19 ± 0.13) with p< 0.01, 6h (0.18 ± 0.12) and 24h (0.33 ± 0.51) with p< 0.05.

Figure 57: Relative expression level of KLF4 in PAECs sheared at 20 dyne/cm² for 2h, 4h, 6h, 12h and 24h in response to gene silencing with a combination of KLF2 ABC siRNA and KLF4 BC siRNA (total 300 nM), represented as a grey straight line in comparison to non-impaired wild type (WT) control represented as black dotted line. Data was assessed by qPCR and normalised to HPRT1. Significant differences between WT and KLF2 ABC/KLF4 BC as analysed by two-way ANOVA are shown as triangles (p< 0.05=Δ, p< 0.01=ΔΔ and p< 0.001=ΔΔΔ). Error bars represent the standard deviation (n=3).
KLF6 relative expression levels were also not affected by mutual gene silencing of KLF2 and KLF4 mRNA, even at a longer time scale. The initial hypothesis that KLF6 might possibly play a role in the interaction of KLFs after a 6h observation window can therefore not be validated.

**Figure 58:** Relative expression level of KLF6 in PAECs sheared at 20 dyne/cm² for 2h, 4h, 6h, 12h and 24h in response to gene silencing with a combination of KLF2 ABC siRNA and KLF4 BC siRNA (total 300 nM), represented as a grey straight line in comparison to non-impaired wild type (WT) control represented as black dotted line. Data was assessed by qPCR and normalised to HPRT1. Significant differences between WT and KLF2 ABC/KLF4 BC as analysed by two-way ANOVA are shown as triangles (p< 0.05=△, p< 0.01=△△ and p< 0.001=△△△). Error bars represent the standard deviation (n=3).
Surprisingly, eNOS expression levels were also not impaired by gene silencing of KLF2 and KLF4 mRNA. Figure 59 shows that the expression levels were slightly reduced, but due to variations in the dataset, the standard deviations were too large for statistical significance. Additional experiments to increase the number of data points could have possibly helped to gain significantly different gene expression levels as a decreasing trend could be observed. Furthermore, other transcriptional activators could also compensate for the lack of KLF2 and KLF4. The promoter region of eNOS has many binding sites and some of them are not shear-related elements. It can be hypothesised that the KLF network, which interacts with different pathway, might be replaced by other transcription factors compensating for the induction of eNOS.

For the time points 0-6h a statistical two-way ANOVA analysis was performed, comparing single gene silencing with the double silencing to account for possible additive effects. A comparison of the relative expression levels for each gene for the conditions KLF2 ABC versus (vs.) KLF2 ABC/KLF4 BC, KLF4 BC vs. KLF2 ABC/KLF4 BC and KLF2 ABC vs. KLF4 BC did not result in a statistically significant difference (> 0.05). Therefore, additive effects using single or double siRNA could not be determined as no significant expression differences could be detected by statistical comparison.
### 4.3.3.8 48h high shear with KLF2 and KLF4 gene silencing and phenotypical observations

In addition to the time course discussed in Chapter 4.3.2.1, shear experiments were also conducted for longer time periods of up to 48h. Silencing of KLF2 and KLF4 was performed to investigate the long term expression response of KLF2, KLF4, KLF6 and eNOS. In the following section gene expression data, analysed by qPCR, is presented and the observed changes in phenotype are described. Furthermore, microarray analysis of a selection of these samples is shown in the following Chapter 5.

Gene silencing of KLF2 and KLF4 resulted in a change of endothelial phenotype. Exposure to 20 dyne/cm$^2$ shear for more than 12h is known to result in alignment of cells in the direction of flow as discussed in Chapter 1.2.3. The phenotype of cells transfected with scrambled siRNA for KLF2 (KLF2 M) and KLF4 (KLF4 M) was similarly to the WT shear condition. In comparison to the static control (ctr), these were more compact and oriented in the direction of flow. The conditions transfected with functional siRNA (KLF2 ABC and KLF4 BC) displayed a more polygonal morphology. The cells were not as compact and did not align in the direction of flow (Figure 60). Larger magnifications, as in Figure 61, show that cells transfected with functional siRNA against KLF2 mRNA or KLF4 mRNA had a similar phenotype. They were in tight contact and no cell detachment could be observed. The most striking feature was that they seemed to have lost the ability to align, even after prolonged exposure to shear stress of up to 48h.

It can therefore be concluded that KLF2 and KLF4 must play a major role in shear stress sensing and that they coordinate morphological changes and alignment in response to flow.
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Figure 60: PAEC morphology in gelatine-coated flow channels with a seeding density of 40,000 cells/channel. Cells were transfected with 300 nM KLF2 mock siRNA (KLF2 M), KLF4 mock siRNA (KLF4 M), functional KLF2 siRNA (KLF2 ABC), functional KLF4 siRNA (KLF4 BC) or non-impaired (sheared). After 12h of static culture they were exposed to 20 dyne/cm² shear stress in horizontal direction for 48h or left statically (ctr). The flow system was composed of peristaltic prototype II pump using C-flex tubing and elbow luers. Representative bright field microscopy images at 20x (scale bar =35 µm).

Figure 61: PAEC morphology in gelatine-coated flow channels with a seeding density of 40,000 cells/channel. Cells were transfected with 300 nM KLF2 mock siRNA (KLF2 M), KLF4 mock siRNA (KLF4 M), functional KLF2 siRNA (KLF2 ABC), functional KLF4 siRNA (KLF4 BC) or non-impaired (sheared). After 12h of static culture they were exposed to 20 dyne/cm² shear stress in horizontal direction for 48h or left statically (ctr). The flow system was composed of peristaltic prototype II pump using C-flex tubing and elbow luers. Representative bright field microscopy images at 40x (scale bar =25µm).
Results and Discussion

Figure 62 compares the quantitative expression levels of KLF2, KLF4, KLF6 and eNOS after 48h exposure to high shear stress at 20 dyne/cm² to the static control, meaning cells that were cultured in the same media composition and flow chamber, but excluded from the exposure to shear, set to 1. Cells were either non-impaired WT or electroporated with KLF2 ABC siRNA or KLF4 BC siRNA. The data was normalised to the housekeeping gene HPRT1 and error bars present the standard deviation. For KLF2, the sheared WT condition (5.62 ± 2.15) was statistically different (p< 0.01) from the non-sheared static control (1.0 ± 0.63). This trend followed the observations made at earlier time points (see Figure 40) with relative expression levels at 24h being of similar magnitude. In the condition KLF2 ABC gene silencing, KLF2 was significantly down-regulated in comparison to the WT condition (2.24 ± 0.73 with p< 0.05). This was consistent with previous measures taken at earlier time points (2-6h). The downregulation of KLF2 by inhibition of KLF4 mRNA with KLF4 BC siRNA was also similar. The reduction effects were significant (2.08 ± 0.93 with p< 0.01).

Comparing WT and static condition for KLF4, relative gene expression levels were significantly up-regulated at 48h (5.74 ± 3.39 with p< 0.05). The gene-silenced condition with KLF2 ABC reduced KLF4 expression, but not significantly. In contrast, a large decrease in expression with statistical significance could be observed for KLF4 BC (0.43 ± 0.16 with p< 0.01). This was a continuation of the trend already observed at earlier time points (see Figure 41).

KLF6 expression levels at 48h of shear exposure (1.17 ± 0.47) did not vary from the static condition (1 ± 0.31). This observation was already made at the earlier time points. Gene silencing of KLF2 and KLF4 did also not impair KLF6 expression levels at the 48h time point. It can therefore be concluded that KLF6 expression is not shear-dependent, even after prolonged time of shear exposure.

An interesting observation could be made for the relative expression levels of eNOS. At 0h the expression was normalised to 1 (± 0.36) and it increased with statistical significance (p< 0.05) to 1.62 ± 0.35 after 48h. This increasing trend was in line with measures taken at 2h, 4h, 6h and 24h (Figure 43). Hence, it can be stated that the expression levels of the vasodilator eNOS increased in a time window of 48h, when PAECs were exposed to high shear stress. For eNOS, the silencing of KLF2 did not impair the expression levels significantly, though a reduction in expression levels was observed. This could be due to the remaining KLF2 mRNA molecules, as the gene silencing efficiency was not 100%. In contrast, eNOS was significantly reduced (p< 0.01) in the KLF4-silenced condition with KLF4 BC siRNA (0.95 ± 0.17). This was in line with the previous assessments at 2-6h, where eNOS was also highly impaired (see Figure 55).
Results and Discussion

Figure 62: Relative gene expression for KLF2, KLF4, KLF6 and eNOS in PAECs sheared for 48h at 20 dyne/cm^2 in comparison to gene expression levels in static non-shear exposed control condition at (set to 1). Cells were either non-impaired wild type (WT) or electroporated with KLF2 ABC siRNA or KLF4 BC siRNA prior to exposure to flow. Data was assessed by qPCR and normalised to HPRT1. Significant differences between WT, static, KLF2 ABC and KLF4 BC as analysed by two-way ANOVA are shown as stars (p< 0.5=*, p< 0.01=**) Error bars represent the standard deviation (n=5).

4.4 Conclusion

This chapter covered the establishment, optimisation and application of qPCR as a quantitative method for the evaluation of KLF2, KLF4, KLF4 and eNOS gene expression levels. A broad dynamic range, high efficiency and specificity of the designed primers and the other reaction components were demonstrated.
These required the optimisation of primer concentrations and the individual qPCR melting temperatures. The robust qPCR assay was utilised to evaluate gene expression of the target genes KLF2, KLF4 and KLF6 as well as eNOS, normalised to the housekeeping gene HPRT1. Time course experiments were conducted at 20 dyne/cm\(^2\) and 5 dyne/cm\(^2\) with sampling intervals at 0h, 2h, 4h, 6h, 12h and 24h. Exposed to a high shear regime KLF2 and KLF4 displayed an oscillatory expression pattern with expression maxima at 2h and 6h. KLF6 was not impaired, while the target gene eNOS increased steadily.

Exposure to low shear conditions resulted in a single maximum at 2h for KLF2 with a decreasing trend over time and the single maximum at 2h for KLF4 was followed by an increase in expression. KLF6 did not change significantly and eNOS expression increased with time. The different responses for KLF2 and KLF4 are a very interesting observation and have not been reported previously. Oscillations in the effector genes resulted in a linear response of the target gene as eNOS expression did not follow the pattern. This might be due to a time delay from mRNA transcription to protein synthesis and posttranscriptional modifications.

To further evaluate the relationship between the network components, gene silencing studies using RNAi methodology were conducted. Transfection by electroporation firstly required optimisation of the experimental parameters, which was assessed quantitatively by FACS analysis and qualitatively by confocal microscopy. The ideal setting C3 (1200V, 30ms, 2 pulses) resulted in 91% viability and 96% efficiency, while confocal microscopy validation highlighted a heterogeneous distribution of fluorescently-labelled siRNA. Titration and combinatorial sequence experiments were conducted with the in-house designed siRNAs to reveal the most potent siRNA combination. The cocktails of KLF2 ABC and KLF4 BC at 300 nM concentration were identified as most potent, while the associated mock siRNA (M) did not alter gene expression significantly. Also in line with this finding was the observation that electroporation without any siRNA did not alter expression levels. The electroporation methodology therefore presented an ideal method for the transfection of PAECs.

The functionality of the optimised siRNAs was tested to evaluate the relationship between the network components KLF2, KLF4, KLF6 and eNOS. Within a timeframe of up to 6h exposure to 20 dyne/cm\(^2\) shear stress, KLF2 ABC siRNA transfected cells showed a significant reduction of KLF2 and eNOS with a decreasing trend, while KLF4 expression, with the exception of 2h, and KLF6 expression were not impaired.
Silencing of KLF4 resulted not only in significant downregulation of KLF4, but also KLF2 and eNOS at all time points. KLF6 expression levels were not affected. These results might suggest that KLF4 impairs KLF2 gene levels and sequence studies of the predicted KLF2 promoter revealed four CACCC elements, which could present potential KLF4 binding sites. The observation that eNOS was down-regulated at 2h for KLF2 ABC treatment, as well as at all time points in KLF4-silenced conditions underlines the previous findings that it is a direct target of these two TFs (110). Significance could not be reached at 4h and 6h in KLF2 silenced conditions. This could partially be due to the fact that the gene silencing was not complete, resulting in the activation of eNOS expression through remaining KLF2 protein. In summary, the experimental RNAi results validated eNOS as a target gene to investigate the KLF network behaviour.

In addition, mutual silencing studies of KLF2 and KLF4 with a combination of KLF2 ABC and KLF4 BC (300 nM total) were performed with measures taken at a larger time window of up to 24h. These experiments could have revealed a potential compensatory function of KLF6 for the loss of KLF2 and KLF4 expression. Within the selected time course, the expression of KLF2 remained significantly low (p< 0.001), besides at the 24h time point, where it slightly increased (p< 0.05). Expression of KLF4 was at all time points lower than the static condition, but in comparison to non-impaired WT not always significant. The lowest level of the oscillatory expression pattern reached similar expression values than observed for the static control. At these time points (4h and 12h) significance for KLF4 expression in gene-silenced conditions in comparison to WT could not be obtained. KLF6 expression, in consistency with the single gene silencing experiments, did not alter. Therefore, an initially hypothesised involvement of KLF6 in the absence of KLF2 and KLF4, possibly compensating for their lack, could not be confirmed. The eNOS target gene was surprisingly at none of the time points significantly reduced. Downregulation of the expression levels could be detected, but due to larger variations in the datasets, the observation was not significant. It can be assumed that with an increase in sample number significance might possibly be reached.

To investigate the long term expression of KLF2, KLF4, KLF6 and eNOS in response to high shear at 20 dyne/cm², flow experiments were conducted for 48h. The expression levels of KLF2, KLF4 and their target gene eNOS were significantly up-regulated (p< 0.01), while KLF6 expression did not change in comparison to a static control condition. Furthermore, gene silencing experiments with KLF2 ABC and KLF4 BC siRNA were performed. The 48h time measures confirmed gene silencing of KLF2 and KLF4 with their specific siRNA also at prolonged time (p< 0.01).
This was an important finding as gene silencing by siRNA is only temporarily and the effect normally vanishes with destabilisation of the molecules and cell divisions, but cannot be predicted in advance. KLF2 was significantly reduced (p< 0.05) in the KLF4-silenced condition, but the opposite could not be confirmed. Similarly to earlier time point measures, KLF6 expression did not alter. Surprisingly, eNOS expression was not impaired in response to electroporation with KLF2 ABC siRNA, while silencing of KLF4 reduced its levels significantly (p< 0.01). It is possible that the lack of KLF2 was compensated by maintained KLF4 levels, which accounts for the steady expression. These findings were important as the microarray study discussed in the following section was performed with samples from the 48h time point.

Morphological changes in response to flow and RNAi inhibition could also be observed. PAECs aligned in the direction of flow noticeable as early as 12h of exposure to high shear stress at 20 dyne/cm², while they remained randomly arranged even after 24h in low shear conditions. The gene silencing of KLF2 and KLF4 with siRNA also resulted in a distinct phenotype: cells appeared polygonal, not as compact and did not align in the direction of flow.

Ideally, the time course data should be supported by experiments confirming the results on protein level. Preliminary protein immunoblots, demonstrating the intention to analyse also the protein fraction of the collected samples, are shown in Chapter 7.1. Due to difficulties in antibody specificity and efficiency this experimental series could not be concluded in the time scale of this thesis. Human cells for the verification of the protein levels in sheared conditions over time could not be obtained due to financial and time limitations at the late stage of this project.

In summary, the set of gene silencing experiments highlighted interesting relationships between the hypothesised network components. KLF2 expression is influenced by KLF4 and suggests a possible interaction, supported by the finding of CACCC binding elements in the promoter region. So far only one overexpression study of KLF4 has confirmed a negative relationship resulting in a 2.7-fold downregulation of KLF2 (104). Here, it was demonstrated that the inhibition of KLF4 caused reduction of KLF2 levels. KLF4 might also compensate for the lack of KLF2 expression as eNOS levels were not impaired in KLF2-silenced conditions at 48h. These results emphasise therefore a relationship between KLF2 and KLF4.
CHAPTER MICROARRAY ANALYSIS

5.1 Introduction

DNA microarray genome analysis, as already mentioned in the introduction, is a powerful tool, which allows for the quantification of genes at genome scale. It is commonly used to investigate the functional consequences of loss or gain of function experiments, hereby identifying for example new drug targets and biomarkers. Differentially expressed target molecules and their associated pathways can be revealed by further data processing, leading to in depths understanding of affected signalling cascades.

As the phenotypes for KLF2- and KLF4-silenced conditions were so clearly distinguishable from the WT control, it was assumed that KLF2 and KLF4 have to play a major role in shear stress sensing and the transmission to the cytoskeleton, which coordinates morphological changes. To investigate their role in response to shear stress, microarray analysis was conducted to highlight the functional role of KLF2 and KLF4 in a broader context. RNA samples for the array hybridisation were collected from shear experiments at 20 dyne/cm² for 48h. PAECs were left static, treated either with KLF2 ABC, KLF4 BC siRNA or non-impaired (WT) before exposure to shear stress. The resulting data was processed as outlined in section 2.8.2.

Most published microarrays investigating shear-induced genes were performed with HUVECs, using a variety of different shear regimes and time dependencies (40, 43, 44, 176). Several investigators have focussed their interest on KLF2 and conducted overexpression experiments without shear stress stimulus, thereby revealing some of the KLF2-dependent anti-inflammatory and anti-thrombotic target genes (39, 214). Just one study so far focussed on KLF4. Villarreal et al. used HUVECs to constitutively over-express KLF4 as well as KLF2 and MEK5 and they then performed a comparative genome analysis (180). Studies using arterial cells and gene silencing of either KLF2 or KLF4 have not been conducted so far.
5.2 Hypothesis and Aim

A set of microarray experiments was performed to investigate differentially expressed genes, comparing static, sheared as well as KLF2-, and KLF4-silenced conditions. The hypothesis was that KLF2 and KLF4 have common targets, but also many individual genes, which are important to assess as they contribute to atheroprotection and need to be better understood. Given the functional similarity between KLF2 and KLF4, was of importance to investigate the target genes of both to potentially identify genes exclusively regulated by only one KLF, which will contribute to the understanding of their individual roles. The work therefore builds up on the concept of Villarreal et al., with the difference that gene silencing in combination with shear exposure rather than overexpression in arterial and not venous cells was used. Also other investigators who have assessed genome-wide expression have used overexpression of target genes rather than gene silencing. Therefore the experimental set up presented in this thesis was a novel approach, which contributes to the understanding of the functional roles and the effects resulting from silencing of KLF2 and KLF4.

In the context of network analysis it is also of importance to exclude the interplay of other, so far not considered KLFs. Genome-wide analysis will enable to study the network in a broader context, because entire signalling pathways that depend on KLF2 and/or KLF4 can be identified. The analysis could also provide a possible explanation for the observed expression patterns. It was hypothesised that the oscillatory gene expression of KLF2 and KLF4 in response to high shear stress might result from an indirect negative feedback loop.

5.3 Results and Discussion

5.3.1 Clustering of expression data

The genome-wide transcriptional profiling of PAECs revealed after outlier removal, filtering and batch correction a total of 18,520 annotated genes. Figure 63 displays a pie chart with colour-coded categories to show expression dependencies of the datasets. Comparative studies of sheared and static samples revealed a total of 2,474 shear-dependent genes as their expression levels changed significantly based on FDR with q-value < 0.05. Shear-dependent genes, grouped in the grey-coloured area, can be further divided into a fraction that was additionally dependent on treatment with KLF2 ABC siRNA (yellow), KLF4 BC siRNA (red) or by both (orange).
A total of 477 genes were shown to be differentially expressed in dependence of shear and exclusively KLF2, while less than half of this number, 124 genes, were shear-, and solely KLF4-dependent. 210 genes have been identified to be regulated by shear, KLF2 ABC as well as KLF4 BC.

Previously, Villarreal et al. reported in their microarray study, using constructs over-expressing KLF2 and KLF4 in the absence of shear, that the expression of 1,301 genes was significantly different in response to KLF2 and the expression of 1,899 genes in response to KLF4. An overlapping regulatory role of KLF2 and KLF4 was shown to affect 552 genes (180). Villarreal et al. have used Applied Biosystems, a different array system to the Affymetrix platform used in this study, as well as HUVECs instead of PAECs. The variations in differentially expressed genes between their findings and the results discussed in this work could be explained by these differences. Interesting is the fact that their study suggested KLF4 to have a more extensive transcriptional regulatory role in ECs, while the data presented in this study suggests the contrary. It was therefore important to further analyse the datasets by clustering the affected target genes and analysing specific genes through the generation of gene lists.

![Pie chart displaying the number of genes being significantly expressed in the different microarray conditions.](image)

Figure 63: Pie chart displaying the number of genes being significantly expressed in the different microarray conditions. From a total of 18,533 annotated genes, 2,477 were found to be differentially expressed in dependence of shear (grey). This fraction can be further divided as 811 genes were KLF2-, and/or KLF4-dependent. 477 genes were differentially expressed in response to KLF2 ABC siRNA (yellow), 124 genes were differentially expressed in response to KLF4 BC siRNA (red) and 210 genes were mutually impaired by both (orange).
Results and Discussion

Exploratory data analysis of gene expression patterns was based on SOMs (Figures 64-66). This representation is suited for data analysis, interpretation of the results and to draw conclusions on the structure of the data. Topological ordering, which is the grouping of related genes close to each other, supports the detection of distinct and similar clusters. The SOMs were coloured in warm colours (from yellow to dark red), representing increased expression levels, and cold colours (from green to blue), representing decreased expression patterns. Ratios between the expression levels in siRNA treated conditions versus static, sheared versus static, as well as their difference were calculated and log-base2 of these values was used in the SOM representation. By investigating local relations of the data on the map, distinct clusters (I-IV) could be identified (Figure 64). For the difference of the two maps (siRNA treatment-sheared/static), these distinct areas grouped genes, which are (I) genes that were up-regulated with siRNA silencing and down-regulated in response to shear, (II) genes that were down-regulated with siRNA silencing and down-regulated in response to shear, (III) genes that were up-regulated with siRNA silencing and up-regulated in response to shear and (IV) genes that were down-regulated with siRNA silencing and up-regulated in response to shear.

SOMs based on the comparison of the conditions KLF2 ABC siRNA, shear and static are shown in Figure 64 and the comparison for KLF4 BC siRNA, shear and static is displayed in Figure 65. The last set of SOMs presents the differences of KLF2 ABC siRNA and KLF4 BC siRNA in sheared conditions (Figure 66).

Figure 64: Set of three SOMs visualising the comparative gene analysis of the different microarray conditions. The first SOM displays KLF2 ABC treated versus static, the second sheared versus static and the third results from a subtraction of the second from the third. Four distinct gene clusters have been identified in the last SOM: (I) genes that are up-regulated with KLF2 silencing and down-regulated in response to shear, (II) genes that are down-regulated with KLF2 silencing and down-regulated in response to shear, (III) genes that are up-regulated with KLF2 silencing and up-regulated in response to shear and (IV) genes that are down-regulated with KLF2 silencing and up-regulated in response to shear. The colour scale represents the log2 of the ratio where warm colours from yellow to red represent an upregulation and cold colours from green to blue a downregulation. Courtesy of Nataly Maimari.
Results and Discussion

Figure 65: Set of three SOMs visualising the comparative gene analysis of the different microarray conditions. The first SOM displays KLF4 BC treated versus static, the second sheared versus static and the third results from a subtraction of the second from the third. Four distinct gene clusters have been identified in the last SOM: (I) genes that are up-regulated with KLF2 silencing and down-regulated in response to shear, (II) genes that are down-regulated with KLF2 silencing and down-regulated in response to shear, (III) genes that are up-regulated with KLF2 silencing and up-regulated in response to shear and (IV) genes that are down-regulated with KLF2 silencing and up-regulated in response to shear. The colour scale represents the log_2 of the ratio where warm colours from yellow to red represent an upregulation and cold colours from green to blue a downregulation. Courtesy of Nataly Maimari.

Figure 66: Set of three SOMs visualising the comparative gene analysis of the different microarray conditions. The first SOM displays KLF2 ABC treated versus sheared, the second KLF4 BC versus sheared and the third represents the difference of the two. Four distinct gene clusters could be identified in the maps: (A) genes that are down-regulated with KLF2 silencing and, (B) genes that are down-regulated with KLF4 silencing, (C) genes that are up-regulated with KLF2 silencing, and (D) genes that are down-regulated with KLF4 silencing. The colour scale represents the log_2 of the ratio where warm colours from yellow to red represent an upregulation and cold colours from green to blue a downregulation. Courtesy of Nataly Maimari.
5.3.2 Comparative study of differentially expressed genes

A comparison of the maps revealed a distinct pattern of gene groups as differentially expressed. This means that loss of function experiments of KLF2 and KLF4 resulted in genotypic changes which should be further explored. Therefore the analysis was continued with a comparative study of genes differentially expressed in dependence of shear and/or siRNA treatment. Clustering of genes in groups according to an enrichment score (EScr) and functional annotation were firstly performed using DAVID in conjunction with KEGG databases. Comparing sheared with static conditions, the enriched functional clusters were annotated as ubiquitination (EScr=8.01), mitochondrion (EScr=6.61), nuclear lumen (EScr=6.44), cell cycle and protein modification and degradation (EScr=5.78), apoptosis (EScr=4.42) and angiogenesis (EScr=4.21). A query for the most related disease to the data returned atherosclerosis (pulmonary and coronary). These results were in line with current literature findings about the atheroprotective transcriptional program induced by shear stress as discussed in the introduction section 1.2.3. The analysis therefore gave confidence to trust in the obtained data.

Furthermore, genes differentially expressed in KLF2-silenced conditions in comparison to WT could be clustered into categories of nuclear lumen (EScr=16.63), mitochondrion (EScr=9.64), cytoskeleton (EScr=9.18), protein translation (EScr=7.94) and phosphorylation (EScr=6.23), showing that the transcriptional activity as well as ATP production and turn over, possibly through MAPK pathway impairment, were affected. Also, genes belonging to the cluster vascular development and angiogenesis (EScr=1.63) and apoptosis (EScr=1.43) could be identified. The comparison of KLF4 silencing versus sheared conditions returned gene clusters of similar functions related to nuclear lumen (EScr=5.74), angiogenesis (EScr=5.1), ribosomal function (EScr=3.5), lipid biosynthesis (EScr=3.46) and mitochondrion (EScr=3.11). Within the search option of associated diseases wound healing and thrombosis were returned as well as the related cluster coagulation (EScr=1.05). The cluster analysis revealed a functional correlation of KLF2 and KLF4 in angiogenesis, while this was more than twice as much for KLF4. In order to highlight related functions in more detail, the role of individual genes was further investigated.

The dependence on shear stress, KLF2-silencing or/and KLF4-silencing in differential regulation of these genes was summarised in a Venn diagram (Figure 67). In total 787 genes were up-regulated in dependence of shear and examples of those playing a role in cardiovascular biology are listed in the upper half of the grey shaded area, while genes that were down-regulated are shown in the bottom half.
The circles named KLF2 and KLF4 represent genes that were in addition to shear, also affected by gene silencing of KLF2 (yellow) and gene silencing of KLF4 (red). Genes listed within the intersection of the two circles changed their expression levels in dependence of KLF2 and KLF4. Directionality of the change in gene level expression is outlined by a different script, where genes up-regulated in response to silencing of KLF2 and/or KLF4 are printed in bold type and genes down-regulated in italic type. To highlight the role of KLFs and their specific targets, the following paragraphs shortly describes each gene’s function in the context of vascular biology.

**Figure 67:** Venn diagram displaying a list of differentially expressed genes in dependence of shear, KLF2-silencing and/or KLF4-silencing based on FDR with p-value < 0.05. 787 genes were up-regulated and 879 genes down-regulated comparing sheared with static condition. Examples of some genes from this group are listed in the grey shaded area. The circles named KLF2 and KLF4 represent genes that were in addition to shear also affected by gene silencing of KLF2 (yellow) and gene silencing of KLF4 (red). Genes listed within the intersection of the two circles changed their expression levels in dependence of both. Directionality of the change in gene level expression can be distinguished by the script; genes up-regulated in response to silencing of KLF2, KLF4 or both are printed in bold type and genes down-regulated in italic type. Gene names can be found in the list of abbreviations on page 10.
266 genes were shown to be up-regulated with shear and differentially expressed in KLF2-silenced conditions, including KLF2. Amongst these were vasoprotective MAPK pathway components such as p38β and MEK5, which induce ERK5 and MEF2D, the transcriptional activator of KLF2 and KLF4. Some of these were synergistically down-regulated with KLF2-silencing, which could implicate the existence of a feedback loop. The anti-apoptotic gene B-cell CLL/lymphoma (BCL) 2 was down-regulated, while COX genes and SMAD specific E3 ubiquitin protein ligase 1 (SMURF-1), a mediator of SMAD-dependent inflammation, were up-regulated. 211 genes in total were down-regulated in sheared conditions in KLF2-dependency. In the absence of KLF2, the expression of pro-apoptotic factor BCL10, which has also been shown to activate NF-κB via NF-kappa-beta-inducing kinase (NIK=MEKKK4) and inhibitor of kappa light polypeptide gene enhancer (IKK) (215) was significantly increased as well as phosphoglycerate mutase family member (PGAM) 5, a repressor of the vasoprotective Nrf2. In addition, the LDL receptor related protein associated protein (LRPAP) 1, which facilitates lipid uptake in the activated endothelium, was up-regulated. The sum of these factors supports the vasoprotective and anti-inflammatory and anti-apoptotic mediating role of KLF2. An interesting observation was the downregulation of genes functioning in cell adhesion like ALCAM, VCAM1 and CD38 in absence of KLF2 as it has previously been shown that their expression was suppressed by KLF2 (85, 116). As the silencing of KLF2 was not 100%, it could possibly be that the remaining KLF2 molecules contributed to the observed downregulation.

69 genes were identified as up-regulated in sheared and KLF4-silenced conditions, including KLF4. An important regulator of physiological vascular tone, ASS1, was significantly down-regulated in the absence of KLF4. The transcriptional repressor BCL6, which plays a role in the MEF2 activation through HDAC5, was significantly up-regulated, while juxtaposed with another zinc finger gene (JAZF1) was down-regulated. Phosphatase PPM1D mediates a feedback regulation of p38-p53 signalling that contributes to growth and the induction of stress-induced apoptosis. It was highly up-regulated in the KLF4 siRNA-treated condition. The antagonistic serine threonine kinase (STK) 39, which serves as an intermediate in the response to cellular stress by activating the p38 pathway, was down-regulated.

Shear-dependent downregulation and additional dependence on KLF4 has been shown for 55 genes. Within this category was an important pro-apoptotic factor from the caspase family (CASP7). The stress-activated, pro-apoptotic kinase STK4, which induces chromatin condensation followed by inter-nucleosomal DNA fragmentation and phosphorylation of FOXO 3, resulting in cell death initiation, was also up-regulated.
TGFβ1, which belongs to a family of multifunctional peptides that regulates proliferation, differentiation, adhesion and migration and is also known to induce apoptosis in ECs, was up-regulated in absence of KLF4. Another induced gene was LCCL and coagulation factor V/VIII-homology domains protein 1 (DCBLD2), a marker and regulator of cell proliferation in vascular remodelling, which regulates PDGF-induced VSMC differentiation, proliferation and migration.

The group of genes shown to be KLF2-, as well as KLF4-dependent was synergistically regulated. Only a few non-listed antagonistic genes could be identified in the microarray data, but their roles are not within the context of vascular biology. In KLF2- and KLF4-silenced and shear-dependent condition one major glycoprotein of vascular endothelium endoglin (ENG), an accessory TGFβ receptor, was down-regulated. ENG is known to be highly expressed during angiogenesis and to play a role in the development of the cardiovascular system and in vascular remodelling. It has also been shown to be crucial in the binding of ECs to integrins. Downregulation was also observed for Tie2, which is a tyrosine-kinase transmembrane receptor for ANG1. This gene may constitute the earliest mammalian endothelial cell lineage marker and is known to regulate endothelial cell proliferation and differentiation, to guide the proper patterning of ECs during blood vessel formation and to inhibit endothelial permeability.

Also down-regulated in the absence of KLF2 and KLF4 were SMAD2 and SMAD6. These are signal transducers and transcriptional modulators that mediate TGFβ1 signalling and thus regulate multiple cellular processes such as cell proliferation, apoptosis and differentiation. In coherence, also the expression levels of their upstream regulator TGFβ2 were significantly reduced. MMP28, which has been implicated to play a role in the regulation of matrix composition and turnover, was down-regulated as well. In contrast, pro-atherogenic genes that play a role in inflammation such as IL18BP were up-regulated. ApoO, which is involved in the transport and metabolism of HDL, LDL and VLDL lipoproteins, was up-regulated. An interesting finding was the identification of a member from the KLF family, KLF11, which was down-regulated in response to KLF2 and KLF4 silencing. So far, the understanding of KLF11’s role in the vascular endothelium is very limited. Knowledge about its functionality is very sparse, but it is implied to involve induction of apoptosis. It also activates the globin gene promoters and functions as an antagonist for SP1 proteins, which mediate cholesterol-dependent genes as published by Cao et al (216).
The cluster of genes whose expression levels decreased in response to shear, while being modulated by KLF2 and KLF4, included several inflammatory and apoptotic mediators. JUN, a member of the MAPK pathway downstream of JNK, also known as AP-1, was highly up-regulated. The protein has been implicated to play a role in mediating cell growth and transformation as well as pro-inflammatory pathways in a fashion similar to NF-κB activation. In contrast, an inhibitor of NF-κB translocation into the nucleus was down-regulated. The role of NF-κB1α, also known as IKKα, is crucial in the pro-inflammatory signal transduction and its dependence on KLF2 and KLF4 demonstrates their functional role in the initiation of inflammatory processes. In addition to global markers of inflammation, the rather unknown NR3C2 gene, encoding the mineralocorticoid receptor 1 (NR3C), was up-regulated. The NR3C2 protein can signal by binding to other proteins, mainly with transcription factors such as NF-κB, AP-1 or sterol O-acyltransferase (STAT) 1. It also has two physiological ligands, aldosterone and cortisol, that act as critical regulators of cardiovascular tone. A positive mediator of programmed cell death, death-associated protein (DAP), was down-regulated.

The microarray data analysis revealed a total of 2,477 genes with significant differences in gene expression levels comparing sheared conditions with static. Up-regulated genes belong to the category of vasoprotective, anti-inflammatory and anti-apoptotic mediators. Kelch-like ECH-associated protein (KEAP) 1 interacts with nuclear factor (erythroid-derived)-like (Nrf) 2 in a redox-sensitive manner and the dissociation of the proteins in the cytoplasm is followed by transportation of Nrf2 to the nucleus, where it binds to the anti-oxidant response element in the upstream promoter region of many anti-oxidative genes. MAPK9, also known as c-Jun N-terminal kinases, blocks the ubiquitination of tumour suppressor p53 (217) and thus it increases the stability of p53, which is important for apoptosis, genomic stability and inhibition of angiogenesis. eNOS, encoded by the gene NOS3, is a critical mediator of cardiovascular homeostasis through its vasodilating properties and maintenance of an anti-proliferative and anti-apoptotic environment in the vasculature. Nuclear receptor subfamily 2 (2NR2) is a ligand-activated transcription factor which mediates the regulation of the ApoA I gene transcription. The PI3K-dependent pathway is known to acetylate histones, resulting in chromatin remodelling, which allows for the binding of the general transcription machinery (106).
The group of down-regulated genes comprised regulators of angiogenesis, apoptosis and inflammation. ANG2, an antagonist of ANG1 and endothelial Tie2, which was up-regulated in dependence of shear, is known to mediate loosening of cell-matrix contacts and it may induce endothelial cell apoptosis with consequent vascular regression (218). VEGF is a regulator of blood vessel physiology and growth and plays a role in endothelial targeting of lipids to peripheral tissues, while BMP4 governs similar effects related to growth and differentiation. The executioner CASP3 was down-regulated, emphasising the pro-survival effects stimulated by shear stress. An example of the anti-inflammatory effect is the downregulation of toll-like receptor (TLR) 2, whose signalling cascade coupled to toll-like receptor activation, is very similar to that of ILR activation. NF-κB activation and cytokine secretion are triggered as part of an inflammatory cascade in response to lipoproteins and may also occur through TLR-2. The transcriptional activator GATA binding protein (GATA) 2, which regulates ET-1, a vasoconstrictor, but also an inducer of NO production, was down-regulated. The expression of serpine1 mRNA binding protein (SREBP) 1, the thrombogenic stabiliser of PAI-1, which functions as an inhibitor of fibrinolysis, was also regulated by shear stress.

Worth mentioning is that the housekeeping gene HPRT1, which was used to normalise the qPCR data presented in section 4.3, was not differentially regulated by shear, KLF2 or KLF4. This result therefore provides a quality control measure, which justifies the housekeeping role of HPRT1 as it was stably expressed in all conditions. In contrast, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, which are often selected for this purpose, showed dependence on KLF2.

5.3.3 Identification of DUSPs as possible regulators of KLF oscillatory expression pattern

From the list of differentially regulated genes in dependence of KLF2 and KLF4, DUSP7 and DUSP16 were identified as possible components of a regulatory negative feedback loop. DUSPs belong to a class of negative regulators of MAPKs by dephosphorylation of threonine and tyrosine residues TXY in kinase domain regions. In the MAPK pathway strong inhibition of nuclear ERK1/2 by DUSP5 and cytosolic ERK1/2 by DUSP7 have been reported by Jeffrey et al. (219). DUSP7 does also target two of the other main signalling cascades by inhibiting JNK and p38. DUSP16, which has been shown to be active in the nucleus as well as in the cytoplasm, has a preference for JNK and p38 over ERK1/2. To investigate if DUSPs could potentially interact with ERK5, which is the MAPK known to be involved in mechanosensitive activation of MEF2, comparative promoter studies have been performed.
Indeed, these have shown that ERK5 shares a 66% similarity in the kinase domain with ERK1/2. Especially the TEY motif, which is the DUSP recognition sequence, is conserved amongst ERK5 (218-220 aa), ERK1 (202-204 aa) and ERK2 (185-187 aa). Reviewing the literature on ERK5 inhibition by DUSPs, several groups have reported at least indirect interaction. Sarkoezi et al. have postulated ERK1/2-driven negative feed-back regulation of ERK5, which is mediated by DUSP5, DUSP6 and/or DUSP1. They have found that these co-immunoprecipitated with ERK5 and that transient expression of an adenoviral constitutively active DUSP1 construct completely blocked EGF-induced ERK5 phosphorylation (220). Similarly, results from Kamakura et al. show that expression of DUSP1 or DUSP6 potently inhibited the EGF-induced activation of ERK5 in kinase assays (221). As DUSPs share large sequence homology, interactions of DUSP5, DUSP7 or DUSP16 cannot be excluded. Promoter studies revealed the presence of CACCC motif for all three DUSPs, which could be potential KLF binding sites. These interactions would then lead to a negative feedback loop as postulated in Figure 68, which could explain the oscillatory expression behaviour in response to high shear stress for KLF2 and KLF4.

Figure 68: Schematic drawing of a potential negative feedback loop on KLF2 expression through ERK5 inhibition by dephosphorylation triggered by DUSP7 and DUSP16. High laminar shear stress has been shown to activate MAPK pathway through phosphorylation of MEK5 and subsequently ERK5. ERK5 is known as an activator of MEF2, which binds to the KLF2 promoter and initiates its expression. KLF2/4 CACCC binding motifs have been identified on DUSP7/16 promoters and a direct interaction could be hypothesised. Translocated DUSP could potentially bind to the kinase domain of ERK5 and inhibit its activity by dephosphorylation.
5.4 Conclusion

High laminar shear stress is considered to be atheroprotective, stimulating an anti-inflammatory and anti-thrombotic endothelial phenotype. The activated signalling cascades are to some extent regulated by members of the KLF family, which act as exclusively shear-induced transcription factors. KLF2 and KLF4 have both been termed key regulators of endothelial inflammation, but it is not clear to which degree they function cooperatively or act as antagonists. Several microarray studies have been performed with ECs exposed to shear stress and/or inflammatory cytokines, also in conjunction with the overexpression of mechanosensitive genes as detailed in section 1.2.2. This study is the first one performed in primary arterial cells in contrast to venous-derived cells. As plaque development occurs only in arteries of the vascular tree, the in vivo situation should be resembled as closely as possible. In addition, it was decided to individually suppress KLF2 and KLF4 mRNA using RNAi technology in combination with long term exposure to shear stress rather than viral overexpression as done by other research groups. This strategy allowed to differentiate between direct transcriptional targets of KLF2, KLF4 or both and to assign functional roles to KLF2 and KLF4 in more detail.

The genome-wide microarray data analysis revealed 18,533 annotated genes of which 2,477 were differentially expressed comparing sheared versus static conditions. Amongst these genes were targets that are known to be differentially regulated by atheroprotective shear stress. The focus of this study was to further investigate the 32.71% of genes that were additionally dependent on KLF2 and/or KLF4 expression. Comparative analysis of the identified gene lists was used to determine functional clusters. Within the group of 477 exclusively KLF2-regulated transcripts, analysis of the main clusters revealed affiliation to the categories of transcriptional activation, cytoskeleton and ATP turnover. Functional analysis of each individual gene within these clusters was then performed. It could be shown that many members of phosphorylation-dependent MAPK signalling pathway, such as MEK5 and p38 were differentially expressed in the absence of KLF2. Interestingly, MEF2, a strong activator of KLF2 transcription, was also down-regulated. This observation could implement the existence of a direct or indirect negative feedback loop from KLF2 to its inducer. Genes playing a role in cytoskeletal arrangement and cell adhesion, such as ALCAM, CD38 and VCAM1, were differentially regulated.
This is in agreement with the phenotypical observations showing that cell alignment, which depends on adhesion molecules and rearrangement of the cytoskeleton, in KLF2-silenced condition was strongly impaired even after 48h shear exposure (section 4.3.3.8). KLF2 seemed to also regulate factors governing leukocyte rolling and attachment, supporting its role as anti-inflammatory mediator. An interesting observation that needs to be mentioned is the non-differential expression of eNOS in KLF2-silenced conditions. Also the qPCR analysis of eNOS expression at 48h (Figure 62) revealed that it was slightly down-regulated, but without significance. eNOS has previously been established as a direct target and it was shown that eNOS was significantly up-regulated (p<0.05) in WT shear versus static conditions after 48h (Figure 62). It could therefore be possible that in the presented model other eNOS inducers compensated for the lack of KLF2.

The cluster analysis of genes listed as shear-induced and KLF4-dependent revealed a strong link to wound healing and thrombosis as disease indicators and returned genes important for angiogenesis as highly enriched. These associations could not be made for KLF2 and represent hence an important difference in function comparing the two transcription factors. Further detailed analysis of individual genes revealed a new target of KLF4, DCBLD2, which acts as a marker for vascular remodelling and could be a key downstream target, regulating processes within the identified clusters. Several inducers of apoptosis were up-regulated, underlining the pro-survival mediation of KLF4 signalling, which is to a similar extent true for KLF2. An interesting finding was the exclusive regulation of ASS1 through KLF4. In the only published comparison of KLF2 and KLF4 targets by overexpression studies (104), ASS1 was shown to be transcriptional inducible by both factors. Similar to the observations that KLF2 regulates MEF2D activity, BCL6, a repressor of MEF2 activating HDAC5, was shown to be up-regulated in the absence of KLF4, thereby enhancing the inhibition effect. Hence, this cascade, which forms a negative feedback loop, could partially explain the observed oscillatory expression of KLF4.

30.57% of genes regulated by KLF2 were similarly controlled by KLF4, suggesting a significant degree of common downstream targets. The clusters with the highest enrichment scores were associated with nuclear lumen and kinase binding activity. Gene analysis revealed a number of MAPK pathway components to be differentially expressed, including up-regulated JUN and down-regulated inhibitor of NF-κB, DUSP7 and DUSP16, which can all be activated to transmit a signal via phosphorylation. Common targets, such as Tie2, TGFβ and ENG, important vasoprotective factors and mediators of vascular remodelling, were down-regulated.
Within the context of the hypothesised network, KLF6 could not be identified as a target of KLF2 and KLF4 by the genome-wide analysis. This result mirrors the conclusion of the previous chapter based on qPCR analysis that KLF6 was not differentially regulated by shear as well as by RNAi targeting KLF2 and KLF4. Hence, the initially proposed network comprising KLF2, KLF4 and KLF6 could not be confirmed, while a mutual interaction of KLF2 and KLF4 was shown. Within this context it is important to mention that KLF11 arose as one of the gene targets differentially expressed in dependence of shear stress, KLF2 and KLF4. It could be of value to investigate the relationship of these components, to pursue the network hypothesis, as a circuitry of KLF2, KLF4 and KLF5 has been shown to be crucial for ES cell self-renewal (131).

The microarray data analysis revealed for the first time DUSPs as new shear-dependent targets of KLF2 and KLF4. DUSPs have been shown to negatively regulate MAPK pathway signalling cascades by dephosphorylation of components such as ERK1/2, JNK and p38. Comparative promoter analysis resulted in 66% sequence homology between ERK5 and ERK1/2. Most importantly, this work has shown conservation of the DUSP recognition sequence amongst all ERKs.

Evidence suggesting interaction between the differentially regulated DUSP7/16 and ERK5 could not be confirmed by an extensive literature search. Reports mention the possible involvement of DUSP5, 6 and/or 1 in ERK5 inhibition, but current findings are controversial. It could therefore be possible that the effects of DUSP7/16 on ERK5 have just not yet been studied. A direct negative feedback loop through transcriptional activation of DUSP7/16 induced by binding of KLF2/4 to the promoter was proposed. DUSP proteins can subsequently translocate into the cytoplasm to inhibit ERK5 activity, which can therefore not induce the transcriptional activator MEF2, resulting in reduced KLF2 and KLF4 expression levels (Figure 68). This mechanism is only hypothetical and would need to be further investigated, but there is enough theoretical evidence to suggest such an interaction.
An issue that is often addressed when discussing results based on RNAi experiments is the specificity of the siRNA probes. Since the discovery of OTEs in 2004 (222), investigations revealed several determination factors, which can be grouped into these categories:

1. siRNA-induced sequence-dependent regulation of unintended transcripts through partial sequence complementarities to their 3' UTRs (miRNA-like OTEs). It has been shown that most of the OTEs appear to be mediated by siRNAs containing seed matches, predominantly in the guide strand, to conserved regions of the 3' UTR of the mRNAs transcripts. As siRNAs and miRNAs share the same silencing machinery, the sequence-dependent off-target activity of siRNAs seems to result from siRNAs entering the natural miRNA pathway. They function as miRNAs on targets with partial sequence complementarity to the siRNA guide strand (223).

2. siRNA-triggered inflammatory response through activation of the immune system. The RNA-sensing receptors TLR3, TLR7 and TLR8 traffic between the endoplasmic reticulum and intracellular compartments, such as endosomes and lysosomes and trigger the induction of IFN, TNFα and IL6. Transfection methods such as lipofection, which require the endosomal pathway, can potentiate the immune stimulation (224).

3. A novel non-specific effect of RNAi expression relates to saturation of the RNAi machinery. It is suggested that cells have a limited capacity to assemble the RISC complex on exogenous siRNAs, and this might affect miRNA expression levels and function (225).

To address these issues, extensive research revealed strategies to successfully limit the risk of OTEs and thereby the misinterpretation of data. Regarding the siRNA design, minor modifications in the siRNA sequence such as a match at position 16 of the siRNA or avoidance of G and U nucleotides in the seed region, have been shown to reduce OTEs. Additional chemical modification reported by Jackson et al. could reduce the OTEs by 80%. They report that sense strand modifications have been added to impede strand entry into RISC and that they identified key nucleotides on the antisense strand to limit OTEs (148). As the specific siRNA sequence is strongly dependent on the target sequence, many siRNA design algorithms focusing on gene-target specificity and efficiency have been developed. Companies have created online tools to support the custom-design taking into account the above mentioned parameters.
Koenig et al suggested, in addition to an optimally designed siRNA, a strict requirement for multiple potent siRNAs against one gene as the pooling may reduce the likelihood of over-interpreting screen hits resulting from OTEs (226). For the interpretation of the biological effect, due to target-specific gene silencing through siRNA, it should ideally be verified that after reintroduction of the particular gene product in gene-silenced conditions the reverse effect can be observed.

OTEs cannot be completely avoided, but the listed points should be considered as a guideline for RNAi experiments. For the study presented here, the probes were chemically modified and rationally designed with the support of Dharmacon experts. One challenge that had to be overcome was the species specificity, as currently no company offers an algorithm based on the pig genome as background. The siRNAs were therefore designed to target sequences homologous to the human database and were subsequently blasted against the pig genome using several tools to identify possible mismatches. As described in section 4.3.1.1, several KLF2-, and KLF4-specific siRNAs were pooled to minimise the possibility of non-specific differential gene expression results.

To conclude this chapter, the experiments and evaluation of the microarray analysis have been performed according to golden standard rules (see section 1.4.2), giving confidence in the interpretation of the results. Collectively, the observations have revealed novel regulatory processes mediated by KLF2 and KLF4, which contribute to the understanding of vasoprotective mechanisms.
6 CHAPTER GENERAL DISCUSSION

6.1 Summary

Atherosclerosis is a complex multifactorial chronic inflammatory disease, which develops predominantly in branches and curvatures of large arteries, where blood flow is non-laminar (11). The focal distribution of plaques has been explained by differences in shear stress magnitude acting on the endothelium in these regions, which triggers a stimuli-specific mechanosensitive signalling cascade. Ectopic mechanoreceptors are able to sense shear stress and further stimulate an intracellular biochemical response. In atheroprone regions, where disturbed flow dominates, the endothelium is in an inflamed activated state, leading to the upregulation of pro-atherogenic genes. Induced cytokines mediate the attachment and transmigration of monocytes, which dedifferentiate into macrophages sustaining inflammation, oxidative stress and matrix remodelling. Foam cells develop as LDL infiltrates with increasing endothelial permeability in low flow regions, forming the inner core of the plaque in addition to ECM fibres. Growth and migration of VSMCs contribute to plaque progression, which leads to increased stenosis and could eventually result in plaque rupture and thrombus formation associated with a high mortality rate.

Risk factors of atherosclerosis and potential preventative measures have been discussed in Chapter 0. One of the key points in prevention of the disease is the detailed understanding of the underlying molecular mechanisms and their effectors. As the endothelium remains inactivated in regions of high shear stress the mediators, which protect the healthy endothelium and those triggering the inflammatory response, need to be identified and understood. The transcription factors KLF2 and KLF4 have been found to be shear-induced through activation of the ERK5/MEF2D-dependent pathway and already a decade ago the atheroprotective role of KLF2 through induction of vasoprotective factors has been established. Lately emerging reports about KLF4 having an anti-inflammatory role and regulatory function in atheroprotection now question to which degree each component orchestrates the stimulation of atheroprotective signalling (110).
The work presented in this thesis aimed to investigate the role of several shear stress-induced KLFs for the induction of eNOS in a time-dependent manner. It was hypothesised that KLF2, KLF4 and KLF6, which was also shown to be induced through shear stress (41), may form a tightly regulated network. Jiang et al. have reported a network formed by KLF2, KLF4 and KLF5 as the key regulator determining ES self-renewal through the common target Nanog (131). This was a novel approach as so far investigators have only looked at individual KLFs.

The project aimed to additionally identify in a comparative analysis genome-wide targets of KLF2 and/or KLF4 to highlight their individual and overlapping roles as these are mostly unexplored. In vivo studies do not allow for controlled modulations of shear stress in a time-dependent manner to study molecular signalling cascades. Therefore, an in vitro flow system and the necessary techniques to culture isolated primary ECs under shear stress had to be developed. The project required a set of multidisciplinary skills such as engineering, cell-, and molecular biology as well as understanding of bioinformatics.

Chapter 3 detailed the iterative process leading to the development of a functional flow device. This initial phase required substantial improvements of cell isolation and culture conditions, optimisation of the flow chamber coating and cell seeding densities, testing of various materials for tubing and the media chamber as well as the validation of three different pump circuits. The main obstacle encountered was initiated by the formation of gas bubbles that circulated around the system. As a result, cells were scratched off from the surface of the flow chamber, thereby impairing monolayer integrity. Phenotypical changes such as the accumulation of vesicles in the cytoplasm and almost dendritic shape, which eventually resulted in apoptosis, could be observed. After the optimisation period, the final system peristaltic prototype II proved to function reliably for up to 48h at steady shear stress, ranging from 0.5 dyne/cm² to 30 dyne/cm². The cell seeding density was optimised so that a monolayer could be formed over night and that subsequent exposure to flow did not alter the integrity. High shear stress resulted in cell alignment in the direction of flow after approximately 12h. This observation was considered as a quality control of the system as cells in atheroprotected regions in vivo are known to be aligned (12).

The following Chapter 4 described the application of the developed flow system for studying the expression levels of KLF2, KLF4, KLF6 and eNOS in dependence of two different shear rates. Cells were exposed to atheroprotected high shear stress at 20 dyne/cm² and atheroprone shear stress at 5 dyne/cm² for 2h, 4h, 6h, 12h and 24h. Further processing of the sheared samples required the establishment of qPCR methodology, including primer design, validation and optimisation of required reagents.
The fine-tuning of all parameters yielded optimal experimental conditions with efficiencies $>90\%$ over a broad dynamic range, allowing for robust quantification of target gene expression. After normalisation and outlier removal based on z-score analysis, time course experiments of 2-24h exposure to high shear revealed distinct expression patterns for KLF2 and KLF4. These were highly up-regulated in response to shear after 2h and their expression profile showed an oscillatory pattern over time. KLF6 was, despite the hypothesis, not significantly regulated by shear stress and eNOS expression showed a steadily increasing trend. In low shear stress, KLF2 and KLF4 upregulation at 2h was less significant and not different from static at later time points. Similarly, KLF6 and eNOS expression did not significantly change from the static condition. These experiments showed that differences in shear magnitude resulted in distinct expression patterns of KLF2 and KLF4.

High atheroprotective shear stress caused a dynamic upregulation of the two transcription factors, while their target gene eNOS increased steadily. These observations led to the proposal of a network, functioning in a compensatory mode to control eNOS expression. So far researchers have performed studies in which KLF2 or KLF4 were overexpressed to understand their functional roles. The work in this thesis presented a novel approach as the consequences of loss of function using siRNA targeting KLF2 and KLF4 were explored. The data resulting from these siRNA experiments provided new insight to their regulatory roles.

To perform gene silencing experiments based on RNAi, siRNAs were custom-designed and tested for their efficiency and specificity, which involved the development of a suitable transfection method. Primary cells such as PAECs are difficult to transfecit, but with the optimised electroporation parameters, transfection efficiencies of $>90\%$ in conjunction with high viability could be achieved.

Gene silencing of KLF4 impaired the expression of KLF2 at all measured time points, while the reverse observation could not be made for KLF2 silencing. This result suggests an existing dependence of KLF2 on KLF4, which presents a newly postulated concept. KLF6 was not affected by the inhibition of KLF2 and/or KLF4, which disproved the initial hypothesis of it playing a role in a KLF network, though other groups have shown shear-dependence of KLF6 (41). eNOS was at early time points significantly reduced in KLF2- and KLF4-silenced conditions, while this trend was later only significant for KLF4 BC siRNA treatment. This means that the absence of KLF2 over long term might stimulate compensators, amongst those possibly KLF4, which maintain eNOS expression. In addition to the time series of up to 24h, the long term effects of gene silencing were tested. The dependence of KLF2 on KLF4 could be confirmed, while an opposite relationship was not detected.
Even at prolonged shear exposure, KLF6 was not impaired, while eNOS was significantly down-regulated dependent on KLF4 silencing. In summary, this chapter brought new insight on the unidirectional relationship of KLF2 dependence on KLF4. It also revealed that the likelihood of a network involving KLF6 is rather low, but eNOS was confirmed as a direct target of both transcription factors. Initially, the work was meant to be continued by investigating a computational model that could mathematically describe and also predict interactions between the network components. As the involvement of KLF6 could biologically not be confirmed, another strategy had to be implemented. As a network of KLFs comprising KLF2, KLF4 and KLF5 was shown to regulate self-renewal of ES cells (131), the possible interaction of KLF2 and KLF4 with so far not considered KLFs was addressed.

Chapter 5 presented the results obtained from a comparative microarray analysis of samples that were kept statically, exposed to high shear stress or sheared with additional siRNA treatment targeting KLF2 and KLF4 mRNAs. The genome wide analysis with loss of function of KLF2 and KLF4 was novel as so far investigators have only performed overexpression studies and only a single publication is known, which considered KLF2 as well as KLF4 (180).

Gene lists of differentially regulated targets in these conditions were created as a result of initial data processing. The array revealed 2,477 shear-dependent genes, of which 477 were additionally KLF2-dependent and 124 KLF4-dependent, while 210 genes were mutually regulated by both transcription factors. DAVID and KEGG databases were used as tools to investigate the biological meaning behind those gene lists through functional annotation, pathway identification and gene-disease associations. Comparison of sheared versus static conditions revealed mostly known functional annotations such as mitochondrion, reflecting ATP production, vasoprotection and angiogenesis, while atherosclerosis was returned as the associated disease. More in depth analysis of individual genes from this list confirmed several regulatory key factors of the proposed pathways. Shear-dependent downregulation of KLF12 and KLF15 comprised a novel finding and could potentially be of interest for further investigations. Also KLF11 has been shown to be down-regulated in response to shear and siRNA intervention against KLF2mRNA and KLF4mRNA.

New findings arose especially from the analysis of siRNA-treated conditions, as for the first time, this study addressed global consequences of KLF2 and KLF4 gene silencing in conjunction with shear stress. The analysis allowed for the identification of KLF2 and KLF4 specific targets, which has not been made in such detail so far.
Hamik et al. proposed overlapping function in some areas (110), which could be confirmed as the expression levels of 210 genes changed in dependence of both TFs, meaning that KLF2 and KLF4 can regulate the promoter activity of these target genes.

In response to silencing of KLF2, genes associated with nuclear lumen, cytoskeleton, phosphorylation, vascular development and apoptosis were differentially expressed. Individual genes of each category could be identified, most importantly upstream regulators like MEK5, MEF2D and other MAPK components such as p38 and ATF2. As KLF2 can impair the expression of its direct inducers, a feedback mechanism is likely to exist, which could partially explain the oscillatory pattern observed for KLF2 expression over time. Low KLF2 expression levels function as transcriptional activators of the upstream MEK5, which induces KLF2 through ERK5 stimulation. It remains to be explored whether this regulation occurs through direct or indirect interaction.

Upregulation of inflammatory molecules like VCAM-1 and CD38, which mediate adhesion events, supports the role of KLF2 as a regulator of anti-inflammatory processes. Within the set of genes solely dependent on KLF4 and shear, none of the direct MAPK components was differentially expressed, while an indirect link via BCL6, which is a transcriptional repressor of the MEF2D activating HDAC5, could be revealed (227). Regulation of ASS-1 underlines the important role of KLF4 as a mediator of vasoprotection. The entire gene list analysis defined the categories of nuclear lumen, angiogenesis, apoptosis and coagulation as KLF4-regulated functions, while wound healing and thrombosis were suggested as linked diseases. Some of these functions are very distinct from the ones revealed for KLF2, especially the association with angiogenesis and thrombosis. It would be of interest to evaluate these further so that the roles of KLF2 and KLF4 in the context of atherosclerosis progression can be even more distinguished.

A significant degree of transcriptional redundancy between KLF2 and KLF4 can be assumed as 120 genes were differentially regulated by both with the same directionality. These genes can be clustered in processes involving adherent junctions, lipid biosynthesis, TGFβ regulation, transcriptional activation and repression, angiogenesis and vasculature development. As components of the NF-κB activation machinery were also modulated targets, the role of KLF2 and KLF4 as mediators of inflammatory processes could be confirmed. An interesting finding was the mutual regulation of DUSP7/16, which are known to inactivate their targets through dephosphorylation. DUSP family members have been shown to repress signalling cascades of the MAPK pathway and a role of DUSP7/16 in ERK5 signalling was hypothesised (220, 228). The oscillatory pattern of KLF2 and KLF4 could potentially be explained by such negative feedback signalling cascades.
To conclude, the study has given new insight on the regulatory roles of KLF2 and KLF4 resulting in the identification of novel individual as well as common targets. A larger number of genes showed expression dependency on KLF2, which might suggest that its role in transcriptional regulation in PAECs has a wider extend than KLF4. This is in contrast to the findings from Villarreal et al., who detected more genes being influenced by KLF4 overexpression in a genome-wide study. However, in the context of vascular development, KLF 2 (−/−) mice die around age E12.5-14.5 due to haemorrhages as a result of defects in the vessel wall maturation and stability (98), while homozygous KLF4 (−/−) mice show no vascular abnormalities, but die shortly after birth due to loss of the skin barrier function (100). Results of this study suggest a dependency of KLF2 on KLF4 and that possibly another, not yet confirmed member of the KLF family, contributes to a regulatory network that orchestrates processes involved in shear-dependent endothelial vasoprotection.

6.2 Study limitations

The study design contains some limitations, which are discussed in this section. Firstly, the selected flow chamber holds several disadvantages, but also advantages. As the product of choice was not reusable, the perpetual purchasing costs were high, but in comparison to self-made chambers, the sterility was always guaranteed with this commercially available product. The producing company offers a wide product range suitable for different customer needs, but as the flow chambers are constraint to the size of a microscopy slides, the area and therefore the number of cells that can be exposed to shear was limited. For additional processing such as protein analysis, large cell amounts are needed, which can sometimes not be retrieved from a single chamber. The selected peristaltic pumps could only provide unidirectional flow and the perfusion pump additionally oscillatory flow. Although flow rates could be computationally controlled, it was not possible to mimic the flow profile of naturally occurring systolic and diastolic phases. In order to resemble the in vivo situation as closely as possible, this modification in shear stress profile could be considered. In relation to the in vivo situation, the flow system cannot simulate dilation of the vasculature as it occurs naturally and the cells can therefore not experience cyclic stretch as an additional mechanostimulus.

For the time series investigated in Chapter 4, the frequency of sampling presents a limitation. Regular intervals would ideally be preferred, but time constraints dictated the experimental design.
Conclusion

As the phosphorylation cascade in response to an external stimulus such as shear stress is known to be activated within minutes (72), it was decided to sample more frequently at early time points. In terms of expression pattern recognition and modelling efforts, an increased sample size collected at more regular intervals would be beneficial.

The selection of KLFs investigated in the qPCR study was limited to the already known shear-modulated members of the family. As the initial hypothesis predicted a network of KLF2, KLF4 and KLF6, which could not be verified, it would have been advantageous to increase the number of selected KLFs at the beginning. Extending the target genes within the network would contribute to a more detailed analysis, but does also add enhanced complexity.

The study would preferably be completed by protein analysis using protein immunoblot methodology to confirm the observed oscillatory expression for KLF2 and KLF4 and the gene silencing efficiency on protein level. Initial efforts of these studies are outlined in section 7.1.1. One of the main issues why the protein detection could not be completed within the time scale of this thesis was the requirement for optimisation of many reagents. As this study was based on pig-derived samples, none of the reagents such as primers, siRNA, antibodies could be purchased with a working guarantee as companies offer this service only for certain species such as human and mouse. Each new experiment required therefore an extensive testing phase to confirm selectivity and specificity of reagents, which was very time-consuming. On the other hand, the work in this thesis has explored arterial cells in contrast to the commonly used venous cells. It was thought that arterial cells resemble more closely the in vivo situation as plaque development occurs exclusively in large arteries.

The microarray study presented in Chapter 5 has been conducted with three samples for each condition (static, sheared, sheared KLF2-silenced and sheared KLF4-silenced). Some investigators would prefer an increased sample size to better account for biological variability. As microarray analysis is still expensive, the balance between costs and additional benefits had to be evaluated. The technique also presents some limitations as discussed in Chapter 2.8. One of the obstacles in microarray analysis is cross-hybridisation between similar sequences to the non-repetitive fraction of the genome, which complicates analysis of related genes, alternatively spliced transcripts, allelic gene variants and SNPs. Their signal to noise ratio is known to be quite high and could lead to enhanced difficulties when detecting low-abundance sequences and quantitative resolution of changes in frequently occurring sequences (229).
RNA isolation yields were substantial so that the amount needed for hybridisation to the array did not present a challenge as sometimes argued. New generation sequencing could have been selected as a tool for genome analysis as an alternative to microarray technology, but finances were restrictive. A limitation that occurred during the data analysis was again species-related, as the annotations for the Affymetrix pig array were not complete and had to be complemented using an in-house designed database.

6.3 Future work

The results obtained in this thesis have opened up several new lines of enquiry that would be interesting to address in future studies.

6.3.1 Do other KLFs contribute to a shear-dependent regulatory network?

The number of genes assessed by qPCR should be expanded including the KLFs that were shown to be differentially expressed by genome-wide microarray analysis. To date, the shear stress dependent stimulation of KLF11, KLF12 and KLF15 has not been published and it would be of interest to confirm the microarray results with qPCR assays. Possibly one of these KLFs might interact with KLF2 and/or KLF4, contributing to the network hypothesis. Especially KLF11, which was shown to be regulated by shear, KLF2 and KLF4 would present a high potential target in these investigations. To gain further insight on regulatory mechanisms of the network, individual target genes as well as common targets of the investigated KLFs should also be added. Already addressed as a current study limitation, the verification of gene expression data on protein level would be an interesting undertaking. It is therefore a priority to optimise the experimental settings for protein detection and quantification by protein immunoblotting. Preliminary results are shown in the Appendix 7.1.1, but specific antibodies for porcine KLF protein have not been obtained yet. Two complete time series experiments have been conducted and samples, in form of frozen cell pellets, collected to facilitate the continuation of this investigation.

Results of the quantitative gene expression analysis are meant to be used for mathematical modelling purposes based on established differential equation models and a newly developed inductive logic programming (ILP). Current preliminary efforts are summarised in the Appendix 7.1.2, but it would be of interest to further pursue this line of experiments.
Ideally, a computational model could be used to identify and predict important functional relationships *in silico*. Revelation of global gene regulation patterns as well as identification of a KLF cluster would support the biological investigations.

6.3.2 Does a negative feedback loop mechanism control KLF expression?

To verify the proposed feedback loop involving DUSP7/16, direct or indirect interaction with KLF2 and/or KLF4 has to be experimentally addressed. *In silico* promoter studies have confirmed the presence of canonical CACCC binding site within the DUSP7/16 promoters and direct interaction could for example be demonstrated by immunoprecipitation and sequential promoter deletion experiments. In a second step, the interaction of DUSPs with ERK5 has to be shown. As other members of the DUSP family are known to interact with the highly homologous ERK1 or ERK2, similar lines of investigation should be approached (220, 221). Co-immunoprecipitation confirming direct binding, verification of substrate specificity and proof of inactivation of ERK5 by DUSPs *in vitro* and in ECs should be addressed. Furthermore, the mechanism of inactivation, such as retaining ERK5 in the cytoplasm or transcriptional repression, should be explored. As the upstream regulators of KLF2, MEK5 and MEF2D were differentially expressed in dependence of KLF2, a potential interaction, directly or through so far unknown mediators, would also be worthwhile investigating.

6.3.3 May KLFs be useful therapeutic targets?

The research in this thesis contributes to the current knowledge that KLFs mediate vasoprotective mechanisms, underpinning that they have scientific and potentially therapeutic importance. Identified gene targets of KLF2 and KLF4 are known to be key regulators of endothelial homeostasis in health and disease. *In vivo* studies are needed to further confirm the atheroprotective function of KLF2 and KLF4 and to determine their individual roles in depth. It has already been shown that KLF2 is lacking in curvatures or bends of the vasculature, which are considered to be atheropron regions, and this observation should also be confirmed for KLF4 (43, 184). Gain and loss of function studies *in vivo*, should give insight whether KLFs might be potential therapeutic targets in the prevention and treatment of CVDs. Possible strategies could involve the intervention with stents coated with KLF protein to locally release atheroprotective KLF to modulate endothelial biology in stenosed areas. At this stage, there is the potential for KLFs to be further investigated in clinical applications, but more fundamental research needs to contribute to the understanding of these highly interesting and promising key regulators.
7 CHAPTER APPENDIX

7.1 Preliminary data

As discussed in section 6.3 of suggestions for future experiments, two lines of investigation have already been initiated and preliminary data is presented in the following paragraphs.

7.1.1 Protein detection using immunoblotting

The quantification of KLF2 and KLF4 protein levels for time course gene expression data discussed in Chapter 4.3 would be beneficial, as the effect on protein levels due to gene silencing could be demonstrated. It would also be of interest to analyse if the protein pattern changes in a time-dependent manner as observed for the gene expression time-series experiments. Unfortunately, the identification of specific antibodies turned out to be very difficult, as antibodies suitable for detection in pigs are currently not produced and literature reports as references were lacking. Sequences homologues between human and porcine proteins were selected as their respective antibodies were supposed to have binding affinity within these regions.

To test the antibody specificity, recombinant KLF2 and KLF4 (isoform A) protein were generated by Dr. Takayuki Homma, a postdoc in Prof. Krams group, to function as a positive control. Shortly, the protein coding cDNA was selected and amplified from PAECs sheared for 6h at 20 dyne/cm². Ndel and BamHI restriction sites were used to ligate the construct into a pET-16b vector (Novagen), carrying a His-tag coding sequence. Expression of the recombinant proteins was performed in BL21 (DE3) E. coli strain and affinity beats (Invitrogen) for His-tag were used for purification. A dilution series of the recombinant purified proteins KLF2 and KLF4 was preformed to test the detection with anti-His-tag monoclonal antibody (Novagen) and protein specific antibodies. After modification of the experimental conditions, such as changing from nitrocellulose to PVDF membrane, using optimised lysis buffer and varying blocking buffer recipes, an antibody from Abnova was shown to detect KLF2 protein (Figure 69) and an antibody from Santa Cruz KLF4 isoform A protein (Figure 70).
For KLF2, single bands with almost no background could be detected at the expected molecular weight of 40.1 kDa with anti-His-tag antibody, indicated by an arrowhead. Several additional bands can be seen on the blot detecting porcine KLF2 with the specific primary antibody, which suggests that the methodology was unproblematic, but needs further fine-tuning, confirming that each antibody in general is potentially specific for porcine KLF2.

![Image of protein immunoblots]

Figure 69: Protein immunoblots using anti-His-tag antibody and anti-KLF2 antibody from Abnova (H00010365) to detect recombinant porcine His-KLF2 protein (35.42 kDa). 2-16 µL of a 1/5 diluted stock solution of recombinant KLF2 were applied as samples. Courtesy of Dr. Takayuki Homma.
Recombinant porcine KLF4 isoform A with 53.4 kDa could be detected with an anti-His tag antibody as well as with the protein specific antibody. Further optimisation is needed to reduce the unspecific background.

![Image](image.png)

**Figure 70:** Protein immunoblots using anti-His-tag antibody and anti-KLF4 antibody from Santa Cruz (sc-20691) to detect recombinant porcine His-KLF4 protein isoform A (53.4 kDa). 2-16 µL of a 1/5 diluted stock solution of recombinant KLF4 were applied as samples. Courtesy of Dr. Takayuki Homma.

After specific antibodies could be determined, samples derived from PAECs sheared for 4h or left static were tested for protein detection with the anti-KLF4 antibody. The previously tested recombinant porcine KLF4 isoform A protein was used as a positive control. As seen in Figure 71, a weak band at approximately 50 kDa, as indicated by the arrowhead, was observed for the sheared condition, while it was absent in the non-sheared condition. The positives controls, generated from different lysates, were slightly overexposed to enhance the sample band. For future experiments before testing cell samples retrieved from time-course experiments, the dynamic range has to be optimised, including a reduction of protein concentration for the recombinant protein and an increase of sample concentration and marker (M) volume. Similar experiments have to be performed to test the specificity of the KLF2 antibody for sheared cells, which was unfortunately not possible due to time constraints.
Appendix

Figure 71: Protein immunoblot using anti-KLF4 antibody (final dilution 1/1000) to detect KLF4 protein in samples from PAECs sheared for 4h at 20 dyne/cm² (+) or left static (-). Recombinant porcine His-KLF4 isoform A protein was used as a positive control. A weak band can be detected for the sheared sample at marker (M) weight of approximately 50 kDa, which is the expected weight for KLF4, while this band is absent in the non-sheared condition.

7.1.2 Computational model

The observed oscillatory expression patterns of KLFs and the proposed network interactions were analysed using a computational modelling approach based on differential equations. The project is in collaboration with PhD student Borislav Vangelov, who has performed preliminary experiments with a code developed in Matlab. Gene expression data from different time points, discussed in section 4.3.2, showed variability between different replicates for the same time point. An interesting observation was that the comparison of replicates for a continuous time series revealed differences in the dynamics, but not the expression pattern. In the context of a computational model, a modulator explaining the variation between replicates was used to mathematically address this issue. Further investigations are needed to confine the initial model.
The following model describes time-dependent gene expression.

\[ \dot{X}(t) = \alpha + \beta m_j S(t) - \gamma X(t) \]

with

- \( X(t) \) = expression of the gene in time \( t \)
- \( \alpha \) = basal expression rate for the gene
- \( \beta \) = influence of the signal on the expression of the gene
- \( S(t) \) = signal intensity
- \( m_j \) = magnitude for a replicate
- \( \gamma \) = degradation rate of the gene

Equation 9: Mathematical model for time-dependent gene expression based on quantitative PCR data.

\[ \frac{X(t+1) - X(t)}{\Delta t} = \alpha + \beta m_j S(t) - \gamma X(t) \]

with \( \Delta t \) = sampling intervals (h), which are kept constant.

The Error (E) can be determined as shown in part (3) of the equation and is used to determine the quality of the model predictions compared to the observed data.

\[ E = X(t + 1) - X(t) - (\alpha + \beta m_j S(t) - \gamma X(t) - \Delta t)^2 \]

F solved for \( \Delta t \) constant = 266.84

The resulting inferred signals for low and high shear stress with \( \Delta t \) constant are shown in Figure 72.

*Figure 72: Preliminary results of the computed inferred signal for low shear conditions (A) and high shear conditions (B) for time series data discussed in section 4.3.2. Courtesy of Borislav Vangelov.*
7.2 Buffer recipes

Table 17: Buffer recipes.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 Tween 20</td>
<td>In PBS (-)</td>
</tr>
<tr>
<td>FACS Buffer</td>
<td>PBS 1x containing 0.5% BSA and 0.05% sodium azide</td>
</tr>
<tr>
<td>Original lysis buffer</td>
<td>50 mM TrisHCl (pH 8.0), 150 mM NaCl, 10% glycerol, 1% TritonX-100, 1/100 protease inhibitor</td>
</tr>
<tr>
<td>RIPA lysis buffer</td>
<td>150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0. (SIGMA R0278)</td>
</tr>
<tr>
<td>TBE (5x)</td>
<td>108 g Tris, 55g Boric acid, 0.5 M EDTA</td>
</tr>
<tr>
<td>Transfer buffer (10x)</td>
<td>120 g Tris, 576 g glycine and 10% methanol in 4 L</td>
</tr>
<tr>
<td>Trisglycine SDS (10x)</td>
<td>25 mM Tris, 193 mM glycine, 0.1% SDS (pH 8.3) Fermentas</td>
</tr>
<tr>
<td>WB blocking buffer</td>
<td>5% (w/v) non-fat dried powder milk in PBS (-)</td>
</tr>
</tbody>
</table>

7.3 Specification of reagents, materials, chemicals and solutions

Table 18: Reagent kits.

<table>
<thead>
<tr>
<th>Kits</th>
<th>Company</th>
<th>Order nr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA isolation RNAeasy mini kit</td>
<td>Qiagen</td>
<td>74106</td>
</tr>
<tr>
<td>ECL™ Prime Protein immunoblotting Detection Reagent</td>
<td>Applied Biosystems</td>
<td>89168-782</td>
</tr>
<tr>
<td>Fast SYBR® Green Master Mix</td>
<td>Applied Biosystems</td>
<td>4385616</td>
</tr>
<tr>
<td>High Capacity RNA-to-cDNA Kit</td>
<td>Invitrogen</td>
<td>4387406</td>
</tr>
<tr>
<td>Neon™ Transfection System</td>
<td>GE Healthcare</td>
<td>MPK1025</td>
</tr>
</tbody>
</table>

Table 19: Cell culture items.

<table>
<thead>
<tr>
<th>Cell culture items</th>
<th>Company</th>
<th>Order nr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>T25 culture flasks</td>
<td>Corning</td>
<td>734-1712</td>
</tr>
<tr>
<td>10 ml pipettes</td>
<td>Fisher</td>
<td>734-1738</td>
</tr>
<tr>
<td>10 ml syringes</td>
<td>Fisher</td>
<td>613-4919</td>
</tr>
<tr>
<td>20 ml syringes</td>
<td>Fisher</td>
<td>613-5402</td>
</tr>
<tr>
<td>25 ml pipettes</td>
<td>Fisher</td>
<td>734-1739</td>
</tr>
<tr>
<td>4-well, flat bottomed plate</td>
<td>Nunc</td>
<td>179820</td>
</tr>
<tr>
<td>5 ml pipettes</td>
<td>Fisher</td>
<td>734-1737</td>
</tr>
<tr>
<td>6-well plate</td>
<td>Corning</td>
<td>734-1599</td>
</tr>
<tr>
<td>Glass pasteurs</td>
<td>Fisher</td>
<td>612-1702</td>
</tr>
<tr>
<td>Microcentrifuge tubes (0.5 ml)</td>
<td>Fisher</td>
<td>211-0024</td>
</tr>
<tr>
<td>Microfluidic chamber</td>
<td>ibidi</td>
<td>80606</td>
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<tr>
<td>Petri dishes (90 mm)</td>
<td>Sterilin</td>
<td>391-2015</td>
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Table 20: Reagents, materials, chemicals and solutions.

<table>
<thead>
<tr>
<th>Reagents, materials, chemicals and solutions</th>
<th>Company</th>
<th>Order nr.</th>
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<tr>
<td>2-Mercaptoethanol</td>
<td>Sigma</td>
<td>63689</td>
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<tr>
<td>All stars RNA</td>
<td>Qiagen</td>
<td>1027284</td>
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<tr>
<td>Amphotericin (Fungizone)</td>
<td>Sigma</td>
<td>A 9528</td>
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<tr>
<td>BSA (standard Fraction V)</td>
<td>Sigma</td>
<td>A 9418</td>
</tr>
<tr>
<td>Collagenase IV</td>
<td>Sigma</td>
<td>C 9891</td>
</tr>
<tr>
<td>Confocal Oil Glycerol Type G</td>
<td>Leica</td>
<td>11513910</td>
</tr>
<tr>
<td>Confocal Oil Type F</td>
<td>Leica</td>
<td>11513859</td>
</tr>
<tr>
<td>Dimethylsulfoxide (DMSO)</td>
<td>Sigma</td>
<td>D 8418</td>
</tr>
<tr>
<td>DraQ5</td>
<td>Biostatus Limited</td>
<td>DR50200</td>
</tr>
<tr>
<td>Dulbecco`s Modified Eagle Medium (DMEM)</td>
<td>Sigma</td>
<td>D 5546</td>
</tr>
<tr>
<td>ECGF</td>
<td>Sigma</td>
<td>E 2759</td>
</tr>
<tr>
<td>Ethanol</td>
<td>VWR</td>
<td>E/0600DF/17</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Sigma</td>
<td>ED2SS</td>
</tr>
<tr>
<td>Foetal Calf Serum (FCS)</td>
<td>Sigma</td>
<td>F 7524</td>
</tr>
<tr>
<td>Gel drying solution</td>
<td>Invitrogen</td>
<td>LC 4025</td>
</tr>
<tr>
<td>Gelatine</td>
<td>Sigma</td>
<td>G 9382</td>
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<tr>
<td>Gentamycin</td>
<td>Sigma</td>
<td>G 3632</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Sigma</td>
<td>G 8540</td>
</tr>
<tr>
<td>HBSS</td>
<td>Sigma</td>
<td>H 9269</td>
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<tr>
<td>Newborn Calf Serum (6 x 500 ml)</td>
<td>Sigma</td>
<td>N 4637</td>
</tr>
<tr>
<td>Nitrocellulose blotting membrane</td>
<td>Whatman</td>
<td>HS03-0931</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>Promega</td>
<td>P1193</td>
</tr>
<tr>
<td>PBS (10 x)</td>
<td>Sigma</td>
<td>D 1408</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>Sigma</td>
<td>P 4333</td>
</tr>
<tr>
<td>PVDV membrane</td>
<td>Bio-Rad</td>
<td>162-0175</td>
</tr>
<tr>
<td>RNase AWAY</td>
<td>Sigma</td>
<td>83931</td>
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<tr>
<td>Sample buffer</td>
<td>Invitrogen.</td>
<td>LC2676</td>
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<tr>
<td>siGLO Red Transfection Indicator</td>
<td>Thermos Scientific</td>
<td>D-001630-02-05</td>
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<tr>
<td>TO-PRO-3 iodide</td>
<td>Invitrogen</td>
<td>T3605</td>
</tr>
<tr>
<td>Trypan Blue HyClone</td>
<td>Thermo Scientific</td>
<td>SV30084.01</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Sigma</td>
<td>T 4799</td>
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## 7.4 List of equipment

*Table 21: Equipment.*

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Company</th>
<th>Details</th>
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<tbody>
<tr>
<td>Accu Spin 400</td>
<td>Fisher Scientific</td>
<td></td>
</tr>
<tr>
<td>Autoclave ML3375IL</td>
<td>SANYO</td>
<td></td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Hettich Rotanda 460R</td>
<td></td>
</tr>
<tr>
<td>Chromatography paper</td>
<td>Whatman</td>
<td>30306461</td>
</tr>
<tr>
<td>Combs 12 well</td>
<td>Invitrogen</td>
<td></td>
</tr>
<tr>
<td>Confocal microscope</td>
<td>Leica TCS SP5</td>
<td></td>
</tr>
<tr>
<td>DFC290 camera</td>
<td>Leica</td>
<td></td>
</tr>
<tr>
<td>DNA gel Ow3l Easycast B2</td>
<td>Thermo Scientific</td>
<td></td>
</tr>
<tr>
<td>Dryease mini gel drying system</td>
<td>Invitrogen</td>
<td></td>
</tr>
<tr>
<td>Elbow Luer connector</td>
<td>ibidi</td>
<td>10802</td>
</tr>
<tr>
<td>Freezing box (Mr frosty)</td>
<td>Nalgene</td>
<td></td>
</tr>
<tr>
<td>Gel cassettes</td>
<td>Invitrogen</td>
<td>NC 2012</td>
</tr>
<tr>
<td>Incubator</td>
<td>Labnet 311 DS</td>
<td></td>
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<tr>
<td>Incubator Incusafe</td>
<td>SANYO</td>
<td></td>
</tr>
<tr>
<td>Luer Male luer to 200 series Barb Elbow 1/8</td>
<td>The Westgroup</td>
<td>LE 7230 -6</td>
</tr>
<tr>
<td>Microporator MP-100</td>
<td>Digital Bio</td>
<td></td>
</tr>
<tr>
<td>Microscope DM IL LED</td>
<td>Leica</td>
<td></td>
</tr>
<tr>
<td>Mr Frosty</td>
<td>Nalgene</td>
<td>5100-0001</td>
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<tr>
<td>NanoDrop 2000c/2000</td>
<td>Nanodrop</td>
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<tr>
<td>PCR machine</td>
<td>Labnet MultiGene gradient</td>
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<tr>
<td>Perfusion prototype</td>
<td>Iibi</td>
<td>10902</td>
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<tr>
<td>Peristaltic pump prototype I</td>
<td>Gilson Minipuls F1 55001</td>
<td>10 rollers</td>
</tr>
<tr>
<td>Peristaltic pump prototype II</td>
<td>Atto perista SJ1220</td>
<td>6 rollers</td>
</tr>
<tr>
<td>qPCR machine</td>
<td>Eppendorf realplex 4</td>
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<tr>
<td>SDS tank</td>
<td>Novex Minicell 700</td>
<td></td>
</tr>
<tr>
<td>Syringes</td>
<td>Terumo</td>
<td></td>
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<tr>
<td>Table top centrifuge</td>
<td>Technico maxi</td>
<td></td>
</tr>
<tr>
<td>Tissue culture hood Topsafe 1.5</td>
<td>Bioair Euroone devision</td>
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<tr>
<td>Transfer tank</td>
<td>Appleton Woods</td>
<td>BCV510</td>
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<tr>
<td>UV Chamber</td>
<td>UVP Biospectrum</td>
<td></td>
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<tr>
<td>UV-Vis Spectrophotometer</td>
<td>Eppendorf</td>
<td></td>
</tr>
<tr>
<td>Voltage meter</td>
<td>VWR</td>
<td></td>
</tr>
</tbody>
</table>
8 CHAPTER REFERENCES


4. Movva R & Figueredo VM (Alcohol and the heart: To abstain or not to abstain? *International Journal of Cardiology* 01.030).


