Chapter 6

General Discussion and Future Perspectives
Plasticity of endothelial cells under various local environments

The response of vascular endothelial cells to extracellular signals

The local environment along the vascular tree is heterogeneous and dynamic so as to accommodate the different spatiotemporal needs. Also, functions of blood vessels vary temporally and spatially, according to the demand of the body.\(^1\) For instance, under normal physiological conditions, vessels prevent the passage of leukocytes from the bloodstream to the surrounding tissue. However, upon vascular injury, the vessels act as a facilitator by recruiting leukocytes to the site of inflammation.\(^5\) Arteries deliver oxygen and nutrients, capillaries regulate exchange of gases and nutrients, whereas veins transfer deoxygenated blood and waste from tissues and organs. Endothelial cells in both macrovasculatures (arteries and veins) and microvessels (arterioles, capillaries and venules) are highly plastic, being able to adapt to local environmental changes to secure maintenance of vascular homeostasis and execution of relevant physiological functions.\(^1,3\)

Plasticity of endothelial cells is well reflected when comparing arteries with veins. Besides oxygen tension, there are differences in hemodynamic forces, in particular flow direction and fluid shear stress.\(^1\) In arteries, there is a higher oxygen tension and a higher shear stress than in veins. Remarkably, heterogeneity of the local environment is also evident within the arterial tree, as shown by pulmonary arteries that have a low oxygen tension, but a high shear stress. Both arteries and veins are lined by a continuous non-fenestrated endothelium, yet arterial endothelial cells exhibit stronger intercellular junctions than those in the veins.\(^1,4\) Arterial endothelial cells with a narrow and elongated morphology are aligned parallel to the direction of flow. However, venous endothelial cells with a wide polygonal morphology orient randomly without a definite direction.\(^1\) Interestingly, an irregular arterial geometry that brings about heterogeneous hemodynamic forces in different regions of the arterial tree causes variation in endothelial phenotype as well.\(^5\) Indeed, endothelial cells can distinguish and elicit a specific phenotype in response to different magnitudes of shear stress\(^6\) and direction of flow.\(^7\) The distinct endothelial phenotype of a specific organ vanishes when the cells grow in vitro in the absence of blood flow.\(^8\) Hence, blood flow that elicits hemodynamic forces is the primary determinant of endothelial phenotype\(^6,10\) and is an important factor that contributes to cardiovascular disorders, particularly in the arteries.\(^11-13\)

Murine models with venous bypass grafts undergo arterialisation when subjected to arterial flow conditions, as shown by the thickening of circumferential media, higher elasticity of the vessels and lower endothelium-dependent vascular permeability.\(^14\) Similarly, endothelium of human saphenous veins gains arterial properties and express higher levels of endothelial nitric oxide synthase (eNOS) when exposed to arterial circulation.\(^15\) On the other
hand, adaptation of venous endothelial cells to an arterial environment may result in endothelial-to-mesenchymal transition (EndMT), a process that contributes to neointima formation and graft atherosclerosis. In Chapter 2, we suggest that EndMT-associated neointima formation is caused by disturbed arterial flow that elicits low shear stress. In contrast to disturbed flow, high shear stress as a result of laminar flow can counteract the adverse endothelial activation by biochemical stimuli, such as TNF-α and lipopolysaccharide. Our data in Chapter 2 and Chapter 3 show that under static conditions in which cells are not exposed to shear stress, the endothelial phenotype is principally governed by transforming growth factor-β1 (TGF-β1). However, the effects of TGF-β diminished when cells were subjected to flow, suggesting that shear stress is more potent than biochemical stimuli in regulating the endothelial phenotype. Moreover, studies in chick embryos demonstrate that the endothelial phenotype was resolved and arterial-venous patterning was achieved after the exposure to blood flow. Kurniati et al. and Li et al. reported that the loss of flow alters the endothelial gene expression, notably downregulation of TEK tyrosine kinase (TEK) and upregulation of vascular adhesion molecules (SELE and VCAM1). Indeed, endothelial gene transcription is tightly mediated by flow through shear stress-inducible transcription factors that bind to shear stress responsive elements (SSRE) in the gene promoter.

It has been postulated that the arterial and venous endothelial phenotypes are determined during embryogenesis via genetic programming before the onset of blood flow. Consistent with this argument, studies in zebrafish embryos indicate that epigenetic changes precede haemodynamic forces in determining the molecular identity of endothelial cells. Notch signalling that occurs through binding of ligands to Notch receptors during cell-cell interaction also accounts for endothelial cell fate. The lack of sonic hedgehog and vascular endothelial growth factor (VEGF) activity that act upstream of Notch results in loss of arterial gene expression and gain of venous gene expression in endothelial cells. Therefore, endothelial cell fate is not entirely determined by biomechanical signals in the embryo. However, we and others showed that haemodynamic forces play a key role in modulation of the endothelial phenotype in adults, as shown by arterialisation of vein grafts and reliance of arterial endothelial cell function on high shear stresses. Evidently, plasticity of endothelial cells is modulated in a temporal and spatial manner by both biomechanical and biochemical stimuli. These exogenous inducers mediate epigenetics, and gene and protein expression in endothelial cells by triggering different intracellular signal transduction pathways. To date, the mechanisms that relate extracellular signals with the phenotype of endothelial cells remain incomplete. This has led to a surge of interest in mechanobiology and cellular biology to understand how endothelial cells interact with the local environment and to what extent these interactions have an impact on endothelial phenotype and cardiovascular (patho)physiology.
Human umbilical vein endothelial cells as a model for *in vitro* studies of arterial functions

In comparison with *in vivo* studies, *in vitro* studies which are conducted on a specific type of tissue under a defined local environment are less complex and easier to control. Most importantly, *in vitro* studies can provide more explicit molecular information about the interactions between cells and extracellular inducers. Therefore, *in vitro* models have advantages over *in vivo* models in mechanistic studies that relate the phenotype of cells with signal transductions. This is especially the case for those signal transductions that are involved in multiple pathways and/or in a network of signalling crosstalks. This advantage is very much appreciated in understanding the complex intracellular signal transductions by biomechanical forces (mechano-transduction) under varied local environmental conditions. Markedly, *in vitro* studies contributed the earliest direct evidence that haemodynamic forces modulate structure and function of endothelial cells.\(^{10}\) The *in vitro* experiments of this thesis were conducted with human umbilical vein endothelial cells (HUVEC). It has been suggested that HUVEC which are isolated from neonatal tissue may not reflect the phenotypical properties of adult endothelium accurately. Human saphenous vein endothelial cells are proposed to be more functionally similar to arterial endothelial cells than HUVEC, as shown by their response to stimulation of oxidised-low density lipoprotein and pro-inflammatory cytokines, *i.e.* tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β) and interferon γ (IFNγ).\(^{26}\) We (Chapters 2 & 3) and others\(^{5,10,12,27-30}\) demonstrated that, upon exposure to arterial flow, HUVEC exhibit phenotypical properties that are consistent with the adult arterial endothelium, *i.e.* elongation and alignment of cell axes in the direction of flow, ERK5 activation, increased transcript expression for Kruppel-like factor 2 (KLF2), KLF4, eNOS, TEK and kinase insert domain receptor (KDR). We also showed that, as in the case with human aortic endothelial cells, HUVEC underwent endothelial-to-mesenchymal transition (EndMT) upon stimulation with TGF-β1. Shear stress-induced ERK5 activation counteracts EndMT, irrespective of the cell type. These *in vitro* data corroborate our observations in porcine abdominal aorta (Chapter 2), and support the notion that HUVEC is a reliable *in vitro* model to reproduce *in vivo* endothelial plasticity.\(^{31}\) Therefore, HUVEC is suitable to study arterial endothelial functions, in particular the responses to shear stress and TGF-β1.

Cytoskeletal remodelling mediates the response of endothelial cells to biomechanical signals

The cytoskeleton, which is composed of actin filaments (F-actin), microtubules and intermediate filaments, is a durable and flexible scaffold that regulates cell shape and cell migration.\(^{32}\) Activation of intracellular signal transductions by biomechanical forces is critically reliant on the angle between
flow and the cell axis, and suggests a correlation between cytoskeleton and response of endothelial cells to flow. Blood flow comprises two crucial physical factors, i.e. flow direction and flow intensity (shear stress). In the arteries, a disturbed flow as a result of the ever changing flow direction and intensity, yields a low net forward direction and shear stress (<4 dyn/cm²), whereas laminar flow is characterised by unidirectional flow and high shear stress (10–70 dyn/cm²). Endothelial cells exposed to a disturbed flow in the arterial regions orient randomly as if those in the vein which are also exposed to a low shear (1–6 dyn/cm²). Endothelial cells exposed to laminar flow show an explicit alignment in the direction of the flow. Therefore, endothelial cell morphology is often used as an indicator of local flow patterns. An alignment parallel to the flow direction minimises the stresses imposed on cells, thus maintaining intracellular homeostasis. In vivo and in vitro studies have revealed that the loss of endothelial alignment is associated with a pro-inflammatory phenotype.

Endothelial cells in a static environment show dense actin fibers (peripheral bands) around the cell membrane (Figure 1A). When subjected to prolonged laminar flow, these peripheral bands are reorganised into long, central actin fibers (stress fibers) which are parallel to the cell axis (Figure 1B). Indeed, flow is a strong inducer of actin reorganisation (cytoskeletal remodelling), yet it remains elusive if the formation of stress fibers and the alignment of cells are induced by the flow direction, by the intensity of shear stress, or by both. We showed in Chapter 4 that laminar (unidirectional) shear stress at 20 dyn/cm² induced cell alignment parallel to the flow, whereas a shear stress at 2 dyn/cm² failed to do so, despite the flow was laminar. These findings lead to two interesting conclusions. First, shear stress instead of flow direction induces cytoskeletal remodelling. Second, laminar flow works in concert with high levels of shear stress to induce cytoskeletal remodelling. An experiment which exposes endothelial cells to flow with an irregular direction and a high intensity of shear stress (20 dyn/cm²) should reveal which conclusion is true.

Strikingly, as shown in Chapter 5, laminar flow failed to induce alignment in HUVEC when the activity of p38 mitogen activated protein kinase (MAPK) was repressed under pro-fibrotic conditions. This observation is consistent with those in bovine aortic endothelial cells that performed under non-fibrotic conditions. Azuma et al. and Kadohama et al. elucidated that the loss of alignment in response to laminar flow is caused by an impairment in p38 MAPK-mediated cytoskeletal remodelling. Interestingly, such an impairment did not block the response of endothelial cells to shear stress, because expression of the shear stress-responsive KLF2 and KLF4 genes were elevated (Chapter 5). It appears that laminar flow regulates cytoskeletal remodelling and gene expression through two distinct mechanisms. Indeed, the mechanosensory component that senses flow direction is different from which that senses shear
stress. Interruption in either the flow direction or the shear stress sensing machinery alters the downstream response of endothelial cells to flow.\textsuperscript{38,41} p38 MAPK could be a mediator in these signal transductions. Since our endothelial cells respond to shear stress, we suggest that inhibition of p38 MAPK impedes cytoskeletal remodelling by interfering with the signal transductions of flow direction sensing. It is also plausible that p38 MAPK inhibition limits the potential of shear stress in inducing cytoskeletal remodelling, while the signalling that regulate gene expression remains intact. Orientation of cells relative to the flow direction might reflect the state of intracellular signalling. Cells that orient perpendicular (60–90°) to the flow direction correlate with the activation of pro-inflammatory nuclear factor (NF)\textsuperscript{κ}B signalling and attenuation of anti-inflammatory eNOS signalling.\textsuperscript{7} Under pro-fibrotic conditions, 38\% of the cells oriented between 60 and 90° to the flow direction when p38 MAPK pathway was blocked (Chapter 5). However, we were not able to provide direct evidence whether these cells are pro-inflammatory, as blocking of the p38 MAPK pathway with an inhibitor potently downregulated the expression of adhesion molecules and chemotactants (Chapters 3 and 5). Immunostaining for \textit{in situ} detection of NF\textsuperscript{κ}B nuclear translocation and activation of eNOS would clarify whether the cells in Chapter 5 that orient perpendicular to the flow are indeed pro-inflammatory. Of note, sensing and transmission of extracellular forces occurs not only apically from the cell surface to the cytoskeleton, but also from the

\textbf{Figure 1. Prolonged laminar flow induces reorganisation of cytoskeleton.} (A) Under static condition, endothelial cells show peripheral bands around the cell membrane. (B) Endothelial cells show stress fibers which are parallel to the cell axis when exposed to prolonged laminar flow that elicits high shear stress.
basal matrix to the cytoskeleton. The latter requires interaction of integrins with matrix proteins. Shear stress-induced integrin αvβ3 activation and binding to extracellular matrix promotes the transient inactivation of Rho and alignment of endothelial cells in the direction of flow. Future studies looking into the effects of p38 MAPK inhibition on integrin signalling under stimulation of TGF-β should provide further insight into cytoskeletal remodelling under pro-fibrotic conditions.

A transient activation of the p38 MAPK and the c-Jun NH2-terminal kinase (JNK) pathways by flow is required for cytoskeletal remodelling, yet persistent activation of these pathways results in a pro-inflammatory endothelial phenotype. Hahn et al. reported that endothelial cells seeded on fibronectin showed, upon exposure to laminar flow for 45 min, pronounced JNK activation, but not on collagen type I or Matrigel (a solubilised basement membrane). It thus appears that the extracellular matrix affects activation of the haemodynamic force-induced signal transduction in endothelial cells, but this is evident only in cells which are exposed to short-term laminar flow or long-term disturbed flow. All \textit{in vitro} experiments described in this thesis were performed with fibronectin as extracellular matrix. Our data show that long-term (24 h or longer) laminar flow repressed the activation of p38 MAPK and JNK (Chapter 2), as well as downregulated the expression of pro-inflammatory adhesion molecules and chemoattractants (Chapter 3), despite the cells were seeded on fibronectin. This corresponds with earlier findings that prolonged exposure to laminar shear antagonises pro-inflammatory pathways and brings about anti-inflammatory effects. In \textit{in vitro} studies revealed that the pro-inflammatory pathways are indeed activated upon abrupt onset of both laminar and disturbed flow. Long-term laminar flow induces negative feedback mechanisms to block these pathways, but a disturbed flow fails to do so, thus resulting in a pro-inflammatory phenotype. Since extracellular matrix and the exposure time to flow affects the response of endothelial cells to shear stress, the selection of matrix protein for coating and the time course for \textit{in vitro} shear stress experiments should be performed with caution while studying phenotypes of endothelial cells and in relation to mechanotransduction.

**The role of shear stress and TGF-β in regulation of endothelial phenotype**

**TGF-β-induced endothelial-to-mesenchymal transition contributes to vascular remodelling**

TGF-β-induced EndMT is required for the development of heart valves during embryogenesis, but the same process contributes to cardiovascular pathologies in adult life. Markedly, embryonic EndMT occurs at areas of
high shear stress,\textsuperscript{47,53,54} whereas EndMT in adult arteries is prevalent at regions of low shear. Most importantly, high shear suppresses EndMT in adult endothelial cells (Chapter 2). This indicates that the mechanisms by which shear stress regulates EndMT in embryonic and adult endothelial cells are different. Studies in chicken embryos and mouse embryonic endothelial cells demonstrated that shear stress induces the expression of the \textit{KLF2} transcript, which is accompanied by the activation of the TGF-\textbeta/3/ALK5 pathway.\textsuperscript{54} In contrast, we elucidated that shear stress attenuates the activation of the TGF-\textbeta/1/ALK5 pathway while upregulating the \textit{KLF2} expression in HUVEC. Egorova \textit{et al.}\textsuperscript{54} reported that activation of the TGF-\textbeta/3/ALK5 pathway is crucial for the shear stress-induced \textit{KLF2} expression in embryonic endothelial cells but not in HUVEC and human aortic endothelial cells. These contradicting data suggest that the function of TGF-\textbeta/ALK5 signalling under shear stress is different between embryonic and adult endothelial cells. On the other hand, Walshe \textit{et al.}\textsuperscript{55} showed in HUVEC that shear stress induced TGF-\textbeta/3/ALK5 signalling along with \textit{KLF2} induction. This suggests that regulation of endothelial gene expression by TGF-\textbeta/ALK5 signalling under shear stress also depends on the type of the TGF-\textbeta ligand.

In adults, expression of TGF-\textbeta/1 is elevated in arteries upon vascular injury\textsuperscript{56-59} and accounts for cardiovascular pathologies.\textsuperscript{48-52} Prior results\textsuperscript{48,60-62} showed that higher levels of TGF-\textbeta/1 promotes EndMT in endothelial cells under static environment. We provide \textit{in vitro} and \textit{in vivo} evidence that TGF-\textbeta/1 induces EndMT in disturbed flow regions or at places where vascular repair is required (\textit{i.e.} stenotic and atherosclerotic lesions). EndMT generates fibroblasts, myofibroblasts and smooth muscle cells, increases extracellular matrix deposition, and thus contributes to intimal thickening and atherosclerosis (Chapter 2). This observation was recently confirmed in a murine model, namely that EndMT through the pro-fibrotic TGF-\textbeta/SMAD2/3–Slug signalling pathway induces neointima thickening that accounts for vein graft failure.\textsuperscript{16} In Chapter 2, we elucidated that laminar flow activates the protective ERK5 pathway that represses EndMT. Chapter 3 shows that laminar flow attenuates TGF-\textbeta/SMAD2 activation through upregulation of inhibitory-SMADs via an ERK5-independent pathway, which provides further mechanistic evidence why TGF-\textbeta-driven EndMT is inhibited by laminar flow. These novel results, together with the existing conclusions,\textsuperscript{34,35} elucidate that laminar flow is anti-inflammatory and anti-fibrotic, whereas a disturbed flow is pro-inflammatory and pro-fibrotic.

In addition to the TGF-\textbeta/ALK5 signalling, EndMT is provoked by NF\kappaB signalling too\textsuperscript{60} (Chapter 3). Nuclear translocation of NF\kappaB was attenuated by shear stress at 10–25 dyn/cm\textsuperscript{2}, but was enhanced in response to shear stress higher than 25 dyn/cm\textsuperscript{2}.\textsuperscript{6} We showed in Chapter 2 that shear stress at 20 dyn/cm\textsuperscript{2} suppresses EndMT. However, it remains unknown whether a shear stress higher than 25 dyn/cm\textsuperscript{2} induces EndMT. In addition to intensity of shear stress
and direction of flow, endothelial cells are also sensitive to pulsatility in arterial flow.\textsuperscript{64} Li \textit{et al.}\textsuperscript{64} showed that with a mean shear stress of 14 dyn/cm\textsuperscript{2}, medium (pulsatility index = 1.7) and high (pulsatility index = 2.6) flow pulsatility enhanced the expression of selectin E (SELE), intercellular adhesion molecule 1 (ICAM1), and chemokine (C-C motif) ligand 2 (CCL2) in pulmonary arterial endothelial cells. In our studies, steady flow without pulsatility was employed to assess the response of cells to haemodynamic forces. For future studies, examination of the kinetics of endothelial and mesenchymal markers expression under different pulsatility of flow may yield further insights into the regulation of EndMT by blood flow. In the blood vessels, endothelial cells are in contact with smooth muscle cells. Homeostasis and function of smooth muscle cells are tightly governed by endothelium-derived nitric oxide (NO) and cytokines. High flow pulsatility induces endothelial dysfunction and alters the phenotype of pulmonary smooth muscle cells.\textsuperscript{65} We hypothesise that high flow pulsatility alters the phenotype of pulmonary smooth muscle cells via induction of EndMT. Future studies looking into the effects of EndMT on the phenotype of smooth muscle cells may provide a better insight in the role of endothelial cells in pulmonary hypertension.

We showed in Chapter 2 that endothelial cells exposed to a shear stress of 20 dyn/cm\textsuperscript{2} expressed higher levels of PECAM-1 and VE-cadherin in comparison with endothelial cells cultured under static conditions. However, we did not observe these differences in the data of Chapter 4, despite the fact that experiments were performed with endothelial cells at the same passage number and under the same local environment (pro-fibrotic conditions). This unexpected observation might be explained by phenotypic variations between different batches of HUVEC (HUVEC pooled from 50 donors) that we have used in the respective experiments. Despite being obtained from the same vascular bed, endothelial cells isolated from the umbilical cord of different donors apparently exhibit heterogenous reactions to exogenous inducers.\textsuperscript{66}

**Pro-fibrotic stimulation results in endothelial-to-mesenchymal transition and endothelial activation: from the perspective of molecular signal transduction**

The pro-fibrotic stimulation\textsuperscript{61} that we used to induce EndMT disrupts the redox balance in endothelial cells. The cells show an increased generation of reactive oxygen species (ROS) and NO metabolites (nitrite, nitrates and nitroso compounds) as compared with the unstimulated condition (Chapter 3). The increased generation of NO metabolites is attributed to intracellular ROS accumulation. When the level of ROS is high, NO interacts with ROS actively and yield peroxynitrites (ONOO\textsuperscript{−})\textsuperscript{67,68} which then decompose to nitrites and nitrates.\textsuperscript{68} Additionally, peroxynitrites also act as oxidising and nitrosating agents that react with other biomolecules to yield nitroso compounds (RXNOs), such
as N-nitrosamines (RNNOs) and S-nitrosothiols (RSNOs).\textsuperscript{68,69} Decomposition of NO to nitrite, nitrate and nitroso compounds not only reduces bioavailability of NO, but also alters the NO-dependent redox signalling, particularly protein nitrosation and lipid nitration,\textsuperscript{70} which may contribute to the induction of EndMT. Notably, peroxy nitrites which are not decomposed to nitrates and nitrates results in eNOS uncoupling. We showed in Chapter 3 that inhibition of mitochondrial ROS production represses the expression of the mesenchymal marker smooth muscle 22α (SM22α). We anticipate that eNOS uncoupling, as a result of ROS generation and NO depletion, induces EndMT, but it requires further studies whether pro-fibrotic stimulation indeed induces eNOS uncoupling. Maleszewska \textit{et al.}\textsuperscript{63} showed that it is the combined effect of pro-inflammatory (IL-1β) and pro-fibrotic (TGF-β2) stimuli that trigger a pronounced expression of SM22α and calponin in endothelial cells. Indeed, ROS activate the pro-inflammatory NFκB\textsuperscript{71} and p38 MAPK\textsuperscript{72} pathways which are implicated in the induction of SM22α (Chapter 3). Interestingly, the combined influence of IL-1β and TGF-β2 on SM22α expression was regulated by epigenetic mechanisms, in particular via enhancer of zeste homolog-2 (EZH2).\textsuperscript{73} The idea that oxidative stress acts in concert with TGF-β signalling to induce EndMT was confirmed by Montorfano \textit{et al.}\textsuperscript{74} They reported that oxidative stress induces the conversion of endothelial cells into myofibroblasts via activation of the ALK5/SMAD3, NFκB and p38 MAPK pathways. In Chapter 3 we showed that inhibition of TAK1 repressed the transcript and protein expression of SM22α as efficient as inhibition of ALK5, NFκB, p38 MAPK or ROS generation. The TAK1 pathway is involved in the induction of epithelial-to-mesenchymal transition;\textsuperscript{75} here we show that activation of TAK1 by TGF-β can also result in EndMT.

In Chapter 3, we identified endothelial cells that undergo EndMT exhibit features of endothelial activation. Our data elucidate that a high level of TGF-β aggravates the pro-inflammatory effects of oxidative stress through a non-canonical TGF-β signalling pathway via TAK1. The ERK5 pathway, which can be activated by shear stress\textsuperscript{76} and statin,\textsuperscript{77} safeguards the endothelial redox homeostasis (Chapter 3), counteracts endothelial dysfunction\textsuperscript{77} and downregulates pro-inflammatory signalling.\textsuperscript{17} Strikingly, our findings in Chapter 3 demonstrate that activation of ERK5 signalling does not antagonise endothelial activation under conditions of high TGF-β levels (10 ng/ml), suggesting that the ability of KLF2 and KLF4 in repressing the anti-inflammatory response is reliant on the local environment of cells. KLF2\textsuperscript{78} and KLF4\textsuperscript{17} antagonise TNF-α-induced pro-inflammatory response potently, yet they fail to counteract the pro-inflammatory effects provoked by TGF-β that occur via the activation of the TAK1–NFκB/p38 MAPK pathway (Chapter 3). Inhibition of the TGF-β-driven ALK5/SMAD2 pathway had no effects on the expression of adhesion molecules and chemoattractants. These findings suggest that pro-fibrotic stimulation induces endothelial activation via the TAK1 pathway, independent
of the ALK5/SMAD2 signalling. Our in vitro pharmacological intervention studies lead to the interesting conclusion that under pro-fibrotic conditions, high shear stress counteracts endothelial activation predominantly via suppression of either the TAK1 pathway or by ROS generation, and that this is independent of ERK5 signalling. Notably, the local environment not only determines the phenotype of endothelial cells, but also determines the mechanisms that lead to phenotypic alterations. We suggest that high shear stress inactivates TAK1 signalling by interfering either the affinity of ALK5 to TβRI, or by binding of TGF-β1 to TβRII that occurs independent of the ALK5 kinase activity. Future studies investigating the interplay between TAK1 signalling and NADPH oxidases, eNOS as well as guanylate cyclase signalling are needed to unravel the role of TAK1 in the production of ROS and NO.

Although TGF-β1 had no effect on the redox state, it increased the protein expression of VCAM-1 and the secretion of IL-8 (Chapter 3). Of note, the induced protein level of ICAM-1 and gene expression of SELE remained unaltered by TGF-β1. In contrast, prior investigations reported that TGF-β1 at low doses (0.2-2.0 ng/ml) diminishes the expression of E-selectin and IL-8 by HUVEC. Hence, low TGF-β1 level inhibits the recruitment of leukocytes to the endothelium. Furthermore, it has been shown in glomerular endothelial cells that TGF-β1 at a concentration range of 1-25 ng/ml suppresses VCAM-1 expression.81 The discrepancy between our results and others may be attributed to the different concentration of TGF-β1 used in respective experiments and the heterogeneity of endothelial cells from different vascular beds. We suggest that a high dose (5-10 ng/ml) TGF-β1 aggravates the effects of oxidative stress and promotes endothelial activation. Recruitment of leukocytes to the arterial intima initiates the development of atherosclerosis.82,83 E-selectin, VCAM-1 and ICAM-1 are the adhesion molecules that mediate leukocyte extravasation and are highly expressed by endothelium in the atherosclerosis-prone regions.84-86 Markedly, development of atherosclerosis is attributed to the enhanced expression of E-selectin and VCAM-1 in endothelial cells.88,89 ICAM-1 does not play a significant role in atherogenesis.88,89 Besides IL-8, secretion of MCP-1 by endothelial cells also promotes migration of leukocytes into the intima and initiates atherosclerosis.90 We showed that TGF-β1 had no effect on the expression of CCL2 which encodes MCP-1 (Chapter 3). In brief, results in Chapter 3 reveal that pro-fibrotic stimulation and oxidative stress aggravate endothelial activation by enhancing the expression of VCAM-1 and IL-8.

Interestingly, we discovered that the ERK5-mediated TAGLN/SM22α expression is governed by TGF-β1 in a dose-dependent fashion. Upon a challenge with a high dose of TGF-β1 (10 ng/ml), activation of ERK5 pathway (under static condition) did not result in downregulation of TAGLN. In contrast, when compared with the unstimulated condition, an enhanced expression of TAGLN was evident (Chapter 3, Figure 3C). This unexpected result opposes to those
of Chapter 2 which shows a downregulation of mesenchymal markers when ERK5 was activated upon stimulation with TGF-β1 at a lower dose (5 ng/ml). The enhanced expression of TAGLN suggests that ERK5 pathway synergises the transcriptional effects of TGF-β-SMAD2 signalling upon stimulation with a high dose TGF-β1. Indeed, SMAD2 phosphorylation was not downregulated by the ERK5 pathway (Chapter 3). Activation of ERK5 in endothelial cells induces the expression of the transcription factors myocyte enhancer factor-2A (MEF2A) and MEF2C. Since coupling of SMAD2 with MEF2 requires MEF2 phosphorylation by either p38 MAPK or ERK5, we propose that ERK5, together with p38 MAPK activation, enhances the association of SMAD2 with MEF2, resulting in enhanced transcriptional activity of SMAD2 in endothelial cells. This corroborates our results that inhibition of either TAK1 or its downstream regulator p38 MAPK led to down-regulation of TAGLN in MEK5D-transduced cells.

Under static conditions, canonical TGF-β signalling is transduced via the activation of either the SMAD2/3 or the SMAD1/5/8 pathway. To our knowledge, we have provided the first evidence that high shear stress (20 dyn/cm²) attenuates TGF-β-activated ALK5/SMAD2 signalling (Chapter 3). Consistent with disturbed flow, we show that low shear stress (2 dyn/cm²) enhances the activation of SMAD1/5/8 (Chapter 4) which might be phosphorylated by the upstream modulator ALK1. In addition to ALK1, ALK2, 3 or 6 may also induce activation of SMAD1/5/6. Signalling through ALK1 and ALK5 are vital in angiogenesis. Activation of ALK1/SMAD1/5/8 pathway to an appropriate level promotes angiogenesis by triggering proliferation and migration of endothelial cells. In contrast, activation of the ALK5/SMAD2/3 pathway inhibits cell proliferation and migration, thus imposing anti-angiogenic effects. Interestingly, the enhanced activation of ALK1/SMAD1/5/8 pathway not only brings about cellular effects opposite to that of the ALK5/SMAD2/3 pathway, but also antagonises the signal transduction of ALK5 directly. This agrees with the idea that the endothelial cells require a balance between ALK1/SMAD1/5/8 and ALK5/SMAD2/3 signalling for maintenance of homeostasis. Nuclear translocation of SMAD1 is optimally induced by a shear stress of 10–20 dyn/cm², but inefficiently at intensities higher than this range. We showed in Chapter 4 that phosphorylation of SMAD1/5/8 at 2 dyn/cm² is higher than under static conditions or at 20 dyn/cm². To confirm the transcriptional role of SMAD1/5/8, further study should be performed to assess if phosphorylation of these SMAD proteins are translocated into the nucleus. Further studies should examine whether the shear stress-induced SMAD1/5/8 pathway antagonises SMAD2/3 and attenuates EndMT. Gain- and loss-of-function experiments that introduce genes encoding constitutively active mutant, short hairpins or siRNA of ALK1 should be carried out to validate the role of ALK1/SMAD1/5/8 pathway in SMAD2/3-driven EndMT. In addition
to TGF-β, bone morphogenetic proteins (BMP) also activate SMAD1/5/8 via ALK1 under static environments. Intriguingly, activation of SMAD1/5/8 by shear stress is dispensable of ligands. This suggests that shear stress activates SMAD1/5/8 in endothelial cells via mechanisms different from that under static environment. Since the underlying mechanisms remain unknown, future studies examining mechanosensors or molecular pathways that act upstream of SMAD1/5/8 are needed.

**Senescence that associates with ageing alters the plastic nature of endothelial cells**

Isolated HUVEC that are cultured in vitro transform from a quiescent state (0.1% replication per day) into an activated state (1‒10% replication per day). This transformation requires activation of pro-inflammatory pathways, such as p38 MAPK and JNK signalling are implicated. Awad et al. reported that a low temperature (8°C) induces the generation of ROS, leading to endothelial activation and induction of the pro-inflammatory NFκB pathway. During cell passaging, we repeatedly subjected HUVEC to centrifugation at 4°C. This condition potentially enhances ROS generation and triggers the NFκB pathway in endothelial cells. Interestingly, we showed in Chapter 3 that activation of inflammatory pathways and/or ROS generation upregulates the expression of pro-inflammatory and mesenchymal molecules. These findings implicate that repeated passaging might induce expression of ICAM1 and augments EndMT in endothelial cells as described in Chapter 4.

HUVEC in culture are not only persistently exposed to extracellular and intracellular stresses, they also constantly experiences telomere shortening which ultimately brings about senescence. Both telomere shortening and excessive exposure to stresses provoke a DNA damage response that triggers the activation of p53, p21 and p16 cell cycle arrest pathways. The DNA damage response is sustained through a positive feedback regulation which involves the p53–p21-induced mitochondrial dysfunction and ROS generation via the activation of the GADD45/p38MAPK/GRB2/TGFBR2/TGF-β pathway. This ongoing DNA damage response maintains cells in a senescent and pro-inflammatory state with enhanced mitochondrial ROS generation. Therefore, telomere shortening can enhance the expression of ICAM1 and induction of EndMT as well. As delineated in Chapter 3, induction of EndMT is associated with a pro-inflammatory endothelial phenotype and an increased generation of mitochondrial ROS. These findings imply that both endothelial senescence and EndMT may share common cellular mechanisms. Although senescent endothelial cells do not proliferate, they are metabolically active with altered expression and secretion of bioactive molecules, referred to as senescence-associated secretory phenotype (SASP) or senescence messaging secretome. SASP reinforces the feature of senescence and erodes the plastic
nature of endothelial cells. Upon acquisition of SASP, senescent cells actively secrete biomolecules, particularly IL-1α, IL-1β, IL-6, IL-8, plasminogen activator inhibitor-1 and ROS to their surroundings. Therefore, senescence not only induces intracellular changes, but also remodels the local environment.\textsuperscript{106-108}

In agreement with previous studies,\textsuperscript{109,110} we showed that HUVEC which grow with their normal cobblestone morphology acquired a spindle shape upon senescence. $CDH5$, $PECAM1$ and $VWF$ are typical markers to define endothelial lineage. Although senescent HUVEC maintained the expression of $CDH5$ and $PECAM1$, their expression of $VWF$ (a pro-coagulant) declined significantly as compared with the non-senescent cells. This indicates that although the senescent cells retain features of the endothelial lineage, their function, for instance in blood coagulation is hampered. A correlation between induced expression of mesenchymal markers, \textit{i.e.} $TAGLN$, $CNN1$ and $ACTA2$ and senescence was also noted. Together, our study provides evidence that senescent HUVEC show dysfunctional and EndMT properties, confirming earlier observations in human aortic endothelial cells.\textsuperscript{110} The expression of mesenchymal genes increased gradually before the cells cease to proliferate (Chapter 4), which supports the presumption that EndMT precedes endothelial senescence.\textsuperscript{110} However, the exact mechanisms that associate EndMT with senescence are not completely understood. Detailed mechanistic studies may provide further insights if EndMT is a cause or a consequence of senescence. We suggest that a positive feedback exists between EndMT and senescence, because EndMT in senescent endothelial cells could not be rescued by laminar flow. It is plausible that laminar flow could not reverse SASP, and thus fails to counteract EndMT which associate with senescence. Notably, senescent endothelial cells are present at atheroprone regions, but not at atheroprotective regions where the endothelium is continuously exposed to laminar flow.\textsuperscript{111} This corroborates the idea that SASP of endothelial cells promotes a pro-inflammatory local environment that favours the development of atherosclerotic lesions.

Fleenor \textit{et al.}\textsuperscript{110} suggested that the NFκB pathway links EndMT with endothelial senescence. We showed (Chapter 3) that HUVEC with characteristics of EndMT show an enhanced secretion of interleukin-8 (IL-8). Moreover, gene expression of IL-8 was increased in our senescent cells as compared with the non-senescent cells (data not shown). IL-8 not only activates the NFκB signalling,\textsuperscript{112} but also accounts for cell cycle arrest and senescence.\textsuperscript{108,113} This supports the idea that senescence is a result of EndMT. It is plausible that cells which undergo EndMT produce IL-8 and induce endothelial senescence in both autocrine and paracrine manners.\textsuperscript{108,113} However, the mechanisms by which IL-8 induces DNA damage response are poorly understood. On the other hand, NO preserves the activity of telomerase and prevents telomere shortening in endothelial cells.\textsuperscript{114,115} EndMT depletes the expression of eNOS\textsuperscript{63} which is accompanied by a reduced NO bioavailability and an induced intracellular
oxidative stress (Chapter 3) that trigger the cell cycle arrest pathways. Therefore, we suggest that EndMT as induced by cell passaging works in concert with telomere shortening in promoting and maintaining endothelial senescence. Minamino et al.\textsuperscript{111} reported the contribution of telomere shortening-induced endothelial senescence in the development of atherosclerosis. Since EndMT is associated with intimal thickening and atherosclerosis (Chapter 2), we propose that EndMT and endothelial senescence are the mechanisms that contribute to atherogenesis, apart from inflammation.

On the other hand, we showed that shear stress-induced KLF4 expression is inversely correlated with the induction of EndMT (Chapter 2). Overexpression of KLF4 not only enhances the expression of telomerase reverse transcriptase (TERT), an enzyme that elongates telomeres,\textsuperscript{116} but also suppresses the p53 cell cycle arrest pathway.\textsuperscript{117,118} Therefore, KLF4 is a crucial transcription factor that induces an embryonic-like phenotype which grow indefinitely.\textsuperscript{117,119} In cancer cells, KLF4 facilitates β-catenin in promoting the expression TERT.\textsuperscript{120} Minamino et al.,\textsuperscript{111} who detected senescent endothelial cells in atherosclerotic plaques, links decreased TERT activity with endothelial dysfunction. Of note, NO can counteract senescence by activating TERT.\textsuperscript{114} By extrapolation, it is plausible that high shear stress prevents endothelial dysfunction by counteracting EndMT and endothelial senescence via induction of TERT activity. Intriguingly, the role and the mechanisms by which KLF4 modulate TERT activity in endothelial cells remain unknown. It is of great interest to investigate whether high shear stress modulates TERT activity via induction of KLF4, thus counteracting EndMT and endothelial senescence at atheroprotective regions. Studies in this direction can provide further insight in the contribution of EndMT and endothelial senescence to atherogenesis.

Ageing stimulates pro-inflammatory vascular regulatory mechanisms,\textsuperscript{121} and is thus a risk factor for pathogenesis of cardiovascular diseases.\textsuperscript{122-124} In order to study the association between ageing and endothelial senescence, we analysed the aortas of mice which are not exposed to any risk factors of cardiovascular diseases except ageing. Interestingly, we did not detect endothelial cells with features of senescence in the descending aortas. Despite laminar flow fails to counteract the effects of SASP, it does impede the onset of senescence, possibly via induction of KLF4-regulated TERT activity, thus preventing the development of atherosclerosis. Hypertension, hyperglycemia, hyperlipidemia and hypercholesterolemia, accompanied by reduced physical activity, deplete the generation of NO that safeguards endothelial functions. Indeed, prevention is always better than cure. Reduced caloric intake that lowers metabolic burden, and regular exercise that improve the flow of blood are the most ideal preventions to sustain the generation of NO in the arteries, thus preventing ageing-associated cardiovascular diseases.
Conclusions

The plastic nature of endothelial cells acts as a double-edged sword, for it mediates cardiovascular development, homeostasis and remodelling, but also promotes local vascular pathology. The endothelial cells sense and respond to extracellular biomechanical and biochemical signals, which together determine the endothelial phenotype. Unlike laminar flow, the disturbed flow fails to safeguard redox balance and to counteract the effects of pro-fibrotic and pro-inflammatory pathways. Consequently, disturbed flow disrupts endothelial homeostasis and brings about endothelial dysfunction, endothelial activation, endothelial-to-mesenchymal transition and endothelial senescence which share common features and mechanisms. We identified shear stress-mediated EndMT as a novel mechanism that might contribute to neointimal thickening and atherosclerosis. We showed that laminar flow which activates the ERK5 pathway counteracts EndMT potently, whereas disturbed flow fails to do so. The EndMT-derived cells are endowed with properties of endothelial activation which provokes recruitment of leukocytes, being a potential mechanism that promotes the development of atherosclerosis. The effects of TGF-β on endothelial cells are dose-dependent. Low levels of TGF-β elicit anti-inflammatory effects, whereas a high dose of TGF-β elicits pro-fibrotic and pro-inflammatory effects via the activation of canonical (ALK5/SMAD2/3) and non-canonical (TAK1) TGF-β signalling, respectively (Figure 2).
General Discussion and Future Perspectives
2). Senescence is a prominent mechanism that abrogates the plastic nature of endothelial cells. Senescence correlates with endothelial dysfunction and the development of cardiovascular diseases. Phenotypical alterations of endothelial cells by senescence are irreversible. Although laminar flow can counteract the alteration of the endothelial phenotype, it fails to recover the endothelial cells from a senescent phenotype. Notably, ageing might not be the risk factor of endothelial dysfunction if the protective flow mechanisms are in place. Studies presented in this thesis extend the mechanistic knowledge that link endothelial biology with ageing and cardiovascular diseases.

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