Primary cilia on endothelial cells : component of the shear stress sensor localized to athero-prone flow areas

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Chapter 10

General Discussion

Introduction

The aim of this thesis was to demonstrate the presence of primary cilia on endothelial cells and determine their involvement in endothelial shear stress sensing. The occurrence and characteristics of primary cilia on endothelial cells are described, followed by a discussion on confirmed and potential functions of endothelial primary cilia and their involvement in cardiovascular development, health, and disease.

10.1 Endothelial primary cilia

Primary cilia had already been demonstrated on endothelial cells. However, a specific distribution of ciliated endothelial cells, as described in this thesis, was not reported. Primary cilia were demonstrated on the endothelial cells of the embryonic and adult human aorta, where they were either entirely immersed in the cytoplasm or projecting from the abluminal side[1]. Furthermore, they were described to be abundantly present on endothelial cells of the cornea[2]. *In vitro*, luminal primary cilia were observed on cultured senescent bovine aortic endothelial cells[3] and, more recently, on human umbilical vein endothelial cells (HUVEC)[4]. The correlation between shear stress and endothelial ciliation was first touched upon in the latter. Primary cilia disappeared from HUVEC exposed to steady shear stress. We are the first to describe primary cilia on endocardial cells, being the endothelial cells of the heart (Chapter 2). The primary cilia in the chicken embryonic heart are, consistent with Bystrevskaya *et al.*[1], either protruding from the luminal or the abluminal cell surface, or immersed in the cytoplasm (Chapter 2,3). Abluminal primary cilia are only present on endocardial cells overlying cardiac jelly (Chapter 3), which is present in very early developmental stages. A number of primary cilia is located in the cytoplasm (Chapter 2), as primary cilia are internalized for disassembly before the onset of mitosis[5]. However, usually, primary cilia protrude from the luminal endothelial cell surface.

The distribution of endothelial primary cilia in the chicken embryonic and the adult mouse cardiovascular system is spatio-temporally-dependent and can be linked to patterns of shear stress and concomitant patterns of shear-related gene expression. In the chicken embryonic heart the distribution of ciliated endothelial cells is inversely correlated to the expression pattern of *Krüppel-Like Factor-2* (*KLF2* or *LKLF*) (Chapter 2). As expression of *KLF2* is upregulated by high steady and pulsatile shear stress[6] and downregulated by low and oscillatory shear stress[7] it can be concluded that ciliation is restricted to areas of low and oscillatory shear stress. In the adult mouse cardiovascular system primary cilia are present at atherosclerotic predilection sites (Chapter 8). The endothelial cells in these areas are also subjected to low and oscillatory shear stress, termed athero-prone flow[8]. When atherosclerotic lesions have developed at these sites, ciliated endothelial cells are still present (Chapter 8). This is consistent with previous reports on human aortic atherosclerotic lesions[1,9]. We demonstrated that primary cilia are specifically present on endothelial cells overlying the upstream and downstream sides of the lesions (Chapter 8,9).

The shear-related distribution raised the question whether endothelial ciliation is affected by alterations in shear stress. To test this, we disturbed hemodynamics during cardiovascular development by ligating the right lateral vitelline vein (the venous clip model[10]). Venous clip results in altered cardiac flow patterns[10], followed by alterations in gene-expression[11] and eventually cardiovascular malformations[10,12]. Venous clip likely generates an increase in shear stress level in the outflow tract (OFT) of the Hamburger and Hamilton[13] stage 17 (HH17) chicken embryonic heart as determined by gene expression[11]

and micro particle image velocimetry (μPIV) studies (Chapter 3). This apparent increase in shear stress level does not affect ciliation. The question remained whether ciliation is affected by the pattern of shear stress (steady, pulsatile, or oscillatory). In Chapter 5 we demonstrate that ciliation of cultured endocardial cells is induced by flow reversals, which is a characteristic of athero-prone flow. In contrast, exposure of ciliated endocardium to steady or pulsatile shear stress leads to a reduction in ciliation, as was previously reported for HUVEC[4]. Moreover, ciliation is shear stress pattern rather than shear stress level dependent, as an increase in shear stress level does not alter the prevalence of primary cilia (Chapter 5). As endothelial ciliation is not affected in the venous clip model we can conclude that venous clip does not affect the oscillatory shear stress regions (Chapter 3). Endothelial ciliation can also be induced *in vivo*. In Chapter 8 we experimentally-induced various shear stress profiles in a carotid artery that is normally exposed to high pulsatile shear stress and is scarcely ciliated, by placement of a restrictive cast. Cast placement resulted in an induction of endothelial primary cilia in areas of low and oscillatory shear stress. In conclusion, endothelial ciliation is not just shear-related, but it is shear-dependent.

Considering the obvious link between oscillatory shear stress and endothelial ciliation in various conditions, including embryo and adult, *in vivo* and *in vitro*, and in chicken and mouse, the occurrence of ciliated endothelial cells can be used as a marker for the presence of flow reversals. As a consequence, ciliation of endothelial cells overlying the shoulder regions of atherosclerotic lesions in the *Apoe*$^{-/-}$ mice would indicate that flow is oscillatory in these areas, although the shear stress level is presumably higher on the upstream side[14].

If we compare cilia characteristics between the different experimental groups, i.e., the mouse and the chicken, *in vivo* and *in vitro*, we find several dissimilarities. The percentage of ciliation can be deduced from the quantifications and estimations in this thesis. Approximately 80% of endocardial cells of the chicken embryonic atria is ciliated (Chapter 2) as compared with an estimated 50% ciliation in the inner curvature of the adult mouse aortic arch (Chapter 8). The difference in ciliation is most likely due to differences in the degree of flow disturbance at the locations analyzed, but could also be due to the difference in heart rate, as adult mice have a heart rate of 7-8 Hz, as opposed to 2 Hz in the chicken embryo. *In vitro*, 16% of embryonic endocardial cells is ciliated after exposure to oscillatory shear stress with a maximum of 2.5 dyne/cm^2 (Chapter 5). This ciliary incidence of the cultured chicken embryonic endocardial cells is comparable to that of cultured HUVEC, of which 5-35% is ciliated under static conditions[4]. In contrast, 100% ciliation was observed in a static culture of mouse embryonic aortic endothelial cells[15]. However, these cells were arrested in their growth and as only non-proliferating cells can present a primary cilium this increases ciliation (Chapter 5). The lower percentage of ciliation of the chicken embryonic endocardial cells *in vitro* (16%) compared with *in vivo* (80%) might be an effect of culturing. Furthermore, in addition to shear stress, stretch is an important factor, as it also affects endothelial gene expression (Chapter 6). The combination of stress and stretch affects endothelial cells. Despite the fact that stretch-induced gene expression is dependent on actin microfilaments rather than microtubules[16], which form primary cilia, the possibility that ciliation is additionally affected by stretch, which is present *in vivo* but is not incorporated in our *in vitro* system, can not be excluded.

In addition to variations in the prevalence of primary cilia between chicken and mouse, ciliary length and α-tubulin isoform content differ. The endothelial and endocardial primary cilia of the chicken embryo are approximately 5 μm in length (Chapter 2,3,5,6), as opposed to 2 μm in the embryonic (Chapter 7) and adult (Chapter 8,9) mouse. Furthermore, the

chicken primary cilia appear to be more stable than the murine cilia as both α-tubulin isoforms, i.e., acetylated, and the more stable detyrosinated α-tubulin, are present in chicken cilia (Chapter 2), while the murine cilia are negative for detyrosinated α-tubulin (Chapter 8). Moreover, the microtubular cytoskeleton of the chicken endothelial cells is more stable than that of murine endothelial cells, as the former is negative for detyrosinated but positive for acetylated α-tubulin (Chapter 2), while the latter lacks both α-tubulin isoforms. These inter-species variations in ciliary length and microtubule stability are most likely due to differences in heart rate or shear stress level. A maximum shear stress level of 50 dyne/cm^2 was measured in the outflow tract of a chicken embryo[17] as compared to 70 dyne/cm^2 in the human adult vascular network[18]. In adult mice shear stress levels of up to 142 dyne/cm^2 are present[19]. Ciliary length has been shown to be shear stress level, but not frequency dependent[20]. In conclusion, the prevalence of primary cilia is not affected by the levels of shear stress, but length and, possibly, tubulin isoform content are affected.

10.2 Function of endothelial primary cilia

The correlation between shear stress and endothelial primary cilia is obvious. However, the question remains if endothelial primary cilia are functional or merely a marker for the occurrence of flow reversals. In this section, confirmed and potential functions of endothelial primary cilia are described.

10.2.1 Shear stress sensing

On flow-exposed cell types primary cilia were shown to function as shear stress sensors[21-26]. The mechanism behind primary cilia-mediated shear stress sensing was considered to be dependent on a protein complex located in the ciliary membrane, i.e., the polycystin (PC) complex. In this thesis an additional primary cilia-mediated mechanism of shear stress sensing is demonstrated.

Polycystins

PC1, located in the ciliary membrane, is a transmembrane protein with a large extracellular N-terminal domain and an intracellular C-terminal domain. Upon activation, PC1 binds PC2, a Ca^{2+} permeable cation-selective channel[27]. Bending of the cilium results in a Ca^{2+} transient[21] (Fig. 1A), which could generate a shear stress response (reviewed by Torres and Harris[28]). Moreover, PC1 can undergo proteolytic cleavage that releases its C-terminal tail (CTT). The CTT enters the nucleus[29] and activates several signal transduction pathways[30-32]. There is no consensus on whether cleavage occurs upon alterations in flow[15,29] or upon cessation of flow[30].

We postulated that the polycystin complex is also functional in the endothelium as endothelial cells also respond to shear stress with a rise in intracellular Ca^{2+} [33] and both polycystins were detected in these cells[34]. Very recently, Nauli *et al.*[15] confirmed the presence of this mechanism in endothelial primary cilia. They concluded that the primary cilium is the endothelial shear stress sensor. However, not all endothelial cells carry a primary cilium (this thesis). Despite the fact that endothelial ciliation is minimal under steady and pulsatile shear stress (Chapter 2,5,8,9) both affect endothelial phenotype and gene expression. Endothelial cells align in the direction of flow[35] and show an upregulation of *KLF2* under these specific flow patterns[6], indicating that non-ciliated cells are shear stress responsive. *In vivo*, the shear stress responsiveness of non-ciliated endothelial cells of the chicken embryonic and adult mouse cardiovascular system was demonstrated by shear-

related gene expression[36,37]. Therefore, primary cilia/PC-mediated shear stress sensing is not the sole mechanism of shear stress sensing in endothelial cells. The mechanism of endothelial shear stress sensing is more complex.

Cytoskeleton
Endothelial cells elicit a shear stress response through activation of several cell components located at cell-cell and cell-extracellular matrix junctions, and at the cell membrane. These include integrins, the platelet endothelial cell adhesion molecule-1/vascular endothelial-cadherin/vascular endothelial growth factor receptor 2 complex, ion channels, tyrosine kinase receptors, nicotinamide adenine dinucleotide phosphate oxidases, G-proteins, the glycocalyx, and caveolae (reviewed by Helmke and Davies[35], Resnick *et al*.[38], Lehoux and Tedgui[39], Li *et al*.[40]). These components are considered to be shear stress transducers that are activated by the shear stress sensor. All the components are, directly or indirectly, connected to the cytoskeleton[35] (Fig. 1A), which comprises microtubules, actin microfilaments, and intermediate filaments that are interconnected[41]. Therefore, the cytoskeleton is likely to play a central role in the endothelial response to shear stress. It undergoes a conformational change upon exposure to shear stress[35,42,43], which is transduced to the linked cell components[44]. Chapter 6 identifies microtubules as the central cytoskeletal component of the endothelial shear stress response. Moreover, disruption of the endothelial microtubules *in vivo* blocks flow-dependent dilation of arterioles[45]. These data demonstrate that the microtubular cytoskeleton is crucial for endothelial shear stress sensing. In areas of low and oscillatory shear stress primary cilia are physically linked to the microtubular cytoskeleton as cytoskeletal microtubules nucleate from their basal body, i.e., the mother centriole of the centrosome (Fig. 1B). We show that the primary cilium aids in the deformation of the cytoskeleton as ciliated endothelial cells have an enhanced flow response compared with non-ciliated cells (Chapter 6).

Combining these data, a double role for the primary cilium in endothelial shear stress sensing is evident (Chapter 7), as depicted in Figure 1A. (I) In an immediate response to ciliary deformation by flow PCs mediate a Ca^{2+} transient. (II) The cilium amplifies deformation of the cytoskeleton, which leads to a prolonged effect on gene expression. Ciliary sensitivity, as determined by ciliary bending and Ca^{2+} transient, should be considered. Sensitivity is positively correlated to ciliary length[20]. Primary cilia of 2.5 μm respond to shear stresses of as low as 0.007 dyne/cm^2 [46].

Intercellular communication
Endothelial shear stress sensing is a synchronized process rather than a single cell phenomenon, as the signal is not only transduced intracellularly, but also intercellularly. As a consequence, ciliation of all contiguous cells is unnecessary. The Ca^{2+} signal and cytoskeletal deformation can be transduced to neighboring cells via gap-junctions[15,21] and cell-cell contacts[47], respectively (Fig. 1A). A gap junction consists of two hemichannels or connexons, which consist of six connexins (Cxs) each. Endothelial cells possess 3 types of connexins, i.e., Cx37, 40, and 43 [48] that form homo- or heteromeric channels. Lack of one of the endothelial connexins, Cx37, does not alter the number or distribution of ciliated endothelial cells in the adult *Apoe*$^{-/-}$ mouse aortic arch (Chapter 9). These data indicate that either alterations in intercellular communication do not affect ciliation or that intercellular communication is not affected by Cx37 deficiency. The latter is likely due to a redundancy of Cx40 and Cx43, but could also be due to the fact that communication is sustained

through cytoskeletal linkage at the cell-cell contacts. Moreover, the effect of Cx37 deficiency was analyzed in *Apoe*$^{-/-}$ mice with advanced atherosclerotic lesions, while atherosclerosis in itself coincides with altered Cx patterning. Cx37 and Cx40 were shown to be absent from the endothelium in the shoulder area of advanced lesions, while Cx43 expression was increased[49]. This would render the *Gja4*$^{+/+}$ *Apoe*$^{-/-}$ mice unsuitable as a control group as the, highly ciliated (Chapter 8), shoulder areas of atherosclerotic lesions might lack Cx37.

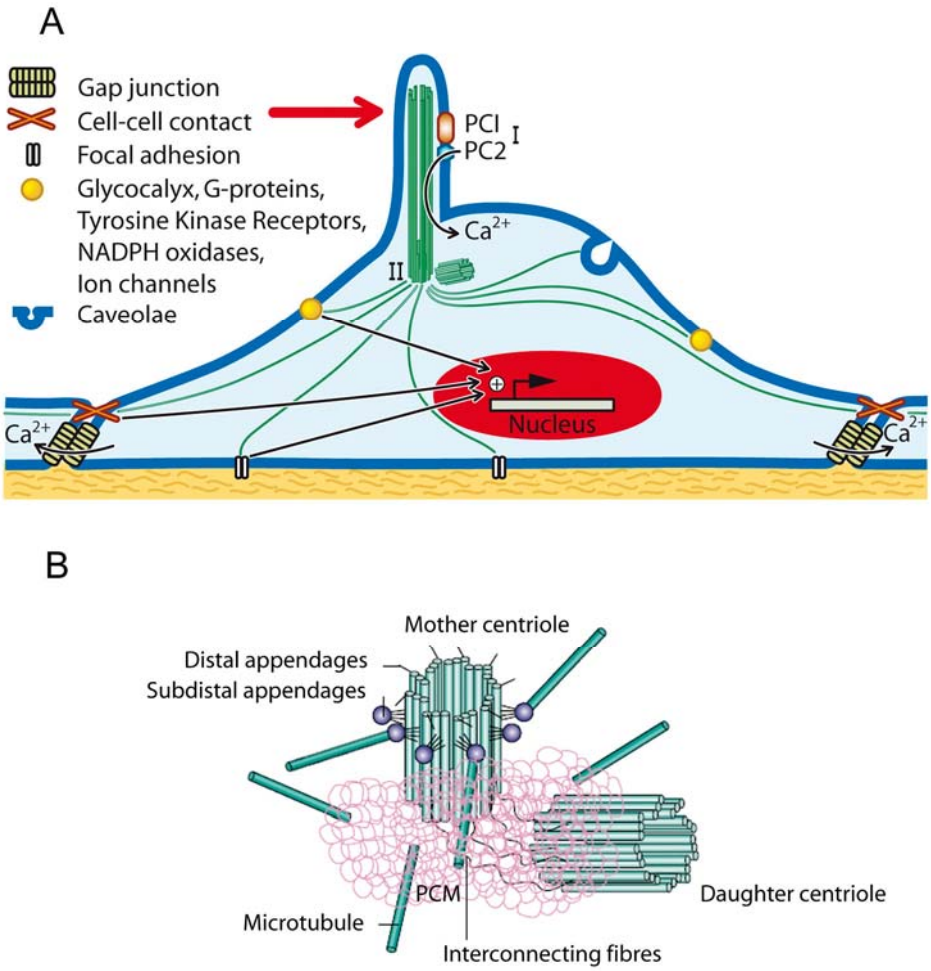

Figure 1. A: Schematic overview demonstrating the double function of primary cilia in endothelial shear stress sensing. In an immediate response to bending of the primary cilium by flow (red arrow) polycystins mediate a Ca^{2+} transient (I). In addition, the cilium aids in deformation of the cytoskeleton (green) (II). The cytoskeleton transduces the force to the cell components it connects. Activation of these components results in a prolonged effect on gene expression (+). Signals can be transduced to neighboring cells via cell-cell contacts and gap junctions. B: Detailed drawing of the connection of the basal body of the primary cilium to the microtubular cytoskeleton (adapted from Doxsey[93]). PC, polycystin; NADPH, nicotinamide adenine dinucleotide phosphate; PCM, pericentriolar material.

10.2.2 Chemosensing

In addition to their double role in mechanosensing, endothelial primary cilia might also act as chemosensors. Several receptors and members of signaling pathways have been demonstrated on or in primary cilia of cell types other than the endothelium. The presence of these receptors in endothelial primary cilia remains to be established.

Wnt signaling

Wnt mediates a canonical (β-catenin) and a non-canonical (planar cell polarity; PCP) pathway. The former regulates cell fate, proliferation and apoptosis, while the latter regulates cell polarization and migration[50]. In the canonical pathway Wnt binds its receptor frizzled, possibly localized in the ciliary membrane[51], which inactivates the β-catenin destruction complex and leads to the stabilization of β-catenin and its translocation to the nucleus where it interacts with transcription factors and activates target gene expression. Inversin is localized to primary cilia of kidney epithelial cells[52] and upregulated by shear stress[53]. Inversin and the cilium-related Kif3a[54] activate the β-catenin destruction complex, shutting down the canonical pathway and consequently activating the non-canonical pathway. However, it is not clear whether primary cilia enhance or suppress PCP signaling, as both loss and gain of PCP generate similar phenotypes[55].

Endothelial cells express Wnt and Frizzled family members[56,57] and respond to Wnt[57], suggesting that the Wnt pathway is active in endothelial cells. Whether it is associated to the endothelial primary cilium remains to be elucidated. However, the PC1 CTT has been shown to activate the canonical Wnt pathway[30,31] and canonical Wnt signaling is observed in the mouse embryonic heart specifically in the endocardial cushions of the outflow tract and atrioventricular canal[58], areas that are non-ciliated (Chapter 2). The non-canonical Wnt pathway appears to be suppressed in ciliated endothelial cells as well, as flow reversals, which induce endothelial ciliation (Chapter 5), induce random PCP[59]. Polarized cell mitosis has important effects on endothelial cell repair[59], which is indeed impaired in areas of disturbed flow[60].

Hedgehog signaling

Hedgehog binds its receptor Patched, which abolishes the inhibitory effect of Patched on Smoothened. This allows Smoothened to translocate into the cilium where it promotes Gli activator formation. Gli activators then move down the cilium and enter the nucleus where they regulate expression of Hedgehog target genes. Patched[61], Smoothened[62], and Gli transcription factors[63] are present in the primary cilium of fibroblasts and kidney epithelial cells.

In adult mice, endothelial cells express Patched[64] and release NO upon stimulation with Hedgehog[65], indicating that this pathway is active in endothelial cells. Whether it is related to the endothelial primary cilium is not clear. Endothelial Hedgehog signaling is involved in vasculogenesis and angiogenesis[66]. A target gene of the Hedgehog signaling pathway is TGFβ, which plays a major role in cardiovascular development[67] and atherosclerosis[68]. Furthermore, Hedgehog signaling has been shown to protect against atherosclerosis in *Apoe*$^{-/-}$ mice[69].

There is no evidence provided for the presence of other ciliary receptors on endothelial primary cilia. Platelet-derived growth factor receptor α, which localizes to fibroblast primary cilia[70], was never detected in the endothelium or endocardium[71]. In addition, the SST_3 somatostatin and 5-HT_6 serotonin receptors present in neuronal primary cilia[72,73] and

the extracellular matrix (ECM) receptors on chondrocyte primary cilia[74] are not to be expected on luminal endothelial primary cilia.

In summary, polycystin-mediated shear stress sensing[15] and cytoskeleton-mediated shear stress sensing (Chapter 6) are confirmed mechanisms of endothelial primary cilia function. The presence of receptors and mediators of signaling pathways in endothelial primary cilia remains to be established.

10.3 Endothelial primary cilia in health and disease

Primary cilia are present on endothelial cells during embryonic and adult life. Through mechanosensation (and possibly chemosensation) they will affect cardiovascular development and postnatal vascular function. As a consequence, they will be involved in the formation of cardiovascular anomalies and pathologies.

10.3.1 Endothelial primary cilia in development

We demonstrated that primary cilia are present in areas of low and oscillatory shear stress (Chapter 2) and render endothelial cells more responsive to shear stress (Chapter 6). This leads us to postulate that endothelial cells in these areas require a primary cilium to sense shear stress. Ciliated and non-ciliated endothelial cells translate alterations in shear stress into a biological response, i.e., altered gene expression, which in turn regulates cardiovascular development. Endothelial primary cilia can be involved in the formation of cardiovascular anomalies for instance through sensing of (pathological) disturbances in hemodynamics during development. The model used in this thesis to alter hemodynamics during development is the venous clip model (Chapter 3). Venous clip induces a raise in shear stress level in specific regions the heart, as determined by gene expression[11] and μPIV (Chapter 3) studies, eventually leading to alterations in cardiovascular function[75-77] and the formation of structural abnormalities[10,12]. More specifically, venous clip shifts blood flow towards the inner curvature of the heart, which results in an increase in *KLF2* expression in the endocardial cells lining the inner curvature. Compaction of the inner curvature is crucial for the second phase of heart looping and shear stress is an important mediator in this process. The venous-clip induced alterations in shear stress in the inner curvature result in cardiovascular abnormalities that are all related to abnormal cardiac looping (Chapter 4). These abnormalities, however, are most probably not initiated by alterations in ciliated areas, as changes in shear-related gene expression upon clip[11] only occur in the non-ciliated areas (Chapter 3). Venous clip does not directly affect the distribution or amount of endothelial primary cilia before the onset of structural malformations (Chapter 3). However, clip-induced changes in gene expression will affect the function and geometry, which will alter shear stress patterns and consequently ciliation, leading to a vicious circle, as altered ciliation will lead to alterations in gene expression. Thus primary cilia are indirectly involved in venous clip-induced malformations.

10.3.2 Ciliopathies

In addition to the registration of (pathological) alterations in shear stress, primary cilia can cause cardiovascular abnormalities or pathology by being dysfunctional. Defects in primary cilia have been associated to polycystic kidney diseases (PKDs), nephronophthisis (NPHP), Senior-Loken, Alström, Orofaciodigital, Jeune, Usher, Joubert, Meckel(-Gruber), and Bardet-Biedl syndrome (BBS). A characteristic of several ciliary syndromes is situs

inversus, in which cardiac looping is randomized[78]. This is most likely caused by defective primary cilia on the epithelial cells of Hensen's node[79], which are involved in determining left-right asymmetry[22,23,80,81]. Whether the endothelial primary cilia are also defective in these syndromes is yet unclear. However, cardiovascular defects occur in NPHP, BBS, and PKD[82] and defects in the gene for PC1 have been shown to cause aortic aneurysms[83]. In addition, PKD patients display endothelial dysfunction and increased carotid intima-media thickness[84]. Nevertheless, it should be noted that the cardiovascular abnormalities observed can be secondary effects of the syndromes rather than the result of dysfunctional endothelial primary cilia.

10.3.3 Endothelial primary cilia in adult life

Steady and pulsatile shear stress induce expression of *KLF2*[6,85], which plays a major role in maintaining a constant shear stress level as it regulates expression of the vasoconstrictor *endothelin-1* (*ET-1*), and *endothelial nitric oxide synthase* (*eNOS*), which produces the vasodilator nitric oxide (NO)[6]. *KLF2* induces anti-thrombotic, anti-proliferative, and anti-inflammatory pathways[86,87], thereby protecting against atherosclerosis. Oscillatory shear stress, on the other hand, predisposes the endothelium for atherosclerosis. The presence of ciliated endothelial cells at atherosclerotic predilection sites suggests that they are involved in atherogenesis. The endothelium at these sites is activated or primed; to be precise it shows an inflammatory response. Primary cilia might be involved in this priming. In the presence of cardiovascular risk factors, primed endothelium can become dysfunctional, which is the start of lesion formation[88]. The key regulatory pathway involved in inflammation is the NF-κB pathway[89,90], which is activated by disturbed flow. Strikingly, PC1 appears to play a role in this process, as its CTT can activate the NF-κB pathway[32]. NF-κB is a transcription factor that regulates expression of several adhesion molecules, such as VCAM-1, ICAM-1, and E-selectin, responsible for the adherence, migration, and accumulation of monocytes and lymphocytes, and thus for progression of the inflammatory status of the vessel wall. In addition to the inflammatory gene profile, the glyocalyx is very thin at the atherosclerotic predilection sites[91] and reactive oxygen species are present[92]. In the presence of cardiovascular risk factors these aspects lead to atherosclerosis. Whether primary cilia are involved in the above mentioned aspects, and thus potentially pathogenic, is unclear. On the other hand, ciliated endothelial cells express more *KLF2* than non-ciliated cells upon flow exposure (Chapter 6). Therefore, endothelial ciliation might be a protective mechanism of endothelial cells to enhance their response to shear stress which fails in the presence of cardiovascular risk factors.

In conclusion, understanding the true capacity of the endothelial primary cilium will have broad implications for cardiovascular development and disease.

10.4 References

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