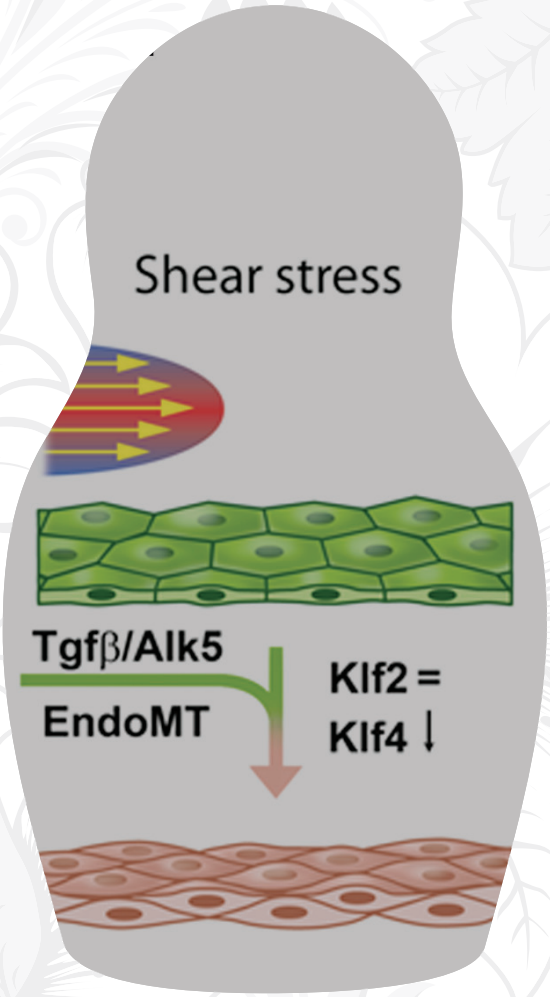
Shear stress
Tgfβ/Alk5
EndoMT
Klf2 =
Klf4 ↓

Chapter 7

Primary Cilia as Biomechanical Sensors in Regulating Endothelial Function

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Abstract

Depending on the pattern of blood flow to which they are exposed and the irproliferative status, vascular endothelial cells can present a primary cilium in to the flow compartment of a blood vessel. The cilium modifies the response of endothelial cells to biomechanical forces. Shear stress, which is the drag force exerted by blood flow, is best studied in this respect. Here we review the structural composition of the endothelial cilia and the current status of knowledge about the relation between the presence of primary cilia on endothelial cells and the shear stress to which they are exposed.

Review

Most cells in our body can bear monocilia or primary cilia, depending on e.g. their proliferative status. Cilia on the epithelial cells of the collecting ducts in the nephron are a classical example of monocilia (reviewed previously)[1]. A decade ago attention in the developmental biology field was drawn to cilia by the groups of Hamada and Brueckner[2,3] who showed that the endodermal epithelial cells of the embryonic organizing center utilized a combination of motile and primary cilia to generate and sense nodal flow. This has an instrumental role in breaking the symmetry of the embryo. Since endothelial cells (ECs) comprise a specialized population of epithelial cells which are continuously exposed to fluid flow and are highly responsive to hemodynamic forces it is not surprising that these cells can bear primary cilia as well (reviewed earlier)[4]. To date, there are no reports of ECs bearing motile cilia which have functions in blood vessels, but ciliary protrusions on ECs have been described for over 40 years[5-7]. It is now clear that endothelial cilia truly belong to the subpopulation of primary cilia since they are composed of a 9+0 bundle core of microtubule doublets, and extend from the basal body of the cell (Figure 7.1). Through the basal body they connect to the cytoskeletal microtubules of the cells (Figure 7.1G and H). They are present on many types of endothelial cells, like Human Umbilical Vein Endothelial Cells (HUVECs)[8,9], mouse aorta ECs[10], and embryonic ECs from various species[11-13]. Depending on the species and the location in the cardiovascular system the typical length of endothelial cilia varies between 1 and 5 μm, making them significantly shorter than cilia on other (epithelial) cells. A video which shows that, despite their short length, fluid flow is able to bend the endothelial cilium is available and accompanies the electronic version of this manuscript. A selection of still pictures from this video is shown in Figure 7.1A. A cilium typically protrudes from the luminal side of the cell into the lumen of the vessel although it sometimes appears to protrude from the basal side into the basement membrane and underlying extracellular matrix (Figure 7.2A). Interestingly, many cells found on the ventral luminal side of the embryonic aorta present primary cilia (Figure 7.2B). These cells most probably represent endothelium-derived hematopoietic stem cells[14]. Functional consequences of the presence of cilia on these cells are yet unclear. Nauli and colleagues first demonstrated that primary cilia are necessary for calcium and nitric oxide signaling in ECs[11]. In contrast to ciliated cells, ECs without a cilium were not able to translate mechanical stimulation of the cilium into an intracellular calcium transient. This function depends on the presence of Polycystic Kidney Disease (PKD) proteins which are localized in the cilium. This is very interesting since it adds a vascular component to the large spectrum of ciliopathies which often present with various variants of cystic kidney diseases (reviewed in[4,15,16]).

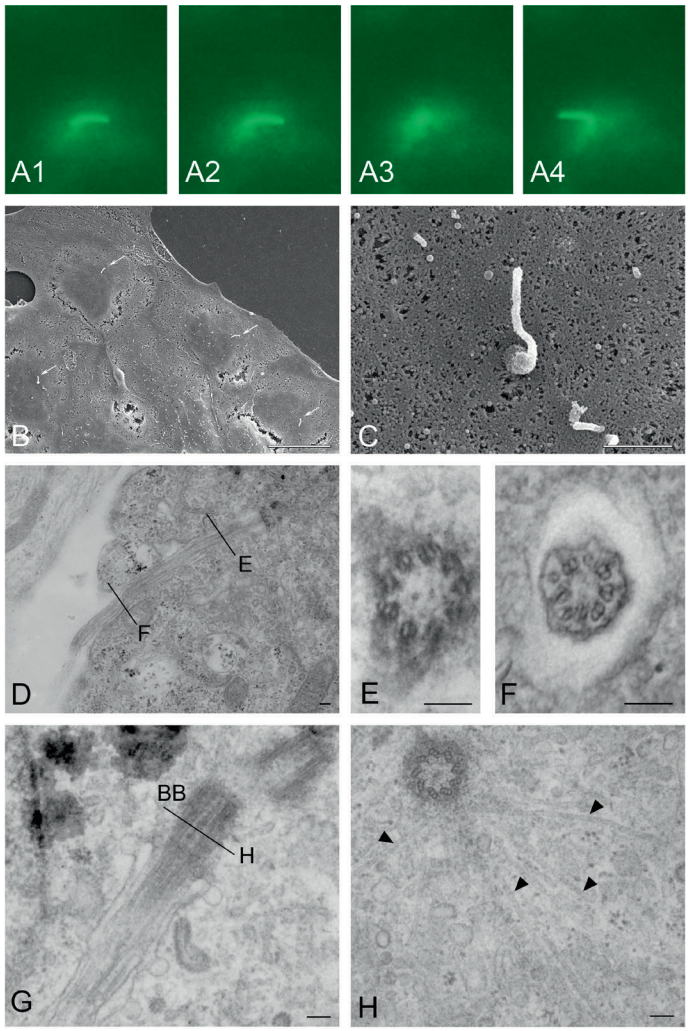

Figure 7.1. **Structural characteristics of the endothelial primary cilium.** Panels A1 to A4 show fluorescent micrographs of a time series of ECs which are stably transfected with a tubulin-eGFP fusion construct and are exposed to oscillatory flow. Note that the fluorescent fusion protein localizes to the cilium which bends from right to left into the flow. Panels B and C show field emission scanning electron micrographs of primary cultures of chicken embryonic endothelial cells. The arrows (B) point to the primary cilia which show a typical perinuclear localization. Transmission electron micrographs (D–H) show longitudinal (D,G) and cross sections (E,F,H) through the cilium. Endothelial cilia are 1–5 µm in length and 200 nm in diameter. They protrude from the surface (A–D), have a 9+0 configuration of microtubules in their core (E,F), and are connected through the basal body (BB, G) to the microtubular cytoskeleton (arrowheads in H). Scale bars: 10 µm (B), 1 µm (C), 100nm (D-H).

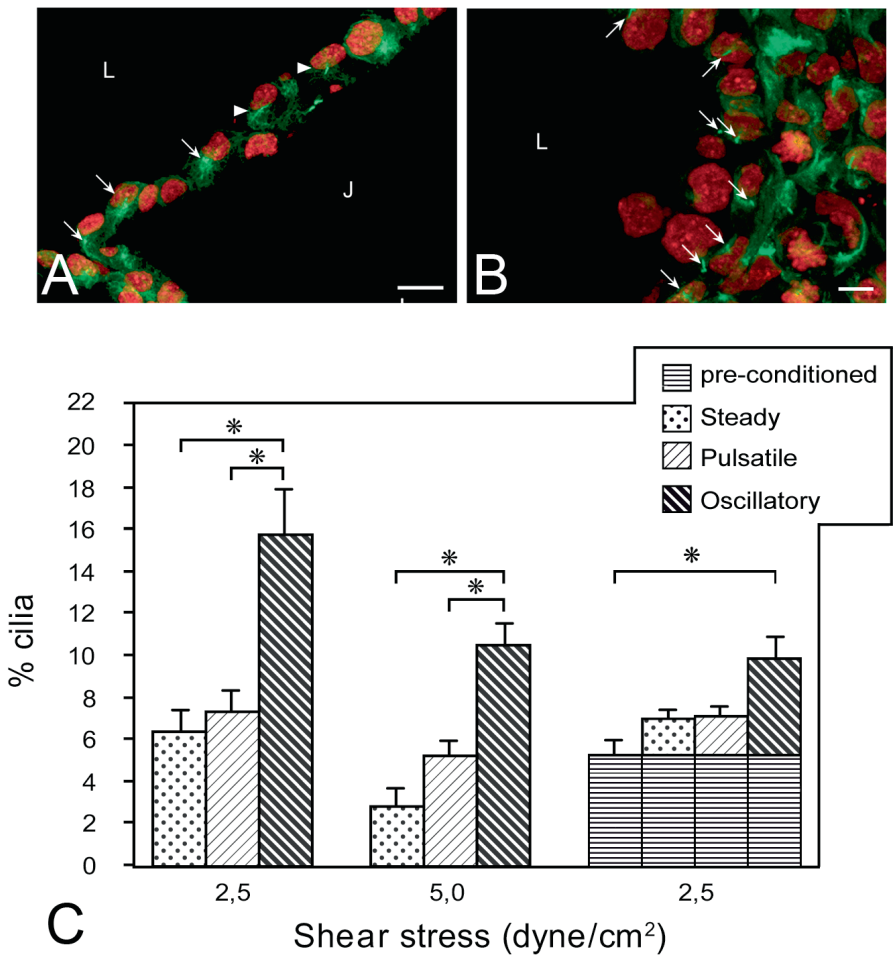

Figure 7.2. **Endothelial cilia are induced by oscillatory flow.** Confocal Laser Scanning Micrographs (A,B) of luminal (arrows) and abluminal (arrowheads) cilia on ECs of a stage HH17 chicken embryo. Panel A shows a section through the ventricle and panel B one through the aorta. Note that cilia project into the lumen (L) as well as into the basement membrane/cardiac jelly (J) and that ciliated hematopoietic stem cells are located at the ventral side in the lumen of the aorta (panel B). Panel C shows the percentage of ciliated primary ECs after exposure to various flow patterns. Cells were exposed to a maximum shear stress level of 2.5 or 5.0 dyne/ cm^2 (0.25 or 0.5 Pa, respectively) for 5 hours, with or without a preconditioning period of 2 hours at 25dyne/cm^2 (2.5 Pa) in order to deprive the cells of cilia. Note that significantly more ciliated cells are present under oscillatory flow conditions, regardless of the level of shear stress, and that this flow pattern induces the presence of primary cilia on ECs after preconditioning. The asterisks indicates $p<0.05$ and power≥80%. Error bars represent SEM values. Acetylated α-tubulin (green; FITC), nuclei (red; propidium iodide). Scale bar:10 μm.

ECs are highly responsive to (changes in) blood flow. In fact, they are able to sense the friction force or drag which is exerted on the cells of the vessel wall by the blood flow. This force is called shear stress and it depends on the viscosity and velocity profile of local blood flow. The unit of shear stress is Pascal (Pa) although it is often represented in dyne/cm^2 (10 dyne/cm^2=1 Pa). Blood flow and shear stress play decisive roles in e.g. the development of the cardiovascular system[17,18]. Rapidly changing geometries are intricately related to changes in shear stress patterning. High shear stress usually between 1.5 and 5 Pa renders human ECs anti-thrombotic, anti-proliferative, and anti-inflammatory[19]. This quiescent state is largely coordinated through the family of Krüppel-like factor transcription factors (KLFs). Expression levels of *KLF2* and *KLF4* are induced by shear stress, resulting in the activation or repression of a large spectrum of signaling pathways, and in ''healthy'' ECs[20-22]. On the other hand, low and oscillating flow, often called disturbed flow, fails to induce *KLF2* expression and leads to activation of an inflammatory program in ECs[23,24]. In human arteries these flow profiles are found on valves, on the inner side of curved vessels, and at branch points which are typical predilection sites for atherosclerosis. The relation between the presence of primary cilia on ECs and the expression of *Klf2* is complex. Ciliated ECs show a stronger induction of *Klf2* in response, to shear stress, compared to non-ciliated ECs[12,25]. On the other hand, exposure to high shear stress results in the internalization of cilia in ECs[8,13,25]. This appears to be a feedback mechanism the consequences of which are yet unclear. Figure 7.2C shows that the presence of primary cilia on ECs is related to oscillatory flow with cyclic flow reversals, regardless of the level of shear stress. In fact, upon de-ciliation of ECs by high levels of flow, oscillatory flow demonstrated to be the only flow pattern which was able to cause ECs to regain their cilia. This demonstrates that ciliation of ECs depends on flow pattern rather than flow level, and that flow reversals induce ciliation. It is likely to represent a ''rescue'' mechanism to prevent endothelial activation and transdifferentiation in areas of oscillatory flow, since endothelial ciliation coincides with increased Klf2 signaling and subsequent stabilization of the endothelial phenotype.

Although the cilium of ECs is involved in the intracellular calcium transient[11] and in the regulation of *Klf2* gene expression (Figure 7.3), these two mechanisms appear to be independent from each other[4,26]. When the calcium transient is prevented fluid flow still induces the expression of *KLF2* in ciliated ECs (Figure 7.3A). On the other hand, induction of the calcium transient, without exposure to fluid flow, does not induce expression of *KLF2*. An explanation for this phenomenon can most probably be found in the timing of these responses. The calcium transient can be observed within seconds from the initiation of fluid flow, and leads to activation of endothelial Nitric Oxide Synthase (eNOS/NOS3) which converts L-arginine into NO. Nitric oxide then diffuses into the vessel wall and induces relaxation of the vascular smooth muscle cells which leads to vasodilatation. This, in turn, causes an immediate decrease in shear stress. Induction of KLF2 expression takes the ECs at least 1–2 hours. Although KLF2 induces the expression of the *eNOS* gene and thereby increases the dilatation potential of the vessel wall, this is rather considered to be an adaptive response to e.g. hypertension.

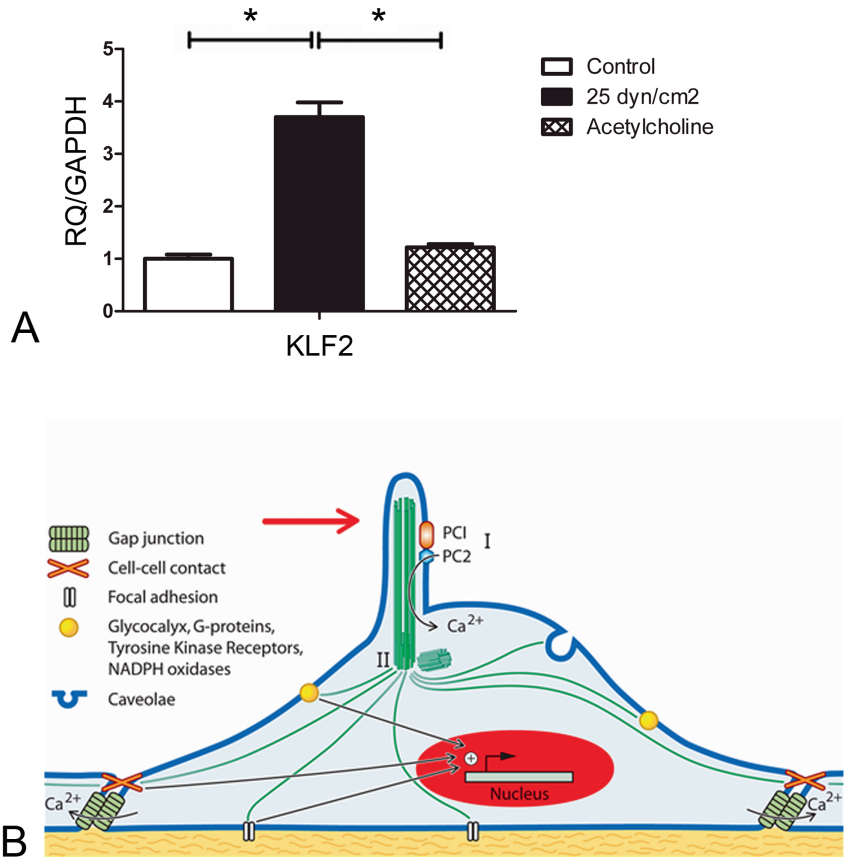

Figure 7.3. **Flow induced calcium transients and gene expression are independent.** Ciliated primary chicken ECs were exposed for 5 hours to 25 dyne/cm^2 (2.5 Pa) shear stress in calcium-free medium which prevents the flow-induced intracellular calcium transient, or to 1µm acetylcholine (Ach) which induces a calcium transient without a flow trigger (panel A). Q-PCR shows exclusive induction of *KLF2* in ECs which were exposed to fluid flow. Induction of calcium transient without flow did not result in activation of *KLF2* transcription. Panel B shows a schematic representation of the immediate response of ECs to fluid flow (i.e., intracellular calcium transient, I), and their prolonged response (i.e., induction of gene expression, II). Both depend on the presence of a primary cilium, but they represent independent signaling pathways. Panel B is reprinted with permission from[4]. The asterisks indicates $p<0.05$ and power≥80%. Error bars represent SEM values.

As mentioned above, the distribution of ciliated ECs in the cardiovascular system depends on local blood flow patterns. In adult mice ciliated ECs are typically found on the aortic side of the semilunar valves, in the inner curvature of the aorta, and at the branch points of e.g. the common, carotid, and subclavian arteries[10,27,28]. These are all predilection sites for the development of atherosclerosis. In fact, in *ApoE*$^{-/-}$ mice with well-developed atherosclerotic plaques the number of ciliated ECs was increased, probably due to an increase in plaque-induced flow disturbance[10]. ECs in the high-shear

areas, like in the outer curve of the aorta, were devoid of primary cilia. The developing heart also shows intricate blood flow and shear stress patterning which is instrumental for proper embryonic development[17]. Already in these very early stages of development a similar relation between blood flow and endothelial ciliation can be found[13,29]. Ciliated ECs are present in areas of oscillatory flow, like in the atrium and on the ventricular trabeculations, whereas ECs which are exposed to high shear stress do not bear cilia. It is yet unclear which cellular mechanism is involved in this process. Among other mechanisms activation of Protein Kinase A signaling has been suggested in other epithelial cells[30-33].

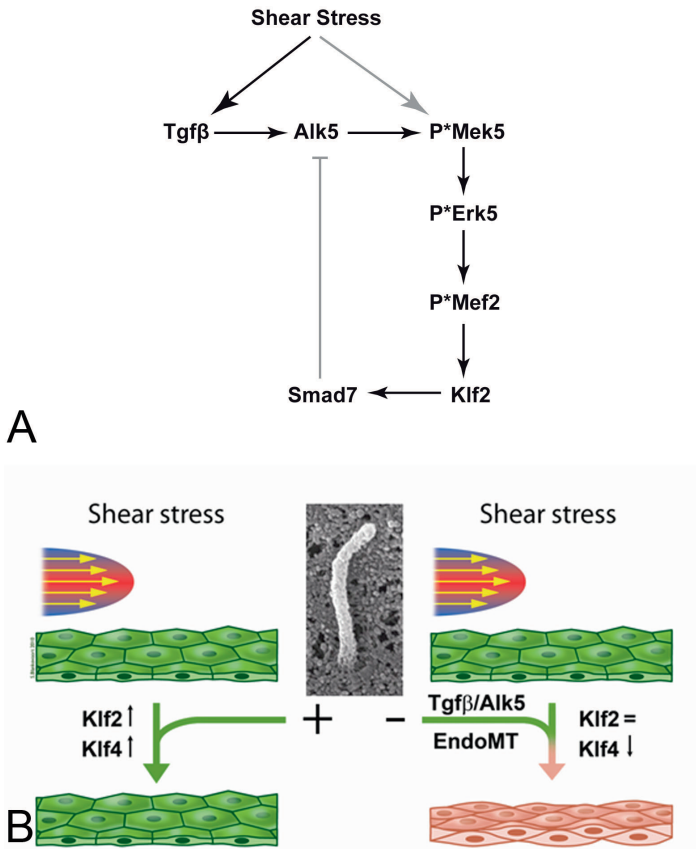

Figure 7.4. Shear stress activates Tgfβ signaling and EndoMT in embryonic ECs. Panel A shows the mechanism by which shear stress induces *Klf2* expression in embryonic ECs (black arrows). This is distinct from the mechanism in mature ECs (grey arrows), which bypasses Tgfβ/Alk5 and results in inactivation of Tgfβ signaling through Smad7. This figure is a schematic representation of data from[43]. Panel B is reprinted with permission[25], and schematically shows the differentiating role which the primary cilium has on the response of ECs to shear stress. In the presence of cilia shear stress induces the expression of *Klf2* and *Klf4*, which results in a retention of the endothelial phenotype. In the absence of primary cilia shear stress does not induce *Klf2*, represses the expression of *Klf4* and causes EndoMT in a Tgfβ/Alk5 dependent manner.

High shear stress and absence of ciliated ECs coincide in the area of the endocardial cushions in the embryonic heart. These cushions consist of extracellular matrix or cardiac jelly and are situated between the myocardial and endothelial layers in the atrioventricular canal and in the outflow tract. They form the anlagen for the tricuspid and mitral valves, and for the semilunar valves, respectively. Shear stresses can exceed 5 Pa in these areas, even in chicken embryos of around 2 days of development[34]. For proper development into functional valves, these cushions need to cellularize. Although cells from other sources like cardiac neural crest cells[35-37] and epicardial-derived cells[38] enter the cushions, the first wave of cells delaminates fromthe endothelial layer in a process called endothelial-to-mesenchymal transition (EndoMT)[39]. This is one of the most studied processes in heart development and has been shown to be depen- dent on the bioavailability of active members of the Transforming Growth Factor β (Tgfβ) family of proteins[40]. This family consists of, e.g., Tgfβ1-3, Bone Morphogenetic Proteins, and Activins. During EndoMT these growth factors bind to their receptors on ECs which results in a loss of endothelial phenotype and delamination into the cushion extracellular matrix. A role for shear stress in the activation of TGFβ signaling has been suggested[41], but debated by others[42]. Boon and colleagues[42] described that in HUVECs induction of *KLF2* by shear stress caused the induction of SMAD7, which subsequently inactivated TGFβ signaling. Recently, we confirmed these results in HUVECs and human aortic ECs, but also showed that this relation is distinct in embryonic ECs[43]. In these cells shear stress activates Tgfβ signaling through the Tgfβ Activin-like kinase-5 (Alk5), which in turn induces the expression of *Klf2* through activation of MAPK signaling (Figure 7.4A). Klf2 activates expression of Smad7, but this does not result in the inactivation of this signaling pathway. Both Tgfβ1 and Tgfβ3 ligand expression was induced by shear stress[43], which suggests autocrine activation of Alk5. However, rapid phosphorylation and nuclear translocation of Smad2 shows that other cell autonomous mechanisms might be involved[43]. Shear stress- dependent activation of TGFβ signaling was also demonstrated *in vivo*. Ligation of the right lateral viteline vein in a chicken embryo results in a local increase in shear stress which is accompanied by induction of *KLF2* expression[44] and in activation of Alk5 signaling (Smad2 phosphorylation) in the ECs covering the cushions[43]. By this mechanism high shear stress in the cushion area of the heart could be the driving force for cushion EndoMT through activation of Tgfβ signaling. We also showed that the absence of a cilium on the cushion ECs is a prerequisite for EndoMT[25]. Shear stress failed to initiate mesenchymal transition in ciliated ECs *in vitro*, whereas it induced EndoMT in non-ciliated ECs. An exception to that rule was exposure of ciliated cells to high levels of unidirectional shear stress (2.5 Pa). EndoMT was induced in this flow regime in a Tgfβ-dependent dependent manner[25]. However, these high shear levels also resulted in the deciliation of the ECs which rendered them sensitive for EndoMT, like the genetically modified unciliated ECs were at low levels of shear stress (0.5 Pa, see below). EndoMT was critically dependent on Tgfβ signaling, as it was prevented by inhibition of Alk5 kinase activity (Figure 7.4B). Interestingly, Klf4 which is a functional counterpart of Klf2 in ECs[22] was induced by shear stress in ciliated cells, but downregulated in non-ciliated cells[25]. Flow independent overexpression of Klf4 prevented EndoMT by shear stress. This adds to a pivotal role for Klf transcription factors in the transduction of mechanical stimuli into a cellular response.

In addition, rescue of the mutated gene for Intraflagellar Transport protein-88 (Ift88/Polaris) in the non-ciliated *Tg737*$^{orpk/orpk}$ ECs re-established the primary cilium and prevented flow-induced EndoMT. Although shear stress and the absence of primary cilia on the cushion ECs are instrumental for proper EndoMT other yet unknown factors modulate this process *in vivo*. In the *Tg737*$^{orpk/orpk}$ mouse model Tgfβ signaling activation is enhanced in the ECs which would be ciliated in wildtype mice, but other factors prevent excessive EndoMT in these areas[25]. Whether EndoMT plays a role in the development or progression of athero- sclerosis is unclear. Especially in neointima formation, the earliest onset of atherogenesis, Tgfβ, and Klf4 signaling and EndoMT could play a role[25,45]. Further plaque progression also involves homing of blood borne cells into the lesions. It is tempting to suggest a role for endothelial cilia in inducing an anti-atherogenic program at sites of disturbed flow through increasing Klf2 signaling, but strong experimental evidence lacks to date.

The mechanosensory role of primary cilia on ECs has been well established. Cilia modify the response to biomechanical forces. Of these forces shear stress is most important for ECs. However, the function of cilia goes beyond ‘‘just’’ being a mechanosensor. Cilia have prime roles in intracellular, and perhaps even intercellular, signaling. This puts forward possible functions for ECs in cilio-pathies or ciliopathy-related phenotypes. An example is hypertension from which many patients with cystic kidney diseases suffer[16]. Increased blood pressures in these patients can be primarily caused by cyst-related kidney failure, or could perhaps be related to endothelial dysfunction. Though small, cilia will no longer be overlooked in vascular biology.

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