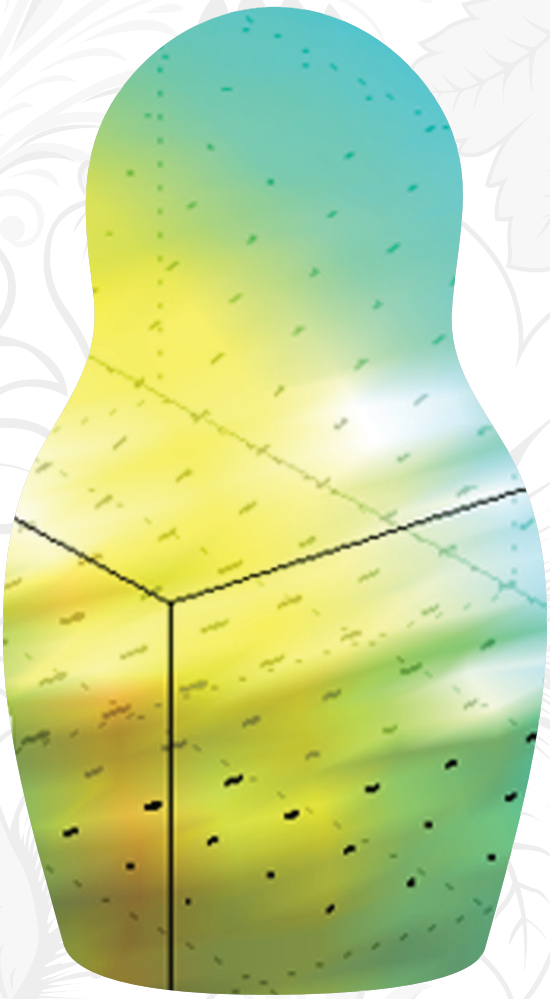

Chapter 9

General Discussion

Endothelial response to shear stress

Endothelial cells (ECs) are highly responsive to changes in blood flow and concomitant shear stress. These hemodynamic forces play decisive roles in the development of the cardiovascular system and maintenance of homeostasis in adult life[1,2]. The large and rapid changes in volume and geometry of cardiac compartments during development are translated into local changes in shear stress and gene expression patterns of e.g. Krüppel like factor 2 (*KLF2*)[3]. KLF2 and KLF4 are shear responsive transcription factors that are essential in establishing and maintaining endothelial function by regulating the expression of many downstream genes[4-9]. Together these factors regulate a transcriptional program to induce an anti-thrombotic, anti-proliferative, and anti-inflammatory endothelial phenotype[9,10]. Low and oscillating flow fails to induce KLF2 expression and leads to activation of an inflammatory program in ECs[8,11], in part through activation of transforming growth factor-β (Tgfβ) signaling[12]. In Chapter 3 we describe the capacity of endothelial progenitors to induce *KLF2* expression in response to shear stress in a manner similar to that of differentiated adult arterial and venous ECs[13]. Our data highlights the universal response of ECs to fluid flow that is apparently independent of their differentiation status. In Chapter 4 we confirm the previously described mechanism by which shear stress-driven KLF2 expression causes the induction of inhibitory SMAD7, which inactivates TGFβ signaling in adult ECs[12,14]. As endothelial progenitors isolated from adult cord blood show a mature response to shear stress, this KLF2/TGFβ-inhibitory mechanism is probably active in endothelial progenitors early during differentiation and can therefore contribute towards vascular repair[15].

In the developing embryo, ECs lining the endocardial cushions of the heart are exposed to high shear stress and show high expression of *KLF2*[8]. For proper development into functional valves, these cushions need to cellularize through the process of endothelial-to-mesenchymal transition (EndoMT). During this transition ECs loose expression of endothelial markers, delaminate from the surrounding monolayer, and migrate into the cardiac jelly[16]. Tgfβ signaling has been described to drive EndoMT[17] and therefore the role for shear stress in the modulation of Tgfβ signaling has been the subject of scientific controversy[12,18]. Our data shows that Tgfβ signaling and *Klf2* expression are regulated through an alternative mechanism in embryonic ECs. Shear stress induces Tgfβ ligand expression and activates signaling through the Tgfβ receptor activin receptor-like kinase-5 (Alk5) which subsequently induces the expression of *Klf2* through activation of mitogen activated protein kinase (MAPK) signaling. We show that Mek5/Erk5/Mef2C activation is required for *Klf2* induction under shear stress, suggesting a relation between Tgfβ and Mek5/Erk5 similar to that described for other cell types[19,20]. Enhanced phosphorylation and nuclear translocation of Smad2, already within hours after the onset of shear stress, suggests that other mechanisms might also contribute to Alk5 mediated *Klf2* induction[14]. Alternatively, an Alk5 independent mechanism by which AKT can phosphorylate the linker region of Smad2 resulting in its activation under shear stress has recently been described[21]. Further studies will need to elucidate the contribution of other signaling mechanisms (e.g. AKT) to Smad activation under shear in embryonic ECs. Taken together, our data

suggests that a switch between shear-induced Tgfβ-dependent and independent induction of Klf2 should take place sometime between the embryonic and the early postnatal phases. It is possible that these embryonic mechanisms are recapitulated in the adult under pathological conditions, where high shear stress might activate Tgfβ signaling and lead to plaque progression or cardiac fibrosis[22,23], discussed later in this chapter.

ECs lining the cardiac cushions are devoid of primary cilia as a consequence of high shear stress in these areas[24,25]. In Chapter 5, Chapter 6, and Chapter 8 we show that the absence of cilia on cushion endothelium is instrumental in rendering these cells prone to shear-induced EndoMT[26]. Lack of primary cilia results in high *Tgfβ2* and Gli1 expression, both of which are prerequisites for cardiac cushion EndoMT (Chapter 8). Shear stress-enhanced Tgfβ2 ligand signaling through its specific TgfβR3/betaglycan receptor results in activation of Alk5/ Smad2/3 mediated transcription program and EndoMT (Chapter 8)[14,26]. The mechanism of Gli1 signaling is not yet clear, however, it appears to influence Tgfβ-mediated EndoMT through regulation of a common factor, presumably Snail (Chapter 8)[27].

Of the three Tgfβ ligand-deficient mouse models, only *Tgfβ2*$^{-/-}$ mice exhibit congenital heart defects[28-32] and it is therefore not surprising that Tgfβ2, and not Tgfβ1 or Tgfβ3 is required for shear-induced EndoMT in our model (Chapter 8). This is in line with reports on requirement of TgfβR3 for endocardial transformation in the heart[33,34] and has been confirmed *in vivo* as *Tgfβ2*$^{-/-}$ embryos show defective cardiac cushion formation[35,36]. Azhar and colleagues have recently identified novel roles of Tgfβ2 in post-EndoMT endocardial cushion remodeling and mesenchymal cell differentiation[37]. The impact of ciliation phenotype of endothelial and/or mesenchymal cells in post-transitional remodeling remains to be elucidated.

We confirmed the significance of shear-stress driven activation of Alk5-mediated Tgfβ signaling *in vivo*[14]. Altered venous return into the heart due to ligation of the right lateral vitelline vein of a chicken embryo results in a local increase in shear stress in the cardiac cushion area[38] (Figure 9.1). This process is accompanied by induction of *KLF2* expression[39] and enhanced Smad2 phosphorylation in the ECs lining the cushions[14]. This mechanism is likely to be responsible for driving EndoMT in the high shear stress area of the cardiac cushions. In line with the necessity of adequate endothelial signaling for normal cardiac development, venous clipping eventually results in a spectrum of cardiac malformations including atrioventricular canal (AVC) and outflow tract anomalies (OFT)[38,40]. Figure 9.1 illustrates how altered intracardiac shear stress results in perturbed EndoMT and a cardiac cushion volume that is significantly reduced in clipped embryos. Interestingly, enhanced Alk5/Smad2 signaling activation was also observed in ECs lining the hearts of *Tg737*$^{orpk/orpk}$ embryos. ECs that would normally be ciliated due to their hemodynamic environment[24] lack the capacity for ciliogenesis in the *Tg737*$^{orpk/orpk}$ model and show enhanced activation of Alk5 signaling. This is in line with reports of elevated epithelial Tgfβ/Smad2 signaling in other models with defective cilia function (e.g. PKD1

mutant)[41]. Convergence on the level of Alk5 signaling in the venous clip model and *Tg737*$^{orpk/orpk}$ embryos highlights that locally increased shear stress or lack of primary cilia both influence EndoMT through altered Tgfβ signaling activation. Perturbed, rather than enhanced EndoMT in the venous clip model can probably be explained by the necessity for balanced Alk1 (BMP ligand-associated) and Alk5 (Tgfβ ligand-associated) mediated signaling for efficient transformation. We showed that shear stress preferentially activates Tgfβ signaling through its Alk5 receptor and inhibits Alk1 mediated effects[14]. The requirement for functional Alk1 pathway is best illustrated by the need of Smad1/5 signaling for robust AVC EndoMT[42,43]. BMP and Tgfβ2 signaling in cushion EndoMT were recently both described to rely on signaling through TgfβR3[42]. Augmented TgfβR3-mediated signaling in *Tg737*$^{orpk/orpk}$ ECs can therefore explain enhanced EndoMT in nonciliated cells (Chapter 8)[26].

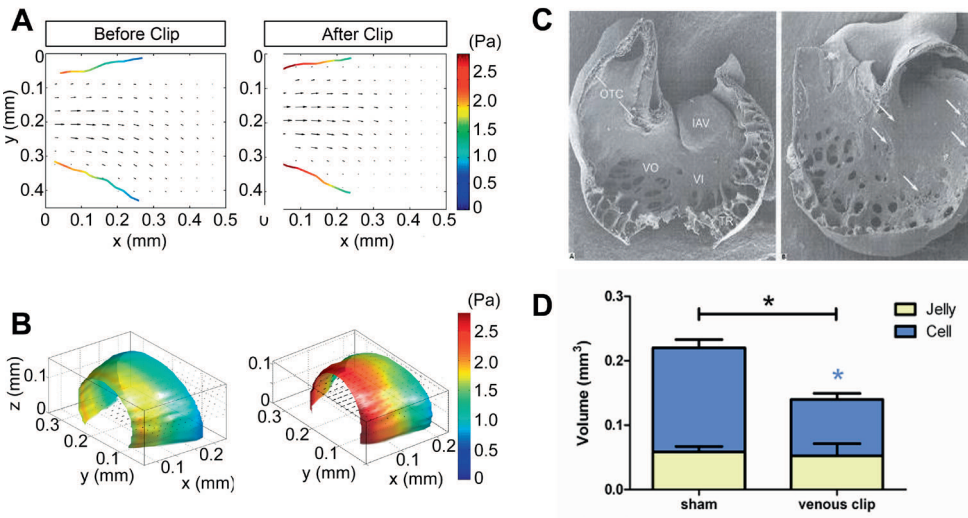

Figure 9.1. Altered intracardiac shear stress leads to perturbed EndoMT *in vivo*. A, Velocity field at the mid-plane of the outflow tract before and immediately after venous clip shows a local increase in wall shear stress levels after intervention. B, Individual velocity fields were used to reconstruct the three-dimensional volumetric data. The wall location is reconstructed and the shear stress levels (Pa) are indicated with the color scheme. The vectors represent the direction of blood flow at peak systole of the cardiac contraction cycle (adapted from Egorova[14]). C, Field emission scanning electron microscopy images showing the morphology of the chicken embryonic heart at HH20. Ventral view of the normal heart (left) and abnormal heart after right vitelline vein ligation at HH17 (right)[38]. OTC indicates outflow tract cushions; IAV, inferior AV-cushion; VO, ventricular outlet segment; VI, ventricular inlet segment; TR, ventricular trabeculations. The volume of AV and OFT cushions is dramatically reduced in the clipped embryo. Note the abnormal appearance of the trabeculations and the numerous intercellular clefts in the endocardial surface after venous clipping. D, Morphometry volume estimates of cardiac jelly and cellularized cushion of HH24 chicken hearts after venous clip (right) procedure compared to sham embryos (left). Note that the reduction in total volume is caused by a loss of volume of cellularized cushion and not by the loss of cardiac jelly.

KLF4 is a functional counterpart of KLF2 in ECs[9] and its induction under shear stress is mediated by Mek5/Erk5[44,45]. In our model Klf4 was induced in ciliated cells, but was downregulated in nonciliated cells under shear stress[26]. Overexpression of Klf4 inhibits EndoMT, suggesting a role for Klf4 in retention of endothelial phenotype and cellular quiescence[46]. This corresponds with findings on the role of Klf4 in the regulation of the endothelial marker VE-Cadherin[47], and in repression of several smooth muscle differentiation markers[48]. Biological effects of KLF4 can best be summarized as a repressor of growth and differentiation[49]. Smooth muscle cell (SMC) differentiation largely depends on Tgfβ signaling and probably involves a similar transition mechanism as observed in *Tg737*$^{orpk/orpk}$ ECs under flow. It could therefore well be that the downregulation of Klf4 expression observed in nonciliated cells signifies differentiation towards mesenchymal cells with SMC characteristics under shear. This allows to speculate on the potential role of Klf4 and Tgfβ signaling in the onset of atherosclerosis, i.e. neointima formation[50], discussed later in this chapter.

Oak Ridge Polycystic Kidney mouse

The Oak Ridge Polycystic Kidney (ORPK, *Tg737*$^{orpk/orpk}$) mouse was described over 15 years ago as a model for human recessive polycystic kidney disease[51]. The *Tg737*$^{orpk/orpk}$ mouse model is characterized by integrational mutation into an intron of the *Ift88* gene resulting in a hypomorphic allele (*Ift88Tg737Rpw*)[51]. This partially disrupts the expression and function of intraflagellar transport 88 protein (Ift88, Tg737, polaris). Ift88 is required for bidirectional transport of proteins along the ciliary axonemes, a process initially described in *C.reinhardtii* flagella[52]. The hypomorphic *Ift88* allele leads to impaired assembly of motile and immotile cilia resulting in cilia that are stunted and malformed, but not entirely absent. *Ift88* null mutations are embryonic lethal in the organogenesis stages. In contrast, the Ift88 allele in the *Tg737*$^{orpk/orpk}$ mouse allows these embryos to survive to young adulthood. This makes the *Tg737*$^{orpk/orpk}$ mouse a good model for studying the function of primary cilia in a large variety of tissues and evaluating the role of ciliary dysfunction in disease. As such, the *Tg737*$^{orpk/orpk}$ mouse was the first mammalian model to establish a connection between ciliary dysfunction and cystic kidney disease. Besides the cystic renal phenotype, analysis of *Tg737*$^{orpk/orpk}$ mice revealed ao. hepatic and pancreatic ductal abnormalities and cysts, retinal degeneration, skeletal defects, cerebellar hypoplasia, hydrocephalus, and laterality defects[51,53-58].

The function of Ift88 goes beyond its critical role in ciliogenesis. Ift88 is implicated to have direct effects on transcriptional and signaling regulation, cell cycle control, and was proposed to be a putative tumor suppression gene[59-61]. Contribution of Ift88 protein-specific effects can therefore not be entirely excluded. However, the loss of primary cilia in wild type (WT) ECs under high shear stress and the concomitant induction of EndoMT strongly suggests the central role for primary cilia, and not Ift88 in this phenotypic transition[26]. Analysis of mouse models, such as Ift172 and Kif3a mutants, for (sub-)endothelial Tgfβ signaling activation and cardiovascular phenotypes should be performed to confirm the cilia (and not Ift88 protein) -specific effects observed in the *Tg737*$^{orpk/orpk}$ embryos.

The *Tg737*$^{orpk/orpk}$ mutation bred on different genetic backgrounds shows a broad range of phenotypic variations. *Tg737*$^{orpk/orpk}$ on the C3HeB/FeJLe background are described to survive for longer than a year and have relatively mild and slow progressing renal and hepatic phenotypes[62]. In contrast, *Tg737*$^{orpk/orpk}$ mutants bred on the FVB/N background are generally embryo-lethal due to severe hydrocephalus, renal cysts, bile duct hyperplasia, dilated pancreatic ducts and acini atrophy[51,63]. Genetic analyses of *Tg737*$^{orpk/orpk}$ mice bred on different backgrounds will probably provide further insight into the phenotypic variations observed and identify potential modifier genes associated with ciliary dysfunction.

Primary cilia as shear sensors

The presence of cilia on ECs is spatiotemporally linked to the blood flow profile. Endothelial primary cilia are present in the areas of low and oscillatory flow and render these cells more sensitive to shear stress[24,25]. Specifically, ciliated ECs show a stronger induction of the transcription factor *Klf2* in response to shear[25,26]. Induction of endothelial ciliation by oscillatory flow with cyclic flow reversals[64] suggests a protective role for primary cilia in preventing endothelial activation in areas of disturbed flow through increased Klf2 levels and establishment of concomitant endothelial quiescence, reviewed in Chapter 2[65]. However, the relation between the presence of primary cilia on ECs and the expression of Klf2 is more complex as exposure to high shear stress results in high *Klf2* induction[25,66,67] despite the loss of endothelial cilia[68]. This appears to be an elegant feedback mechanism in which ECs in areas of disturbed flow require primary cilia to sense shear stress and translate it into adequate functional responses by e.g. altering gene expression. Alternatively, cilia-independent mechanisms contribute to the establishment of a quiescent endothelial phenotype under high shear stress conditions[69]. This, however, is not the case in embryonic ECs where loss of endothelial cilia under high shear, and associated loss of protective ciliary effects, results in activation of Tgfβ signaling and primes for EndoMT[24,26]. The discrepancy between the embryonic and adult situation is not fully understood, however, divergent interactions between Tgfβ and MAPK/Klf2 signaling are likely to explain these results[14].

Similar to epithelial cells, endothelial primary cilia are involved in establishment of intracellular calcium transient under shear[70,71]. Polycystin-1 and polaris were shown to be crucial mechanosensitive proteins in ECs, mediating the rise in intracellular calcium and biochemical nitric oxide synthesis in response to flow induced deflection of the primary cilium[72]. In Chapter 7 we studied this mechanism in relation with the role of primary cilia in the regulation of *Klf2* expression. Our data showed that induction of *Klf2* under shear is independent of the flow stimulated calcium transient[64]. This is explained by considering the ciliary shear stress sensing mechanism as a two-step process[65,71]. In an immediate response that involves ciliary bending, activation of the polycystins leads to a rise in intracellular calcium and a concomitant synthesis and release of nitric oxide within seconds after the initiation of fluid flow. A prolonged response, which is most probably coordinated by changes in cytoskeletal confirmation, then leads to activation of shear responsive genes, e.g. *KLF2*. This

requires at least 1-2 hours. Both responses (directly or via Klf2 expression) lead to induction and activation of eNOS, which results in increased synthesis of nitric oxide. Nitric oxide diffuses into the vessel wall and induces relaxation of the vascular SMCs leading to adaptive vasodilatation and concomitant decrease in shear stress. Cilia-induced epithelial calcium signaling has recently been implicated to contribute to renal cyst formation and functions as the initial signaling messenger for various cellular pathways including Wnt, cAMP, MAPK, and mTOR[73]. Although we found *Klf2* induction to be independent of calcium signaling in ECs, the regulatory role of calcium transient in other endothelial signaling mechanisms is not ruled out.

Ciliary signaling

The primary cilium is an essential regulator of numerous signaling pathways and disruption of ciliary function is involved in human syndromes collectively called ciliopathies[74,75]. Hedgehog (Hh) signaling represents an evolutionary conserved signal transduction pathway that is classically associated with the presence of a primary cilium. Hh signaling is disrupted by mutations in human congenital malformations and cancers[76], and emerging evidence suggests that induction of Hh signals in adult tissues can stimulate tissue repair[77-79]. Data from several independent *Ift88*$^{-/-}$ models showed that cells devoid of primary cilia are unable to adequately respond to Hh ligands[60,80]. In Chapter 8 we show that ECs lacking primary cilia have high noncanonical Hh signaling activation and *Tgfβ2* expression and fail to further activate canonical Hh mechanisms in response to ligand stimulation. This is in contrast to ciliated ECs where canonical Smoothened-mediated and ligand-dependent mechanisms are active, in accordance with reports in other cell types[81]. Similar results were obtained from studies in *Tg737*$^{orpk/orpk}$ mice where the loss of primary cilia also results in increased Hh activity in the mesenchyme of embryonic jaw primordia and a gain-of-function phenotype[82]. Hh activation is aberrantly upregulated upon ciliary ablation in pancreas tissue where a downstream effect on epithelial cell plasticity and differentiation is reported[83-85]. Together, our data suggests that Hh signaling functions in a non-contagious manner in nonciliated ECs and is involved in regulation of cell plasticity through e.g. EndoMT. An emerging number of noncanonical Hh signaling pathway activation mechanisms is reviewed in[86]. The relevance of such mechanisms for endothelial function will be the topic of further research.

Many of the pleiotrophic effects of Hh signaling during development and carcinogenesis are linked to its involvement in epithelial-to-mesenchymal transition (EMT), a process mimicking EndoMT in epithelial cells of various origins[87-89]. In Chapter 8 we demonstrate that high *Gli1* expression is a prerequisite for shear stress-induced EndoMT in nonciliated ECs and that its expression is associated with areas of EndoMT *in vivo*. In contrast to previous reports of Tgfβ/Smad mediated activation of Gli1 and Gli2 in epithelial cells[90], we show that high *Gli1* expression in nonciliated ECs is not determined by Tgfβ. It therefore may well be that although high *Gli1* levels are associated with high *Tgfβ2* expression in nonciliated ECs, the two are driven by independent mechanisms acting in parallel in ECs lacking primary cilia. Tgfβ2 and Gli1 activated pathways both contribute to EndoMT as they converge at a common factor, probably Snail (Chapter 8)[27].

Hh is not the only pathway that relies on primary cilia for its canonical signal transduction. Wnt signaling is closely linked with primary cilia in epithelial cells[91]. Wnt mediates a β-catenin-dependent (canonical) and β-catenin-independent (noncanonical/ planar cell polarity (PCP)) signaling processes. ECs have been shown to express Wnt family members and respond to Wnt ligand, suggesting that this pathway is functional in endothelium[92,93]. Increased β-catenin signaling has also been associated with Tgfβ activity during endocardial cushion EndoMT, and β-catenin deficient mice fail to efficiently populate AV cushions with mesenchymal cells[94]. However, experiments with Wnt inhibitors have succeeded in blocking endocardial cushion formation through alternative, not Tgfβ-mediated mechanisms[95,96]. Tgfβ and Wnt/β-catenin signaling pathways are recognized as important inducers of endocardial cushion formation, however, the exact regulatory relationships have not yet been defined[18]. Although localization of members of Wnt signaling to endothelial primary cilia remains to be confirmed, it may well be that Wnt signaling contributes to priming for shear-stress induced EndoMT in a manner similar to Hh.

Clinical implications

Congenital heart defects

Cardiovascular defects are the most common congenital defects, occurring in 0.8-5% of all live births[97]. A complex interplay of numerous genetic and epigenetic factors guides normal embryonic cardiogenesis. Shear stress is an important hemodynamic force modulating endothelial function, tissue morphogenesis, and embryonic patterning. Signaling through e.g. Tgfβ, Wnt/β-catenin, Notch, and Hh pathways guides neural crest cell migration and EndoMT, both of which are central processes in cardiac cushion development[94,98-100]. Several animal models illustrate the relevance of fluid flow patterns in valve development. In zebrafish, obstruction of the ventricular OFT results in impaired valve formation, diminished looping, and disturbances in ventricular development[101]. Zebrafish bearing mutations resulting in impaired blood flow secondary to defective myocardial function fail to develop normal AV valves[102]. Lack of seeding of the endocardial cushions within the AVC is observed in mice with defective heartbeat as a result of mutated sodium/calcium exchanger[103]. Ligating the right viteline vein of chick embryos (i.e. venous clip model[38]) leads to a local rise in shear stress, as is measured with μPIV[14] and specific gene expression[39]. Specifically, venous clipping shifts the blood flow towards the inner curvature of the heart, compaction of which is crucial in the process of heart looping[1]. Changes in local hemodynamics lead to altered activation of Tgfβ/Alk5 signaling and disturbed EndoMT (Figure 9.1)[14,26,104].On the long-term, clipped embryos develop a range of EndoMT associated anomalies including ventricular septum defects and valve anomalies[40].

The crucial role of shear stress in driving cardiac development suggests the need for an adequate mechanism for the ECs lining the heart to sense blood flow. Primary cilia function as shear sensors and coordinate the response of ECs to fluid flow[25,70]. Considering the function of cilia in nodal flow it is not surprising that several ciliopathies are characterized by gross cardiac anomalies[105]. This is probably related to the random orientation of heart looping along the left–right axis, corresponding

to the relatively high incidence of *situs inversus* in these patients[106-108]. Less than half of mice with structurally normal, but immotile cilia have cardiac defects. In contrast, almost all of mice with mutations leading to complete loss of cilia (effecting function of both motile and immotile cilia) show cardiac anomalies. This suggests that cilia have a more prominent role in cardiac development than exclusively through their function in node cilia[109]. In Chapter 5, Chapter 6, and Chapter 8 we provide evidence that suggests endothelial contribution to the phenotypes observed in ciliopathy syndromes, where the endothelium-derived defects could in first instance be masked by gross phenotypes of laterality disorders. Nonciliated ECs have enhanced Alk5 and Gli1 activation (Chapter 8)[26], which are critical mediators of Tgfβ and Hh morphogens, respectively. Primary cilia could therefore have an indirect effect on cardiovascular phenotypes of ciliopathy patients through altered Tgfβ and Hh signaling. This requires further confirmation *in vivo*.

Atherosclerosis

Endothelial (dys-)function is associated with risk factors[110-112], shows a correlation with disease progression[113], and can predict clinically significant cardiovascular events[114,115]. The endothelium regulates vascular tone, platelet activity, and leukocyte adhesion, all of which play important roles in the initiation of atherosclerosis[116,117]. Low and oscillatory shear stress results in ECs activation, expression of adhesion proteins and chemokines, and leukocyte rolling and adhesion[116]. It is not surprising that atherosclerotic lesions develop predominantly at branches, bends, and bifurcations in the arterial tree as these sites are exposed to low or disturbed blood flow[118-120]. Endothelial primary cilia are efficient mechanosensors and oscillatory flow leads to ciliation of ECs[121]. It is therefore not surprising that in adult vasculature, ciliated ECs are predominantly found at sites of low or oscillatory shear[122]. Ciliation of ECs results in enhanced *KLF2* and *KLF4* expression[25,26] and induction of a downstream transcription program that normally establishes endothelial quiescence[5,9]. It may therefore well be that endothelial cilia have an atheroprotective role at sites of disturbed flow, preventing endothelial activation during the initial stages of plaque formation.

Contribution of Tgfβ family of ligands to the pathogenesis of atherosclerosis has been the subject of scientific discussions[123,124]. Members of the Tgfβ signaling cascade are expressed at high levels in SMCs, macrophages, and T-cells in human atherosclerotic lesions during fatty streak formation and atheroma development[125]. Tgfβ determines the extent to which developing atherosclerotic lesions are stabilized by a collagen-rich fibrous cap, and SMCs in stable lesions show higher Tgfβ expression compared to unstable lesions[126]. Unstable plaques can rupture with high risk of subsequent thrombus-mediated acute clinical events such as myocardial infarction and stroke. A thick fibrous cap is therefore beneficial[127] and high Tgfβ/Smad2 activation at lesion sites is suggestive of a better clinical prognosis[124]. Primary cilia are located upstream, downstream, and at the shoulders of atherosclerotic lesions[122], i.e. regions known for high prevalence of inflammation and plaque rupture[128]. In Chapter 5 and Chapter 6 we demonstrated that ciliated embryonic ECs show lower Tgfβ signaling activation under shear than their nonciliated counterparts[26]. Embryonic mechanisms

are often recapitulated in the adult under pathological conditions and it may therefore well be that ciliated ECs can contribute to plaque vulnerability due to reduced Tgfβ/Smad2 signaling activation and concomitant scarce interstitial collagen synthesis. On the contrary, nonciliated ECs that are found on the top of the plaque are exposed to high shear stress levels and may enhance plaque stability by several mechanisms. First, local suppression of Klf4 can result in better differentiation of SMCs that are responsible for the bulk of collagen production. This is mediated by alleviation of Klf4-related of smooth muscle differentiation[48]. Secondly, nonciliated ECs can directly contribute to a collagen expressing mesechymal cell population through EndoMT. A similar mechanism is implicated in tissue fibrosis[22,129,130]. Thirdly, ECs can also synthesize collagens[131], and although there is no data on direct effects of ciliation phenotype on collagen synthesis, enhanced Tgfβ activation could well correlate with enhance collagen synthesis in nonciliated endothelium. Enhanced Hh signaling has been posed to be atheroprotective[132]. Downregulation of Hh signaling results in a significant increase in total plaque area[132], suggesting that inhibited Hh activation in ciliated ECs (Chapter 8) at atherosclerotic lesion sites may contribute to plaque progression, rending primary cilia pathogenic.

Carcinogenesis

The primary cilium is anchored in the cell by the basal body and is usually displayed on differentiated cells at G0[133]. The basal body develops from the mother centriole of the centrosome in a way that is coordinately regulated with the cell cycle[133,134]. Abnormal ciliary structure and function has been associated with aberrant cell cycle progression and high proliferation rates in various cell types[135,136]. Specifically, abnormal ciliary function in cystic kidneys has been linked to polyploidy and tissue hyperplasia[137], and Tg737/Polaris has been described as a putative tumor suppressor gene[59]. Polyploidy is the hallmark of neoplastic transformation[138,139] and ciliary dysfunction can contribute to carcinogenesis by inducing genetic instability. It is not yet clear what the consequences of polyploidy are for endothelial function and differentiation. There are, however, suggestions of polyploidy in human ECs being part of the natural aging process[140]. AbouAlawi and colleagues have recently reported aortic ECs isolated from *Tg737*$^{orpk/orpk}$ mice to have increased centrosome amplification and polyploidy compared to WT cells, a phenomenon that progressed with increasing age[141]. It may therefore well be that phenotype severity caused by ciliary dysfunction progresses with age. Unfortunately, *Tg737*$^{orpk/orpk}$ mice on FVB/N background die before reaching adulthood. Studies of *Tg737*$^{orpk/orpk}$ on e.g. C3HeB/FeJLe background should elucidate the long term contribution of polyploidy and increased cellular proliferation to (vascular) health.

Angiogenesis is essential for tumor growth and metastasis, and controlling tumor-associated angiogeneic mechanisms is a promising approach in impeding cancer progression. Hh signaling has recently been reported to drive proangiogenic responses in adult human ECs[142]. Hh proteins promote endothelial tubulogenesis in HUVECs through a Gli-independent, yet Smoothened-mediated mechanism. Activation of Smoothened resulted in activation of the small GTPase RhoA, which mediates the reorganization of actin cytoskeleton into stress fibers, and subsequently leads

to enhanced angiogenesis[142]. RhoA kinase system is also modulated by shear stress and regulates stress fiber assembly under flow[143-145]. A central role for RhoA is implicated in EC migration and orientation of cell movement. These processes have classically been interpreted in light of shear-stress induced alignment[146,147]. However, similar mechanisms are likely to be involved in EMT during development and carcinogenesis and shear-stress induced EndoMT[148,149]. Collectively, this suggests that converging RhoA mediated mechanisms are involved in shear stress induced transition and angiogenic responses e.g. during carcinogenesis. Aberrant activation of signaling molecules and receptors that are important for controlling cellular migration, proliferation, differentiation, and apoptosis can lead to the development of many types of cancer. Platelet-derived growth factor receptor-α (PDGFRα) and Hh signaling are two examples of such signal transduction pathways, multiple components of which localize to the primary cilium[80,150,151]. Inappropriate activation of Hh pathway is associated with e.g. the most common neoplasm in humans – basal cell carcinoma[152], and the most common malignant childhood brain tumor – medulloblastoma[153]. Non-canonical Hh signaling pathways are important contributors to carcinogenesis[86,154] and have recently been described to be active in ECs (Chapter 8)[142]. We show the determinant role of primary cilia in the 'switch' between canonical and non-canonical Hh signaling activation. In light of this, contribution of primary cilia and (paracrine) effects of non-contagious endothelial Hh signaling to cancer progression should be re-examined.

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