Molecular mechanisms of Endothelial-Mesenchymal Transition in coronary artery stenosis and cardiac fibrosis
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CHAPTER 1

Introduction
GLOBAL BURDEN OF CARDIOVASCULAR DISEASE

Cardiovascular diseases (CVD) are the leading cause of mortality worldwide. In 2015, an estimated 17.7 million people died from CVD, representing 31% of all mortalities worldwide. The majority of cardiovascular deaths can be attributed to coronary heart disease and cerebrovascular diseases, representing 42 and 38% of CVD-related deaths, respectively (1) wherein arteriosclerosis is the main underlying pathology.

From epidemiological studies, systemic risk factors for the development of atherosclerosis are identified, including behavioral risk factors such as an unhealthy Western diet, physical inactivity, smoking and the excessive use of alcohol. These behavioral risk factors can culminate in intermediate risk factors such as hyperglycemia, hyperlipidemia, obesity and hypertension (2). The non-modifiable risk factors for arteriosclerosis development are aging, gender and genetic susceptibility (1, 3). Although the whole vasculature is exposed to the above mentioned systemic risk factors, atherosclerosis is a focal disease that primarily develops at the site of vascular branches and the inner curvatures of large vessels (4), implying that focal risk factors are involved in the pathogenesis of atherosclerosis (5).

Atherosclerotic plaques are lesions in the arteries characterized by excessive accumulation of oxidized low-density lipoprotein cholesterol in the vessel wall (6, 7), inflammatory cell infiltration (8), smooth muscle cell proliferation, extracellular matrix accumulation and intimal thickening (9). It is commonly accepted that endothelial dysfunction is the initiating event in atherosclerosis development (10, 11), however, the underlying molecular mechanisms that cause endothelial dysfunction in the so-called atheroprone areas remain elusive. The current dogma revolves around biomechanical forces (figure 1) – fluid shear stress (12) and cyclic strain (13) – that have distinct patterns in areas that are atherosclerosis-prone and areas that are resistant to atherosclerosis development (the so-called atheroprotected areas).

Fluid shear stress, the frictional force per unit area generated by the blood flow, plays a crucial role in endothelial homeostasis and disease (14, 15). Areas exposed to laminar flow are protected from the atherosclerosis. In contrast, disturbed flow can induce endothelial cell activation, oxidative stress and the expression of leukocyte adhesion molecules that might induce an inflammatory reaction in the vessel wall (extensively reviewed (16, 17)) Indeed, in animal models wherein disturbed flow is induced by the constriction of an otherwise healthy vessel, atherosclerotic lesions develop in the absence of systemic atherosclerosis risk factors (18, 19).
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Figure 1. Blood vessels are constantly exposed to biomechanical forces, namely shear stress and cyclic strain. Cyclic strain is the circumferential stretch of the vessel. Shear stress is the frictional force per unit area and its magnitude is measured by dyne/cm$^2$. Depending on the direction of the flow, shear stress can be further classified into laminar shear stress (unidirectional) and oscillatory shear stress (disturbed). High laminar shear stress is atheroprotective low, whereas low oscillatory shear stress is considered as atheroprone flow, present at sites where atherosclerotic lesions preferentially develop.

ENDOTHELIAL CELLS IN VASCULAR HOMEOSTASIS AND THE DEVELOPMENT OF CVD

The blood vessels serve as the conduits of circulation, transporting nutrients and oxygen and removing catabolites and carbon dioxide from the tissues. Besides the lymph system, there are 3 major type of blood vessels that differ in morphology and function, namely arteries, veins and capillaries. The arteries carry blood from the heart, then capillaries enable the exchange of gas, nutrients, and catabolites in the tissues, whereas veins transport the blood back to the heart. From the aorta to the smallest capillaries and back through the venous system, the endothelium covers the entire vasculature and is over 100.000 km in length, weighs about 1 kg and represents approximately 1% of the body mass (20).

The endothelium is the most inner luminal cell layer of all blood vessels. In a landmark experiment, Furchgott demonstrated that the endothelial layer is not just a simple barrier between the blood and the surrounding tissues, but the endothelium has number of crucial functions in vascular homeostasis. The removal of the endothelium from isolated aortas precluded acetylcholine-induced vasorelaxation (21). These data exemplify that the endothelium is not solely a static barrier, but also a key player in vasomotor function. Since, many studies have reported on other pivotal functions of the endothelium in safeguarding vascular homeostasis, such as the semi-permeable regulation of oxygen and nutrient exchange from the blood to the underlying tissues, leukocyte recruitment, platelet adhesion/activation, blood clotting and angiogenesis (22, 23).

Various stimuli, such as oxidative stress and oscillatory shear stress can disrupt endothelial homeostasis, which results in endothelial dysfunction. Endothelial dysfunction is a comprehensive concept referring to the reduction of the endothelium-
derived relaxing factors (EDRFs) - in particular nitric oxide (NO) - while, the endothelial-derived contracting factors (EDCFs) are increased.

Endothelial dysfunction not only impairs vasodilation, but also comprises pro-thrombotic, proliferative and pro-inflammatory phenotypes (Figure 2). As a result, the dysfunctional endothelium facilitates other pathophysiological pathways that might induce atherosclerosis (24, 25).

**Figure 2. Endothelial cells in health and disease.** The healthy quiescent endothelium has anti-atherogenic capacities. Dysfunctional endothelial cells lose their protective capacities and acquire pro-atherogenic functions such as proliferation, vasoconstriction, inflammatory activation, and pro-thrombotic activity which contribute to atherogenesis.

**EPIGENETIC REGULATION OF ENDOTHELIAL GENE EXPRESSION**

As phenotypic heterogeneity is the consequence of differential gene and protein expression patterns, the molecular mechanisms that affect endothelial gene and protein expression are extensively investigated in the context of atherosclerosis (26, 27). Although we have an increasing understanding about the endothelial behavior during atherosclerosis development, the contribution of endothelial biomechanical and epigenetic signaling during atherosclerosis development is still elusive.
Berger et al defined epigenetics as “the stable and heritable changes in genome function resulting from changes in the chromatin without alterations in the underlying DNA sequence” (28). As a blueprint of the human body, the DNA needs to be accessible to transcription factors in a spatiotemporal accurate manner in order to regulate gene expression. Each cell contains approximately 2 meters of DNA, which is folded into a nucleus of less than 10 μm in diameter. To ensure this compaction, the DNA is wrapped around an octamer of core histone proteins (H2A, H2B, H3, H4 - two times each) which forms the nucleosomes (Figure 3). The nucleosomes are further compacted by the exterior histone H1 into 10nm fibers and complexed into 30nm fibers by scaffolding proteins forming the chromatin (29).

![Figure 3. The nucleosome is a subunit of chromatin.](image)

This high-order folding or 3D arrangement of the nucleosomes has distinct chromatin states in the genome; euchromatin is the state wherein a portion of the DNA is loosely wrapped and more accessible to the transcriptional machinery. In contrast, heterochromatin is densely packed and less accessible state (Figure 4). This higher-order folding is not merely in place to compact the DNA, but is also pivotal in the regulation of gene expression (30). Despite having the identical genomic information, a single fertilized egg can give rise over 200 types of morphologically and functionally distinct cell types. These structural and functional diversities are the consequence of differential gene expression profiles. Epigenetic regulation poses a layer of transcriptional regulation that culminates in phenotypic diversities between cells (31).
**DNA methylation** is a process where the addition of methyl group on a cytosine nucleotide forms 5-methylcytosine. DNA methylation mostly occurs on CpG islands, which are DNA sequences enriched in cytosine nucleotide followed by a guanine nucleotide coupled via phosphate bonds. CpG islands are mostly found in gene promoter areas (32). In mammals, de novo DNA methylation is performed by the methyltransferases DNMT3a and DNMT3b (33), whereas DNMT1 recognizes hemi-methylated DNA and copies the methylation to the secondary locus thereby allowing the daughter cells keep the same DNA methylation pattern.

![Figure 4. Heterochromatin versus Euchromatin.](image)

**Histone modifications:** Each core histone molecules has N-terminal tail (29) which protrudes out of the histone protein and can undergo a number of modifications (Figure 3). Histone modifications include methylation, acetylation, ubiquitination, phosphorylation, sumoylation, ribosylation and others. The orchestrated arrangement of the histone modifications is regulated by epigenetic enzymes. Depending on the histone modification produced, epigenetic enzymes are divided into epigenetic writers, readers and erasers. Lysine acetylation on histone tails in general results in chromatin opening and thus enhanced gene expression, whereas the tri-methylation of lysine 9 and 27 on histone 3 (H3K9me3 and H3K27me3, respectively) culminate in chromatin closure and gene silencing.

**Polycomb Repressive Complex mediated gene silencing:** The Polycomb repressive complex is an evolutionary preserved transcriptional silencing system that plays a crucial role in stem cell identity and differentiation (34). Two multiprotein Polycomb complexes are identified; Polycomb Repressive Complex 2 (PRC 2) consists of Enhancer of Zeste Homologue 2 (EZH2) or its homologue (EZH1), Embryonic Ectoderm Development (EED), Suppressor of Zeste 12 (SUZ12) and Retinoblastoma binding protein 48 (RbAP48).
Among these proteins, EZH2 and its homologue EZH1 have SET domain holding methyltransferase activity, which enables the writing of the tri-methylation on the 27th lysine residue on the tail of Histone 3 (H3k27me3). H3K27me3 acts as docking site for the Polycomb Repressive Complex 1 (PRC1), which writes a mono-ubiquitination on the 119th lysine residue on the tail of Histone 2A (H2AK119ub1) (35, 36). PRC1 precludes the activation of the RNA polymerase II complex thereby silencing gene expression(37).

**Post-transcriptional silencing - microRNAs:** MicroRNAs are around 23 nucleotides in length, non-protein coding RNAs. They induce posttranscriptional repression by pairing to the transcripts of the protein coding genes (mRNAs). The microRNA precursors are transcribed from the DNA by the RNA polymerase II as longer transcript, the pri-microRNA. The precursor miRNAs undergo a series of alterations to finally form the mature microRNA that is loaded into the RNA induced silencing complex (RISC). The RISC complex can bind to the 3’ UTR of a target mRNA, causing cleavage, destabilization or inhibition of mRNA translation. A single microRNA can target multiple mRNAs simultaneously, if their 3’ UTR sequence match with the seed sequence of the microRNA (38, 39).

**PHENOTYPE SWITCHING OF ENDOTHELIAL CELLS: A DRIVING FORCE FOR DEVELOPING CARDIOVASCULAR DISEASE?**

As mentioned above, the healthy endothelium is quiescent and performs valuable functions for vascular homeostasis, such as the regulation of vascular permeability and the prevention of vasospasms, inflammation, thrombosis and platelet activation. In this thesis, we investigated the influence of a specific epigenetic modification (i.e. H3K27Me3) on the development of endothelial dysfunction and Endothelial-Mesenchymal Transition (EndMT).

**Endothelial-mesenchymal transition (EndMT)** is a specific subtype of endothelial dysfunction wherein endothelial cells lose their endothelial specific markers and morphology while acquiring a mesenchymal phenotype. The loss of endothelial specific markers, such as VE-cadherin, CD31, Tie1/2 and VEGFRII and the concurrent gain in expression of mesenchymal marker proteins αSMA, SM22α, calponin, PAI and vimentin is prominent during EndMT. Functionally, endothelial cells acquire contractile behavior while their angiogenic and anti-thrombogenic capacities are constrained. Moreover, extracellular matrix (ECM) production by endothelial cells is increased during EndMT, culminating in the adaptation of a pro-atherogenic endothelial phenotype (16).

EndMT was first described during the formation of the cardiac cushions and valves during cardiac development (40). In the adult, EndMT contributes to the development of various chronic diseases such as cancer (41), fibrosis (42) (43) (44) (45), cerebral cavernous malformations (46), and endocardial fibroelastosis (47). Recent studies show that EndMT also contributes to atherosclerosis (48, 49) and neointima formation (50, 51).
Figure 5. Endothelial-Mesenchymal transition (EndMT). The healthy quiescent endothelium is a main mediator of vascular homeostasis. During EndMT, the expression of endothelial cell-specific markers such as VE-cadherin and CD31 is reduced whereas mesenchymal cell-specific markers such as αSMA and Calponin is induced. TGFβ, inflammation and oxidative stress induce EndMT, conversely BMP7, laminar flow-mediated pMAPK7 activity and FGF2 inhibit EndMT. The TGFβ, WNT, NOTCH signaling, histone modifications, transcription factors and post-transcriptional modifications modulate EndMT.

Postnatal EndMT is predominantly induced in a TGFβ- or inflammation and oxidative stress-driven manner (Figure 5). TGFβ-driven EndMT is extensively investigated in the context of fibrotic diseases. Canonical TGF-β signaling activates its downstream intermediates SMAD2/3, thereby inducing mesenchymal transcription factors and gene expression. Non-canonical TGF-β signaling can activate downstream molecules such as ERK1/2 and p38 MAPK, which activate the transcription factor SNAIL, the classical transcription factor for the induction of EndMT(16).
On the other hand, inflammation-driven EndMT is initiated by the signaling actions of inflammatory cytokines such as TNFα and IL1β or reactive oxygen species (ROS). This inflammation driven pathway might blend into the TGF-β-driven pathway, since inflammatory-activated endothelial cells induce the endogenous expression of TGF-β (16). Interestingly, the inflammatory cytokine IL1β and TGFβ can synergistically induce EndMT in vitro (52). These data indicate that these two distinct pathways are somehow interwined and synergize each other at the certain points (5).

Potent TGF-β antagonists such as BMP7 (53) and FGF2 (54) inhibit EndMT. Also high laminar shear stress inhibits EndMT via the activation of MAPK7 signaling (50). Systemic administration bone morphogenic protein 7 (BMP7) significantly inhibites EndMT and the progression of fibrosis in the heart and kidney (42), and the endothelial cell-specific ablation of FGFR1 culminates in the activation of TGFβ signaling and the development of EndMT in vitro and in vivo (55).

AIM OF THIS THESIS

Significant advancements have been made in the development of new treatments of cardiovascular diseases, yet CVD are still the leading cause of mortality worldwide. Although the endothelium and atherosclerosis are extensively studied and Endothelial-mesenchymal transition is established as a key component of atherosclerosis, the relative contribution, specific form of-, and functional contribution of endothelial-mesenchymal transition is elusive. The aim of this thesis is to elucidate the molecular and epigenetic mechanisms of how uniform laminar shear stress might modulate endothelial homeostasis and how these mechanisms are disrupted during intimal hyperplasia and cardiac fibrosis.

OUTLINE OF THIS THESIS

A general introduction to the topics under investigation is presented here (Chapter 1). The altered function of the endothelium is an important component of atherosclerosis yet no current anti-atherosclerosis therapies are specifically focused on the amelioration of endothelial dysfunction. Hence, in Chapter 2, we review the current atherosclerosis treatments and investigate how some epigenetic enzymes might be beneficial to normalize endothelial function in disturbed flow areas to preclude atherosclerosis development and its progression.

In healthy blood vessels, the vascular lumen is lined with a quiescent endothelium. In contrast, during vascular pathologies such as atherosclerosis, the endothelium has a fibro-proliferative phenotype, characterized by intimal hyperplasia and mesenchymal phenotype. Uniform laminar flow activates MAPK7 signaling thereby inhibiting Endothelial-Mesenchymal transition. However, how this protective mechanism is overruled in the atheroprone regions is unknown. In Chapter 3, we investigate the mechanism by which TGFβ reduces the expression and activity of MAPK7 signaling during intimal hyperplasia with a focus on TGFβ-sensitive microRNAs that might target the MAPK7 signaling cascade.
Next, we investigate how endothelial quiescence is governed by fluid shear stress and how the histone methyltransferase EZH2, as a pivotal epigenetic mediator, regulates endothelial quiescence. In Chapter 4, we investigate how high laminar shear stress and EZH2 modulate the gene expression profile in endothelial cells by using an RNA sequencing approach. We focus on genes that regulate the cell cycle in endothelial cells and investigate how these are regulated by fluid shear stress and EZH2. Surprisingly, we uncovered that the epigenetic enzyme EZH2 crosstalk’s with MAPK7 signaling in a reciprocal fashion. This finding intrigued us to investigate the crosstalk between these two molecules in Chapter 5. Also, we questioned whether this reciprocity is in imbalance during coronary artery stenosis.

EndMT plays a pivotal role in the development of cardiac fibrosis. Zeisberg et al found that around 30% of myofibroblasts in cardiac fibrosis are derived from the endothelium (42). Meanwhile, Galectin 3 was identified as a key initiator of cardiac fibrosis, and plasma Galectin 3 levels associate with the increased risk of heart failure and mortality (56). However, the molecular mechanism of Galectin 3-induced cardiac fibrosis is elusive. In Chapter 6 we assessed if Galectin 3-induced cardiac fibrosis might originate from EndMT. In Chapter 7, we summarize our main findings of this thesis, describing the complex and multilayered regulation of endothelial-mesenchymal transition by epigenetic and post-transcriptional silencing mechanisms (i.e. EZH2 and microRNAs) and how these are influenced by fluid shear stress. Moreover, we describe how these mechanisms are in imbalance during the development of intimal hyperplasia and cardiac fibrosis. In Chapter 8, we propose future perspectives resulting from our findings.
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REFERENCES

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