Chapter 1

General Introduction
The endothelium and its functions

Angioblasts and hemangioblasts originating from the mesoderm are the precursors of endothelial cells during embryonic development. Endothelium, which is composed of a single layer of endothelial cells, forms the inner lining of blood vessels which are present throughout the body. Blood vessels vary from large vessels, the macrovasculature, such as arteries and veins to smaller and smallest blood vessels that include capillaries, arterioles and venules. Besides, the body contains a lymphatic system that serves to return excessive tissue fluid to the circulation. The structure of endothelium varies between different vascular beds. Depending on the composition of intercellular junctional complexes, endothelium may appear in a continuous or discontinuous form. Continuous endothelium is characterised by tight intercellular connections, whereas the discontinuous endothelium is endowed with loose intercellular junctions and the formation of clefts between cells. The continuous endothelium is subdivided into non-fenestrated and fenestrated categories. Endothelium in the arteries, veins and capillaries of brain, skin, heart and lung is non-fenestrated. In contrast, the capillaries of glands, mucosas, choroid plexus, glomeruli, liver sinusoid and renal tubules are lined by fenestrated continuous endothelium. In the descending part of arteries, endothelial cells with tight intercellular junctions, elongate and align in the direction of blood flow. However, endothelial cells in the veins, at the branch points and large curvatures of arteries appear shorter and wider without a preferential orientation in response to blood flow. Despite the fact that all endothelial cells are derived from the same origin, the endothelium along the vascular tree displays distinct structural and functional differences. These differences are evident in both macrovasculature and microvasculature.

The blood vessel endothelium is a semi-permeable lining that regulates the passage of liquid, solutes, plasma proteins, oxygen, nutrients and waste products between blood and surrounding tissues. The physical barrier function of endothelium is crucial to filtration in kidney glomeruli and liver sinusoid, as well as diffusion in blood brain barrier. In addition, the endothelium is also a synthetic and metabolic tissue that mediates several physiological functions in the vasculatures, including leukocyte recruitment, inflammation (response to injury), regulation of vasomotor tone, haemostasis and formation of blood vessels. Upon vascular injury, the endothelium expresses adhesion molecules and chemoattractants to facilitate migration of leukocytes from blood to site of inflammation (refer to “Endothelial activation” section below for details). In regard to regulation of vasomotor tone, the endothelium acts as a producer of different regulatory factors, such as nitric oxide (NO) and prostacyclin that induce vasodilation, as well as endothelin, prostanoids and angiotensin II that stimulate vasoconstriction. NO and prostacyclin, together with other endothelium-derived anti-coagulants, i.e. heparan and thrombomodulin, as
well as pro-coagulants, *i.e.* plasminogen activator inhibitor type 1 (PAI-1) and von Willebrand factor (vWF) mediate haemostasis. Moreover, NO promotes the quiescent state of endothelial cells and suppresses vascular inflammation. Proliferation of endothelial cells is essential for the development of the vascular system *i.e.* the vasculogenesis in the early embryos. Yet, in adults this endothelial proliferation underlies angiogenesis of the pre-existing vasculature, wound healing and rebuilding of uterine mucosa. Figure 1 summarises the competencies of endothelial cells which are pivotal in regulation of vascular homeostasis, remodelling and development.

**Plasticity of endothelial cells drives vascular homeostasis and pathogenesis of cardiovascular diseases**

Plasticity is defined as the flexibility of a single genotype to exhibit different phenotypes in different environments. Although all endothelial cells in the circulatory system arise from the same progenitor cells and have an identical genome within an individual, these differ in phenotype in the diverse vascular beds. This endothelial heterogeneity is observed as differences in morphology, function, intracellular mechanism, epigenetic profile, gene and protein expression. Underlying to this endothelial heterogeneity is that these cells are able to sense and respond to extracellular biochemical and biomechanical stimulation, which facilitates their adaptation to comply to or counteract changes in the local environment. This plastic nature of endothelial cells ensures optimal *in situ* performance and homeostasis of vasculatures. Oxygenation, pH, growth factors, cytokines, chemokines, hormones, lipoproteins and subendothelial extracellular matrix are examples of biochemical inducers.

![Figure 1. Functions of endothelium.](image-url)

The endothelium regulates vascular permeability, leukocyte recruitment, vascular inflammation, vasomotor tone, haemostasis and formation of new blood vessel.
Haemodynamic forces, particularly fluid shear stress, hydrostatic pressure and cyclic strains (stretch) are the biomechanical inducers. Of note, the phenotype of endothelial cells is strictly regulated in space and time for maintenance of vascular homeostasis and function. Under normal physiological conditions, the endothelial cells prevent coagulation and are quiescent, they do no proliferate. Furthermore, in this state, endothelial cells do not interact with leukocytes, while no pro-inflammatory mediators are produced either, i.e. these cells are anti-inflammatory. This is the normal, non-activated phenotype. However, during vascular injury, when the endothelial cells are activated by the induced expression of inflammatory cytokines, a pro-adhesive, pro-inflammatory, pro-coagulant and proliferative phenotype is evident. This activated endothelial phenotype is required for the recruitment of leukocytes to the site of inflammation (refer to “Endothelial activation” for details) and for vascular repair. The switch from a non-activated phenotype into an activated phenotype, and vice versa is not an on-or-off process. Indeed, a gradient of response exists between these two phenotypes. Unhealthy lifestyle that exposes the endothelium excessively and persistently to cardiovascular risk factors, such as cigarette smoke, hypertension, hyperglycemia, hyperlipidemia and hypercholesterolemia pose pathophysiological threats to the endothelium and sustain the activated phenotype of endothelial cells. This ultimately compromises vascular homeostasis and promote the development of cardiovascular diseases, such as atherosclerosis (Figure 2). Endothelial dysfunction and endothelial activation that result in an activated endothelial phenotype have been studied extensively for their contributions to the pathogenesis of cardiovascular diseases. Markedly, endothelial-to-mesenchymal transition (EndMT) and endothelial senescence that alter phenotype of endothelial cells have been implicated in vascular homeostasis and development of cardiovascular diseases as well.

**Endothelial dysfunction**

Seminal studies of Furchgott, Murad and Ignarro revealed that NO is a pivotal endothelium-derived vascular relaxation factor that safeguards vascular homeostasis and function. Moreover, NO also mediates a wide range of endothelial functions. Therefore, decreased synthesis, release and activity of NO are recognised as the hallmark of endothelial dysfunction. Endothelial dysfunction disrupts the homeostasis of endothelium and promotes the pathogenesis of cardiovascular diseases, in particular atherosclerosis by increasing leukocyte recruitment, cell permeability, low-density lipoprotein oxidation, platelet aggregation, vascular smooth muscle cell proliferation and migration, as well as inflammation. Markedly, endothelial homeostasis and function are rely on a delicate balance of intracellular reactive oxygen species (ROS) and NO. Chronic exposure to cardiovascular risk factors such as cigarette
smoke, hypertension, hyperglycemia, hyperlipidemia and hypercholesterolemia stimulates ROS generation. Mechanistically, NO bioavailability is reduced when endothelial cells are repeatedly exposed to oxidative stress and/or stimuli that attenuate NO synthesising system. In addition to increasing the degradation of NO, excessive ROS can react with NO to produce peroxynitrite. Peroxynitrite is a cytotoxic pro-oxidant that impairs functions of endothelial cells by modifying proteins, nucleic acids and lipids through oxidation and nitration. Peroxynitrite causes uncoupling of endothelial NO synthase (eNOS) and impedes NO synthesis by oxidising tetrahydrobioterin (BH4). Also, ROS can attenuate NO synthesis by stimulating inflammatory responses in endothelial cells. In addition, the elevation of C-reactive protein (CRP) that associates with ROS and inflammation, accounts for the diminished NO synthesis too. The mechanisms for generation of ROS and NO are detailed in “Oxidative stress and bioavailability of nitric oxide” section.
Endothelial activation

Inflammation is a principal response of the immune system to physical and chemical injuries, as well as to invasion of pathological microbes and parasites. Recruitment of leukocytes to the site of injury is a crucial step in inflammatory reactions. The endothelial lining acts as a dynamic barrier that mediates the passage of immune cells from the blood stream to the underlying tissues. Activation of endothelial cells by pro-inflammatory cytokines, such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), as well as bacterial endotoxins, such as lipopolysaccharides (LPS), haemodynamic forces and thrombin promotes leukocyte recruitment. Endothelial activation is characterised by enhanced expression of cell surface adhesion molecules, notably selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), as well as chemoattractants, such as monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) that promote migration of leukocytes from the blood stream through the endothelium to the tissues. Leukocyte recruitment is a sequential process that involves three main events. The first event involves the rolling of leukocytes on the endothelium which requires a transient and reversible binding of leukocyte integrins to vascular selectins, particularly E- and P-selectins. The second event includes the firm adhesion of leukocyte on the endothelium through interaction of leukocytes integrins with ICAM-1 and VCAM-1. Activation of integrins on leukocyte is stimulated by chemoattractants. The third event is a transmigration process of which the leukocytes migrate across the endothelium via a paracellular route through the cell junctions or a transcellular route through the endothelial cells. Transendothelial leukocyte migration is facilitated by chemoattractant secreted by the activated endothelial cells.

Although inflammation is a beneficial process, prolonged activation of endothelium and excessive leukocyte recruitment initiates cardiovascular disorders, in particular atherosclerosis which is a chronic inflammatory disease. The onset of atherosclerosis is multifactorial and complex. The development of atherosclerotic lesions is characterised by chronic endothelial activation and leukocyte recruitment. During progression of atherosclerotic plaques, leukocytes are recruited by the activated endothelium to tunica intima (the innermost layer of an artery) and transform in situ into macrophages that uptake lipid, followed by formation of foam cells. In addition to chronic stimulation of pro-inflammatory cytokines, the risk factors for atherosclerosis, such as cigarette smoke, hypertension, hyperglycemia and hyperlipidemia contribute to endothelial activation too. In addition, NO is also a key component in regulation of endothelial activation. Kubes et al. showed that attenuation of NO synthesis promotes recruitment and migration of leukocytes across the vascular endothelial barrier. This is consistent with mechanistic study of De Caterina et al. that NO suppresses cytokine-stimulated expression of adhesion molecules and chemoattractants by endothelial cells.
**Endothelial-to-mesenchymal transition**

EndMT is one of the mechanisms that best reflects the plasticity of endothelial cells. It is a pivotal mechanism for vascular remodelling. During embryogenesis, EndMT contributes to the formation of endocardial cushion tissue, heart valves and septa for cardiac development. However, in adult life, EndMT associates with cardiovascular pathologies, particularly cardiac fibrosis, pulmonary hypertension, atherosclerosis and intimal thickening. EndMT occurs when endothelial cells are exposed to chronic pro-fibrotic stimulation such as transforming growth factor-β (TGF-β) and/or pro-inflammatory cytokine, such as IL-1β and TNF-α. Oxidative stress that stimulates expression of TGF-β and activation of inflammatory pathways in endothelial cells also induces EndMT. During EndMT, endothelial cells show loss of cell-cell and cell-matrix interaction, gain increased migratory capacity, acquire a spindle-shape morphology and adopt a mesenchymal-like phenotype. Loss of cell-cell interaction is proposed as the initial event in the process of EndMT. Hence, down-regulation of junctional proteins, such as vascular endothelial-cadherin (VE-cadherin) and platelet endothelial cell adhesion molecule 1 (PECAM-1) is evident. In addition, EndMT is also characterised by down-regulation of critical endothelial genes that encode for vWF, Tie-1, Tie-2 and Type IV collagen, together with up-regulation of mesenchymal genes that encode for α-smooth muscle actin (αSMA), smooth muscle 22α (SM22α), vimentin, fibronectin, Type I and Type III collagen. Of note, cells in the process of EndMT may express both endothelial and mesenchymal phenotype. EndMT results in increased number of fibroblasts, myofibroblast and smooth muscle cells, as well as deposition of extracellular matrix.

EndMT is promoted essentially by both canonical and non-canonical TGF-β signalling via extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase B (PKB, also known as AKT), p38 mitogen-activated protein kinase (MAPK) and nuclear factor κ-light-chain-enhancer of activated B cell (NFκB) pathways. Concurrent Snail overexpression and glycogen synthase kinase-3β (GSK-3β) inhibition is required for the induction of EndMT.

The process of EndMT can be suppressed by inhibiting the TGF-β and bone morphogenetic protein 6 (BMP6) signalling with specific inhibitors, or by activating the BMP receptor type II (BMPR2) signalling, while fibroblast growth factor (FGF) signalling has a similar suppressing influence. Recently, Cooley et al. demonstrated in murine model that EndMT contributes to intimal thickening via the activation of SMAD2/3–Slug pathway during adaptation of vein to arterial circulation. This finding suggests a correlation between blood flow and the induction of EndMT. Indeed, EndMT is mediated by shear stress from blood flow. We showed that EndMT is prevalent at regions of disturbed flow which imposes low shear stress, whereas laminar flow which imposes high shear stress and activates the ERK5 pathway counteracts EndMT. Although
fibroblasts seem to be capable to acquire endothelial features,\textsuperscript{40} it remains elusive if the process of EndMT is reversible, namely whether endothelial-derived mesenchymal cells have the potential to regain an endothelial cell fate. Chen \textit{et al.}\textsuperscript{37} found that \textit{let-7} miRNA decreases the induction of mesenchymal genes in EndMT-derived cells potently, implies a possibility of EndMT reversibility. Interestingly, a similar TGF-β-driven process, EMT or epithelial to mesenchymal transition, is fully reversible \textit{in vitro} and \textit{in vivo}.

**Endothelial senescence**

In the 1960s, Leonard Hayflick and Paul Moorhead revealed human somatic cells that grow \textit{in vitro} only proliferate in a limited number of times (termed as the Hayflick limit),\textsuperscript{51} after which the cells cease to proliferate, yet remain viable and metabolic active.\textsuperscript{52} This phenomenon is known as replicative senescence, due to the lack of replication of the cells.\textsuperscript{53} During replication, telomeres progressively shorten after each division. If the telomeres shorten to a critical length, a DNA damage response is triggered in the cell,\textsuperscript{53,54} which comprises of activation of p53 and/or p21 cell cycle arrest pathways among others.\textsuperscript{55} These changes bring about a transient arrest in cell replication to allow DNA repair to take place. However, if the damage is excessive or irreversible, cells are driven to either apoptosis or senescence.\textsuperscript{53} Telomerase contains a telomerase reverse transcriptase (TERT) that maintains the length of telomere by adding telomeric DNA repeats to the end of chromosomes.\textsuperscript{52} Somatic cells, such as endothelial cells do not express or insufficiently express telomerase, and cannot prevent telomere shortening. In contrast, genuine stem cells, germ-line cells but also cancer cells maintain telomere length via high levels of telomerase and thus prevent ageing.\textsuperscript{52,53} The molecular mechanisms involved in induction of senescence vary depending on the cell type, the form and the extent of damage, as well as the local (micro)environment.\textsuperscript{52} Studies in human epithelial cells\textsuperscript{56} and keratinocytes\textsuperscript{57} demonstrate telomere shortening worked in concert with cyclin-dependent kinase inhibitor 2A (CDKN2A/p16) to induce senescence, suggesting that cell cycle arrest is not mediated exclusively by p53 and/or p21 pathway, a role of p16 is implicated. Besides telomere shortening, persistent telomeric and non-telomeric DNA damage by irradiation, genotoxic and oxidative stress stimulates cell cycle arrest and results in senescence too.\textsuperscript{58} This phenomenon is identified as stress-induced premature senescence, as it arises prior to exhaustion of cell proliferative capacity.\textsuperscript{53}

Despite the differences in mechanisms, both replicative and stress-induced premature senescence lead to DNA damage and alter phenotype of endothelial cells irreversibly.\textsuperscript{59} Phenotypically, replicatively or stress-induced senescent endothelial cells do not differ markedly. But, in contrast to quiescent cells, senescent endothelial cells are pro-inflammatory, pro-thrombotic, pro-atherogenic\textsuperscript{59} and are also prone to undergo apoptosis.\textsuperscript{60} Endothelial
senescence is not a process that restricts to in vitro models, but is common in living organisms. In the blood vessels, endothelial senescence occurs upon excessive and/or persistent cellular proliferation and extracellular stresses. Endothelial cells at atherosclerotic prone regions, where vascular injury and chronic pathophysiological stimulation are prevalent, exhibit features of senescence. Ageing is associated with chronic inflammation, oxidative stress, enhanced expression of TGF-β1, collagen and fibronectin in intimal and adventitial arterial layers. Hence, ageing is an independent risk factor of cardiovascular diseases, which may associate with age-related induction of endothelial senescence. Like senescence, ageing compromises endothelial functions and correlate with arterial stiffening, vascular inflammation, intimal thickening and atherosclerosis. Mechanisms that lead to ageing, such as oxidative stress, irreparable DNA damage, decreased telomerase activity and enhanced expression of p53, p21 and p16 are implicated in senescence. Accordingly, endothelial senescence is thought to be the factor that links ageing with cardiovascular disorders. This corroborates studies which show that wound healing and angiogenesis in the elderly is impaired as a result of endothelial senescence. Intriguingly, senescent endothelial cells are also endowed with the features of endothelial dysfunction, endothelial activation and EndMT. These features are reinforced in senescent endothelial cells via an endogenous positive feedback regulation upon sustained exposure to inflammation and oxidative stress during ageing.

The effects of exogenous and endogenous inducers on endothelial cells

TGF-β as a pro-fibrotic and anti-inflammatory stimulus

The TGF-β superfamily comprises over 30 structurally and functionally similar ligands. These ligands which are categorised into two subfamilies, i.e. TGF-β/activin/nodal and BMP/growth & differentiation factor (GDF)/Muellerian inhibiting substance (MIS) regulate multiple cellular processes during embryogenesis and homeostasis in adult tissues, for instance proliferation, differentiation, apoptosis and synthesis of extracellular matrix. Older studies show that in normal non-atherosclerotic aortas, the intima expresses detectable TGF-β1 by immunohistochemistry, while TGF-β3 is expressed at low levels and TGF-β2 is not detectable. Markedly, the level of in situ TGF-β1 is enhanced during vascular injury, as shown by the elevation of TGF-β1 in atherosclerotic lesions of human subjects. In addition, it was shown in human subjects and animal models that balloon angioplasty and stenting bring about higher systemic level of active TGF-β1 which could be released from platelet and leukocytes that accumulate at the injured arterial wall. These findings
suggest a critical role of TGF-β1 in regulation of vascular homeostasis and pathogenesis of cardiovascular diseases. Since the endothelium is the primary modulator of vascular homeostasis and functions, it is of interest to investigate the molecular effects of TGF-β on endothelial cells upon vascular injury, to provide further insights in understanding the role of TGF-β in pathogenesis of cardiovascular disorders.

The dimer of TGF-β ligand is initially produced as a latent complex that requires activation at appropriate time and space. Heat, acidic pH, ROS, proteases, thrombospondin-1, shear stress and integrins are the activators of TGF-β ligands. Binding of activated TGF-β causes the formation of a heterotetrameric receptor complex consists of two type II receptors (TβRII) and two type I receptors [TβRI, which is also termed activin receptor-like kinase 1 or 5 (ALK1 or ALK5)] at cell membrane. Quiescent endothelial cells predominantly express ALK1. In the ligand-induced TGF-β receptor heteromeric complex, the TβRII kinase phosphorylates TβRI. The activated TβRI subsequently transduces the signal intracellularly by phosphorylating receptor-regulated small mothers against decapentaplegic (R-SMADs), i.e. SMAD2 and SMAD3 at the two carboxy terminal serine residue. R-SMADs are transcription factors in the canonical TGF-β signalling which transduces the activating signal to nucleus. SMAD4 is a common mediator SMAD (Co-SMAD) protein that binds to and acts cooperatively with R-SMADs for transcriptional activity. On the other hand, the inhibitory SMADs (I-SMADs), i.e. SMAD6 and SMAD7 inhibit the activation of R-SMADs. In endothelial cells, TGF-β-activated ALK1 causes activation of ALK1–SMAD1/5 axis, while high levels of TGF-β activate the ALK5–SMAD2/3 axis in a dose-dependent manner. Goumans et al. revealed that both signalling cascades elicit opposite effects on regulation of angiogenesis. Of note, upon stimulation with TGF-β1 higher than 0.5 ng/ml, endothelial cells show up-regulation of ALK5–SMAD2/3 axis, but down-regulation of ALK1–SMAD1/5 axis.

TGF-β1 is a potent inducer of EndMT (refer to “Endothelial-to-mesenchymal transition” as described in previous section for details) and extracellular matrix production. In addition to its pro-fibrotic effects, TGF-β1 also elicits anti-inflammatory effects on endothelial cells. In vitro, TGF-β1 at low doses (0.2-2.0 ng/ml) down-regulates E-selectin and IL-8 expression in endothelial cells, which causes a decreased leukocyte adhesion and inhibits neutrophils transmigration across the endothelium. Interestingly, low dose TGF-β1 does not influence the expression of VCAM-1 and ICAM-1. TGF-β-induced SMAD activation and IL-1β-induced NFκB activation elicit their transcriptional effects by interacting with a coactivator, cyclic AMP response element-binding protein (CREB)-binding protein (CBP). The anti-inflammatory effect of TGF-β1 might result from the competition of SMAD proteins with NFκB for CBP. On the other hand, higher doses (5-10 ng/ml) of TGF-β1 are not effective in attenuation
of E-selectin and IL-8 expression, indicating that the anti-inflammatory effect of TGF-β1 on endothelial cells are dose-dependent. It was shown in epithelial malignancy that high dose TGF-β1 (10 ng/ml) induces activation of NFκB signaling and inflammation through stimulation of TGF-β activated kinase 1 (TAK1). Moreover, Gardner et al. reported TGF-β1-induced TAK1 signalling enhances epithelial-to-mesenchymal transition (EMT) via activation of NFκB and c-Jun NH2-terminal kinase (JNK). As mentioned above, EMT and EndMT are related processes that both are triggered by TGF-β. Intriguingly, the pro-inflammatory role of TGF-β1 and TAK1 signalling in regulation of EndMT and inflammation in endothelial cells are poorly understood.

**Oxidative stress and bioavailability of nitric oxide**

Oxygen, which is required for the generation of adenosine triphosphate (ATP), is potentially turned into ROS, namely hydrogen peroxide (H2O2), hydroxyl radicals (•OH) and superoxide anions (O2•-) in all eukaryotic cells. In addition to the exogenous sources, ROS are generated endogenously during intracellular metabolism by mitochondria, cytochrome p450 and enzymes, particularly nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase and uncoupled eNOS in vascular cells. Indeed, ROS is an important mediator in signal transduction and gene transcription. However, it is cytotoxic if the level of ROS overwhelms the antioxidant defence and results in oxidative stress. Like for many other eukaryotic cells, the phenotype of endothelial cells depends on the level intracellular ROS. Continuous and/or excessive exposure to oxidative stress alters cellular functions and contributes to pathogenesis of cardiovascular disorders. As shown in Figure 3, low levels of ROS stimulate nuclear factor erythroid 2–related factor 2 (NRF2) to activate genes encoding for anti-oxidant enzymes. A moderate level of oxidative stress reduces NO bioavailability and activates inflammatory transcription factors, NFκB and activator protein 1 (AP-1), which account for endothelial dysfunction, endothelial activation, endothelial-to-mesenchymal transition and endothelial senescence. High level of oxidative stress disrupts mitochondrial structure and interferes with the intracellular redox reaction which ultimately give rise to apoptosis and necrosis.

Type III NO synthase (NOS) or endothelial-NOS (eNOS) is the main NOS in endothelial cells that generates NO. In the presence of NADPH, oxygen, Ca2+/calmodulin and BH4, the intact eNOS dimers synthesise NO by oxidising guanidine nitrogen of L-arginine. The flow of NADPH-derived electrons from the reductase domain to the oxygenase domain of the homodimeric eNOS yields NO and L-citrulline from L-arginine and oxygen. Depletion of NO bioavailability can be caused by a lack of or inactivation of eNOS, insufficient substrate or cofactors for eNOS synthesis or excessive NO breakdown by ROS. Interestingly, different types of ROS bring about different effects
on eNOS, for example superoxide anions reduce eNOS expression, while hydrogen peroxide induces eNOS expression and activity.\textsuperscript{97} The enzymatic activity of eNOS highly depends on the phosphorylation of specific serine and threonine residues. Phosphorylation of Ser1179 by PKB activates eNOS, whereas phosphorylation of Thr497 by PKC inactivates eNOS. Interestingly, the phosphorylation of Ser1179 and Thr497 is reciprocal, namely phosphorylation of Ser1179 is accompanied by dephosphorylation of Thr497.

Stimulation with vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), fluid shear stress and 3-hydroxy-3methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (also known as statins) activates eNOS at Ser1179 and increases synthesis of NO.\textsuperscript{98} In endothelial cells, NADPH oxidase (NOX2) and eNOS compete for both NADPH and oxygen. Stimulation with inflammatory cytokines or angiotensin II tilts the balance in favour of NOX2 and causes the production of superoxide anions.\textsuperscript{100} In general, the aberrant generation of NO and ROS tips intracellular redox balance, compromises endothelial homeostasis and brings about vascular pathologies. Under pathophysiological condition, excessive superoxide anions react rapidly with NO and yield peroxynitrite (ONOO\textsuperscript{–}).\textsuperscript{16,101} Peroxynitrite leads to uncoupling of eNOS dimers by oxidising BH\textsubscript{4}, a cofactor that catalyses the enzymatic action of eNOS. Uncoupling of eNOS produces ROS rather than NO as a consequence of disruption in
electron transfer for L-arginine oxidation. Uncoupling of eNOS stimulates a positive feedback mechanism that accentuates oxidative stress. Taken together, dysfunctional eNOS goes hand in hand with endothelial dysfunction, thus normalization of eNOS is essential for a normal vascular homeostasis.

**Fluid shear stress**

Fluid shear stress is a frictional force exerted by the blood flow on the vascular wall. As expected, endothelium is the prime cellular ‘sensor’ that monitors and responds to changes in shear stress. These responses causes phenotypic adjustments and are a key to the endothelium in maintenance of vascular homeostasis and function. Shear stress regulates endothelial cell phenotype through a series of sequential mechano-responsive processes, namely mechanotransduction. Mechanotransduction, which converts biomechanical signals into biochemical signals governs various downstream physiological events, such as activation/inactivation of molecular pathways and upregulation/downregulation of gene and protein expression. Endothelial cells sense shear stress through mechanosensors, which comprise of the glycocalyx, primary cilia, caveolae, ion channels, membrane receptor kinases, focal adhesions/integrins, heterotrimeric G proteins, intercellular junctional proteins and even nuclei. The cytoskeleton serves as a scaffold for transmission of shear forces from apical mechanosensors to the basal and lateral domains where the physical forces are converted into biochemical signals. Thus, disruption of the cytoskeleton interferes with mechanotransduction and alters the response of endothelial cells to shear stress. Vartanian et al. demonstrated that micropattern-induced cytoskeletal remodelling i.e. independent of shear stress, induces the expression of anti-inflammatory Kruppel-like factor 2 (KLF2), but suppresses the expression of pro-inflammatory VCAM-1 in endothelial cells. This finding corroborates with the idea that cytoskeletal remodelling is crucial in regulation of endothelial phenotype by shear stress. Cytoskeletal remodelling that involves reorganisation of actin, microtubules and intermediate filaments requires small guanosine triphosphatases (GTPases) Rho, Rac and cell division control protein 42 homolog (CDC42) signalling. Interference of integrin function alters Rho, Rac and CDC42 activity, thus impedes mechanotransduction. Also, the endothelial cell junctional complex composed of PECAM-1, VE-cadherin and vascular endothelial growth factor receptor 2 (VEGFR-2) is essential in mechanotransduction. This complex, via activation of VEGFR-2 by shear stress stimulates phosphatidylinositol-3-OH kinases (PI3K), brings about eNOS activation and NO synthesis. In addition, PI3K also mediates activity of small GTPases by stimulating binding of integrin to extracellular matrix.
Due to the variation in blood vessel geometry, shear stress in different vascular beds and different organisms varies in magnitude and direction. In humans, the magnitude of shear stress ranges from 1 to 6 dyn/cm\(^2\) in veins and from 10 to 70 dyn/cm\(^2\) in arteries.\(^{110}\) Interestingly, the magnitude of shear stress in mice appears to be higher than that in humans, as explained by the allometric scaling laws which relate shape of blood vessels with size of organism.\(^{111}\) Arteries exposed to laminar flow, particularly the outer curvature and descending part of aorta are characterised by the presence of unidirectional high shear stress. In contrast, regions exposed to disturbed flow, such as the inner curvature, bifurcation and branching points of aortas feature low shear stress with irregular direction. Notably, bypass graft and stent placement following balloon angioplasty also bring about disturbed flow.\(^{110}\) Endothelial cell phenotype is tightly regulated by both direction and intensity of shear stress. Endothelial cells exposed to parallel flow exhibit anti-inflammatory phenotype, whereas cells that exposed to perpendicular flow are pro-inflammatory.\(^{112}\) Endothelial cells from different vascular beds have a preferred level of shear stress, namely shear stress set point which safeguards their phenotype and function. This is evident particularly when comparing human umbilical vein endothelial cells with human dermal lymphatic endothelial cells. Human umbilical vein endothelial cells align in the direction of flow and show inactivation of NFκB pathway upon exposure to shear stress of 10–20 dyn/cm\(^2\), yet human dermal lymphatic endothelial cells require lower intensity of shear stress to have the same response.\(^{113}\)

Of note, the onset of flow activates pro-inflammatory pathways\(^{103,114-116}\) and induces formation of ROS.\(^{14,117}\) This has ramifications for several published studies in which only short term (hours) fluid shear stress was used to assess the responses of endothelial cells. The translation of these studies to ‘in vivo’ obviously has limitations. However, adaptation of endothelial cells to sustained (days) laminar flow triggers negative feedback mechanisms to repress the pro-inflammatory effects.\(^{103}\) In contrast, cells exposed to disturbed flow fail to do so.\(^{14,103,117}\) Therefore, endothelial cells exposed to sustained laminar flow are characterised by a non-adhesive, anti-inflammatory, anti-coagulant, anti-thrombotic and non-proliferative phenotype, as a result of activation of eNOS, manganese-dependent superoxide dismutase and KLF2 signalling.\(^{3,118}\) Sustained laminar flow elicits protective effects on endothelial cells, and is atheroprotective. Conversely, disturbed flow is detrimental and atheroprone.\(^3\) Adverse alteration of endothelial phenotype, for instance endothelial dysfunction that modifies response of endothelium to shear, can impede vascular homeostasis and bring about cardiovascular disorders.\(^3,102,110\) Studies looking into the mechanisms and consequences by which endothelial cells sense and respond to shear stress may provide further insights to link endothelial dysfunction with cardiovascular pathophysiology.\(^{110,119}\)
Concluding remarks

Disruption of homeostasis compromises the anti-adhesive, anti-inflammatory, anti-thrombotic, anti-oxidant and non-proliferative phenotype of endothelial cells, as depicted in endothelial dysfunction, endothelial activation, endothelial-to-mesenchymal transition and endothelial senescence. In the circulatory system, blood flow modulates endothelial homeostasis and phenotype through a network of mechanosensitive pathways. Intriguingly, the mechanism by which endothelial cells sense and respond to physical forces is greatly influenced by exogenous and endogenous biochemical stimuli. The inefficiency of endothelial cells to adapt to changes in their local environment and counteract intracellular stresses contributes to the onset and advancement of cardiovascular diseases. Therefore, there is a surge of interest in mechanistic studies to understand how endothelial cells maintain their homeostasis and why alteration of endothelial phenotype relates with pathophysiological condition. Translational research that links endothelial cell biology with pathogenesis of cardiovascular diseases may provide further insights into prevention, management and treatment of these diseases.

References

11. Forsman A. Rethinking phenotypic
plasticity and its consequences for individuals, populations and species. *Heredity (Edinb)*, 2014; doi: 10.1038/hdy.2014.92. [Epub ahead of print].


31. Arciniegas E, Frid MG, Douglas IS and Stenmark KR. Perspectives on endothelial-to-mesenchymal transition: potential contribution to vascular...


45. He J, Xu Y, Koya D and Kanasaki K. Role of the endothelial-to-mesenchymal transition in renal fibrosis of chronic


63. Franceschi C and Campisi J. Chronic inflammation (inflammaging) and its


73. Wang XL, Liu S-X and Wilcken DEL.


