Lack of Primary Cilia Primes Shear-Induced Endothelial-to-Mesenchymal Transition

Anastasia D. Egorova, Padmini P.S.J. Khedoe, Marie-José T.H. Goumans, Bradley K. Yoder, Surya M. Nauli, Peter ten Dijke, Robert E. Poelmann, Beerend P. Hierck

Rationale: Primary cilia are cellular protrusions that serve as mechanosensors for fluid flow. In endothelial cells (ECs), they function by transducing local blood flow information into functional responses, such as nitric oxide production and initiation of gene expression. Cilia are present on ECs in areas of low or disturbed flow and absent in areas of high flow. In the embryonic heart, high-flow regime applies to the endocardial cushion area, and the absence of cilia here coincides with the process of endothelial-to-mesenchymal transition (EndoMT).

Objective: In this study, we investigated the role of the primary cilium in defining the responses of ECs to fluid shear stress and in EndoMT.

Methods and Results: Nonciliated mouse embryonic ECs with a mutation in Tg737/Ift88 were used to compare the response to fluid shear stress to that of ciliated ECs. In vitro, nonciliated ECs undergo shear-induced EndoMT, which is accompanied by downregulation of Klf4. This Tgfβ/Alk5-dependent transformation is prevented by blocking Tgfβ signaling, overexpression of Klf4, or rescue of the primary cilium. In the hearts of Tg737/orpk embryos, Tgfβ/Alk5 signaling was activated in areas in which ECs would normally be ciliated but now lack cilia because of the mutation. In these areas, ECs show increased Smad2 phosphorylation and expression of α-smooth muscle actin.

Conclusions: This study demonstrates the central role of primary cilia in rendering ECs prone to shear-induced activation of Tgfβ/Alk5 signaling and EndoMT and thereby provides a functional link between primary cilia and flow-related endothelial performance. (Circ Res. 2011;108:1093-1101.)

Key Words: cilia ■ shear stress ■ endothelial cells ■ Tg737/orpk ■ EndoMT ■ Tgfβ/Alk5 ■ Klf4

Cilia are specialized membrane covered rod-like organelles protruding from virtually all mammalian cells. Primary cilia are typically present as solitary cellular extensions and are generally immotile, with the exception of cilia on the primitive node of the vertebrate embryo. Interest in the (dys)function of primary cilia arose because of their role in olfaction, photoreception, chemosensation, and mechanosensation; their association with a number of human ciliopathies; and their function as cellular sensory antennae. Biological effects include coordination of cell proliferation and differentiation. Ciliary structure, assembly, and maintenance is dependent on microtubule-based motor transport of axoneme subunits from the body of the cell into ciliary tips using a bidirectional process termed intraflagellar transport (IFT). Mutations in many of the IFT components lead to defective ciliogenesis and are associated with a range of human pathologies, such as Orofaciodigital, Bardet-Biedl, Usher, Senior-Loken, and Jeune syndromes. These syndromes can include laterality defects, vestibular impairment, and polycystic kidney disease.

Endothelial cells lining the heart and blood vessels are constantly exposed to hemodynamic forces, of which shear stress represents the drag force on the endothelium exerted by blood flow. The differential response of endothelial cells (ECs) to constantly varying flow patterns and velocities requires accurate mechanosensing and mechanotransduction. Primary cilia can sense shear stresses as low as 0.0007 Pa and they function as flow sensors in kidney epithelium, bone matrix lacunae osteocytes and osteoblasts, bile duct epithelium, and vascular endothelium. The function of primary cilia in endothelial cell sensing appears to be biphasic. Fluid flow and the associated ciliary deformation lead to a polycystin-mediated intracellular Ca2+ transient within seconds. Furthermore, the cilium is considered to amplify the...
cytoskeletal strain, resulting in a prolonged effect on gene expression of shear-responsive genes, including Krüppel-like factor (KLF)2,10,12 which further coordinates a major part of the phenotypic response of ECs to shear forces.

KLF2 and KLF4 are shear-sensitive transcription factors that have been described to coordinate the regulation of endothelial function and the establishment of a quiescent, antiinflammatory, and antithrombotic phenotype.13–16 Recent studies point toward a significant degree of mechanistic and functional overlap between KLF2 and KLF4 in ECs.16 Shear stress–induced expression of KLF2 has been shown to be related to the presence of primary cilia,9,10 and recent work suggests that cells with abnormal ciliary function or structure are likely to fail to respond to fluid shear stress appropriately.9

Primary cilia are present on ECs during embryonic17,18 and adult19 life, and their distribution has been described to be spatiotemporally linked to shear stress patterns in vivo. In adult vasculature, primary cilia are located at atherosclerotic predilection sites where flow is low and oscillatory.19 Moreover, ECs in areas where shear stress is high are devoid of cilia.19 In the embryonic heart, the presence of ciliated ECs is restricted to areas of low and oscillatory flow, marked by low levels of KLF2.17,20,21 The endocardial cushions are exposed to high shear stress22 and show high expression of KLF2 in the ECs.20 Interestingly, these ECs are nonciliated17 and undergo transforming growth factor (Tgfβ)-driven endothelial-to-mesenchymal transition (EndoMT), during which ECs transdifferentiate to gain a mesenchymal phenotype and migrate into the cardiac jelly to form the primordia of cardiac valves.23 EndoMT is marked by activation of the Tgfβ type I receptor Alk5,24,25 induction and activation of the transcription factor Snail (Snai1), loss of EC markers like CD31, and gain of expression of mesenchymal markers including α-smooth muscle actin (αSMA) and N-cadherin.26,27

In this study, we used transgenic embryonic ECs from the IFT88Tg737RPW (or Tg737orpk/orpk) mouse, which lack primary cilia.9 These were used to investigate the role of the primary cilium in the response of ECs to fluid shear stress and Tgfβ and in EndoMT. Shear stress triggered EndoMT in nonciliated ECs, but not in ciliated cells, a process that depended on Tgfβ signaling and Klf4 regulation. Furthermore, we analyzed the hearts of wild-type (WT) and Tg737orpk/orpk embryos to address the consequences of the lack of endothelial cilia in vivo. ECs lining areas of low shear stress showed enhanced Tgfβ/Alk5 signaling activation and increased expression of the mesenchymal marker αSMA, suggesting that the lack of cilia primes the ECs for shear-induced activation of Tgfβ signaling and EndoMT.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org. It includes a detailed description of the culture and use of the ECs; shear stress experiments; Tgfβ stimulation; the use of Tgfβ neutralizing antibody and Alk5 kinase inhibitor compound SB431542; generation and use of constructs in transfection experiments; immunofluorescence studies; quantitative polymerase chain reaction (Q-PCR) for a set of shear and EndoMT markers; Western blotting for CD31, αSMA, Klf4, P*Smad2, and Ift88; immunohistochemical analysis of Tg737orpk/orpk embryos; and statistical analysis.

Results

Shear Stress Induces EndoMT in Nonciliated ECs

As demonstrated by immunofluorescent staining for acetylated α-tubulin, WT ECs present with primary cilia, whereas Tg737orpk/orpk ECs do not (Figure 1A and IB). To study the effect of cilia on the response to fluid flow, WT and Tg737orpk/orpk ECs were exposed to 0.5 Pa shear stress for 24 hours. Whereas the WT ECs retained their cobblestone morphology (compare Figure 1C with 1E), nonciliated Tg737orpk/orpk ECs acquired an elongated, fibroblast-like phenotype, randomly oriented with respect to the direction of flow (compare Figure 1D with 1F). Their morphology resembled that of cells in which EndoMT was induced under static conditions with Tgfβ (Online Figure I, A). The ciliation phenotype of the ECs did not affect their capacity to undergo Tgfβ ligand–induced EndoMT, because both cell lines showed identical morphological changes. Tgfβ ligand–induced transformation of ciliated WT ECs under static conditions was characterized by loss of CD31 expression and induction of mesenchymal markers αSMA, P*Smad2, and Ncad (Online Figure I, B). Figure 2 summarizes the effects of shear on Tg737orpk/orpk cells. Shear-induced EndoMT was characterized by the loss of CD31 and gain of αSMA (Figure 2G). Q-PCR analysis confirmed this on the mRNA level and showed that CD31 was downregulated by a factor of 2 and αSMA was induced 26-fold under shear stress, compared to the static controls (Figure 2A and 2B). P*Smad2, which is a downstream activation marker of the type I Tgfβ receptor Alk5, was induced 56-fold under shear (Figure 2C). Snai1, a downstream target of Tgfβ and a marker for EndoMT, and Ncad, a transmembrane protein characteristic for mesenchymal cells, were both significantly induced by shear stress in Tg737orpk/orpk cells (Figure 2D and 2E). In comparison,
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Figure II, B).

and Snai1 concomitant response to fluid flow, WT and Tg737
magnitude of shear on ciliation phenotype of ECs and their
these conditions (Online Figure II, A). To study the effect of
Indeed, the majority of WT cells were still ciliated under

Role of Cilia in Defining the Klf2/Klf4 Response to Shear Stress
The specific response of WT and Tg737^ortionpkECs to shear
stress was investigated by analyzing the expression levels of
Klf2 and Klf4 after exposure to 0.5 Pa shear for 24 hours. WT
ECs showed a 2.3- and 1.8-fold induction of Klf2 and Klf4,
respectively, whereas Tg737^portionpk cells failed to induce
Klf2 (Figure 3A and 3B). Remarkably, the latter cells showed
a marked downregulation of Klf4 by more than 75% under
shear stress (Figure 3B). In Tg737^portionpk cells, shear stress–
related upregulation of Klf2 was restored and downregulation of
Klf4 was prevented when Tgfβ signaling was inhibited with
the SB compound or in the presence of a-Tgfβ (Figure 3A and 3B).
The responses of Klf4 mRNA were accurately reflected at the protein level (Figure 3C).

Klf4 Overexpression Prevents Shear Stress–Induced EndoMT
To investigate whether shear-induced EndoMT in Tg737^portionpk cells was a result of the marked downregulation of Klf4 or is rather the cause of it, Klf4 was transiently overexpressed (Tg737^portionpk-Klf4) and transfected cells were exposed to 0.5 Pa shear stress for 24 hours. Tg737^portionpk with LacZ overexpression (Tg737^portionpk-LacZ) were used as a control. Tg737^portionpk-Klf4 showed a 10-fold overexpression of Klf4 mRNA, an effect that was confirmed at the protein level (Figure 4A and 4B). Under shear stress, Tg737^portionpk-Klf4 ECs retained their cobblestone morphology, CD31 expression, and did not undergo EndoMT (Figure 4C through 4E). Pail induction was reduced from 34- to 9-fold, Snai1 was induced only 1.9-fold, compared to 3.2-fold in the control group, and Ncad was not induced under flow (Figure 4D). Overexpression of Klf4 did not result in the reappearance of the cilium (not shown), demonstrating that prevention of flow induced EndoMT in Tg737^portionpk-Klf4 was not a secondary effect of ciliary rescue.

Rescue of Cilia Prevents Shear Stress–Induced Transdifferentiation of ECs
Tg737^portionpk ECs were stably transfected with Ift88-
mCherry cDNA to generate Tg737^portionpk-Ift88*. Figure 5A
ciliated WT ECs did not show altered CD31 expression under fluid flow; showed only a slight induction of aSMA, Pail1, and Snai1; and did not induce Ncad (Figure 2A through 2E).

Indeed, the majority of WT cells were still ciliated under these conditions (Online Figure II, A). To study the effect of magnitude of shear on ciliation phenotype of ECs and their concomitant response to fluid flow, WT and Tg737^portionpk ECs were exposed to 2.5 Pa shear stress for 24 hours. WT ECs became nonciliated and underwent EndoMT to that observed in Tg737^portionpk cells (Online Figure II, A). These morphological changes were reflected by expression changes of CD31, aSMA, Pail1, Snai1, and Ncad (Online Figure II, B).

EndoMT Is Tgfβ/Alk5 Kinase–Dependent
The role of Tgfβ/Alk5 signaling in shear stress–induced EndoMT was investigated using flow in the presence of either a pan-Tgfβ neutralizing antibody (a-Tgfβ) or the Alk5 kinase inhibitor compound SB431542. Depletion of Tgfβ ligand from the culture medium by binding to a-Tgfβ, as well as inhibiting Alk5 kinase activity, prevented EndoMT under shear stress in Tg737^portionpk ECs (Figure 2A through 2F and 2H). This was marked by the retention of cobblestone morphology (Figure 2H) and CD31 expression and by the lack of aSMA induction (not shown). After shear stress in the presence of a-Tgfβ, Pail1 induction was reduced from 56- to 18-fold, and expression of Snai1, aSMA, Ncad, and CD31 remained unchanged compared to the static controls (Figure 2A through 2E). The response to shear stress in the presence of the SB compound largely mimicked the response in the presence of a-Tgfβ. Pail1 induction under flow was reduced to 26-fold; Snai1, aSMA, and Ncad expression was no longer induced (Figure 2B through 2E). CD31 mRNA expression was induced under these conditions, but this was not reflected on protein level (Figure 2A and 2F). Tgfβ-induced Alk5 kinase activation, and induction of the downstream signaling pathways therefore plays a critical role in the shear stress response and in EndoMT.

Figure 1. Different responses to shear stress of ciliated and nonciliated ECs. A and B, Immunostaining for acetylated α-tubulin showing the presence of cilia in WT ECs and absence of cilia in Tg737^portionpk ECs. C and D, WT and Tg737^portionpk ECs show a cobblestone morphology under static conditions. E and F, WT ECs retain their phenotype under flow, whereas Tg737^portionpk ECs undergo EndoMT. Arrows indicate the direction of flow. Scale bars: 35 μm (A and B); 25 μm (C through F).
and 5B shows that Ift88 mRNA and Ift88* protein levels in rescued cells were comparable to the Ift88 levels in WT ECs. Rescue of Ift88 in Tg737 orpk/orpk ECs led to a structural rescue of the cilia, with a normal distribution of one primary cilium per single cell (Figure 5C). To confirm functional rescue, Tg737 orpk/orpk–Ift88* cells were subjected to 0.5 Pa shear stress for 24 hours, a condition that induced EndoMT in nonrescued cells. Cells now retained their cobblestone morphology and did not undergo EndoMT (Figure 5D). Q-PCR analysis showed induction of Pai1 and αSMA similar in magnitude to that of the WT ECs under shear, without any changes in the expression levels of CD31, Snai1, and Ncad (Figure 5E). The table in Figure 5E shows the ratios of relative (to static controls) expression levels of genes of interest in Tg737 orpk/orpk–Ift88*, compared to WT ECs under shear. The expression profiles of these two cell types under shear stress are comparable and rescue of the cilia led to induction of Klf2 and lack of Klf4 downregulation under flow.
Nonciliated ECs Activate Tgfβ Signaling In Vivo

To assess the in vivo consequences of (non)ciliation of ECs for Tgfβ/Alk5 signaling activation, the hearts of embryonic day (E)11.5 WT and Tg737\textsuperscript{orpk/orpk} mouse embryos were analyzed for endothelial expression of phosphorylated Smad2 (P\textsuperscript{*Smad2}) and \(\alpha\)SMA (Figure 6). ECs of Tg737\textsuperscript{orpk/orpk} embryos lining areas of low shear (ie, in the atria, on the ventricular septum, and trabeculations) show increased expression of P\textsuperscript{*Smad2} compared to their WT littermates, pointing toward enhanced Tgfβ/Alk5 activation. Tg737\textsuperscript{orpk/orpk} embryos also have \(\alpha\)SMA-positive cells in these areas, whereas all ECs in the WT embryos were negative for \(\alpha\)SMA. Moreover, the subendocardial space of the ventricular septum and trabeculations is increased in the mutant embryos, suggesting increased extracellular matrix production and deposition. Western blot analysis of P\textsuperscript{*Smad2} levels in WT and Tg737\textsuperscript{orpk/orpk} ECs after exposure to 2 hours of shear stress showed an increase of P\textsuperscript{*Smad2} levels by 30% in the WT ECs, compared to a 2.2-fold increase in the Tg737\textsuperscript{orpk/orpk} cells, confirming enhanced P\textsuperscript{*Smad2} signaling in nonciliated cells (Online Figure III).

Discussion

In this study, we used ECs deficient in functional Ift88 protein\textsuperscript{28} and thus lacking the ability to form cilia. They provide a robust model to analyze the functional role of primary cilia in response to fluid flow. Although the effects of ciliary dysfunction on kidney epithelial cells and their response to flow has been the subject of numerous studies, the effects of dysfunctional primary cilia on ECs remain largely unknown. In vivo EC primary cilia are present in areas of low and disturbed shear stress,\textsuperscript{17,19} where they have been suggested to play an important role in mechanosensing.\textsuperscript{10} In our model, ciliated ECs retained an endothelial phenotype in response to 0.5 Pa shear stress in vitro, whereas nonciliated Tg737\textsuperscript{orpk/orpk} ECs underwent EndoMT and adopted a fibroblast-like phenotype. When exposed to higher shear levels, which resulted in the deciliation of WT ECs, these cells also underwent EndoMT and gained a phenotype that closely resembled that of Tg737\textsuperscript{orpk/orpk} ECs under flow. It is therefore plausible that WT ECs disassemble their cilia under high laminar shear stress, as has previously been reported for chicken and human ECs,\textsuperscript{17,29} priming the cells for shear-induced EndoMT. In this respect, shear-induced EndoMT of nonciliated cells should be clearly distinguished from flow-induced endothelial alignment, which has been described extensively. Here, the direction of cellular elongation was random with respect to the direction of flow, and the changes in phenotype were accompanied by the loss of endothelial markers and the gain of mesenchymal and transition markers. ECs of various origins have been demonstrated to undergo EndoMT in vitro when exposed to Tgfβ.\textsuperscript{26} We confirmed these findings in the WT and Tg737\textsuperscript{orpk/orpk} ECs and identified Tgfβ/Alk5 kinase activity to be essential in shear stress–mediated EndoMT. Because treatment with either Tgfβ\textsuperscript{26} blocking antibodies or Alk5 kinase inhibitor prevented shear-induced transformation, an autocrine mechanism by which flow activates Tgfβ production or promotes its bioavailability, which, in turn, activates signaling by binding to its receptors on ECs, appears feasible. However, this requires further confirmation.

A typical response of endothelial cells to shear stress is upregulation of the zinc finger transcription factor KLF2. In vivo, expression of KLF2 in the embryonic and adult cardiovascular system is confined to areas of high shear stress.\textsuperscript{20,30} In vitro, KLF2 is induced by flow\textsuperscript{21,31} and acts as a regulator of endothelial function through regulation of multiple
Here, we confirm the shear-dependent upregulation of Klf2 in ciliated ECs but also show that the absence of cilia in Tg737\textsuperscript{orpk/orpk} cells prevents this induction. This is in line with previous results showing attenuation of KLF2 induction in deciliated chicken ECs.\textsuperscript{10} Like KLF2, KLF4 has been reported to be expressed in ECs in a shear-dependent manner.\textsuperscript{32} Ciliated ECs show a similar response in our experimental setting. Nonciliated cells, however, show a dramatic downregulation of this transcription factor. We show that this downregulation of Klf4 not only coincides with EndoMT but is, in fact, required for this transition. Preventing downregulation by artificial overexpression of Klf4 or by blocking Tgfβ/H9252 signaling also prevents shear stress–induced EndoMT, as demonstrated by the retention of CD31 expression and a cobblestone phenotype, and by the lack of induction of transition markers like αSMA, Pai1, Snail, and Ncad. This is in line with studies that show that Klf4 potently represses the expression of smooth muscle differentiation genes.\textsuperscript{14,30} 

![Figure 4: Klf4 overexpression prevents shear stress–induced EndoMT. A and B, Q-PCR and Western blot analysis, respectively, showing Klf4 overexpression in Tg737\textsuperscript{orpk/orpk} ECs that were transfected with either LacZ (Tg737\textsuperscript{orpk/orpk}-LacZ, sham) or Klf4 (Tg737\textsuperscript{orpk/orpk}-Klf4) expression constructs. Transfection with Klf4 results in a 10-fold overexpression on mRNA level and a 2.2-fold overexpression on protein level. C, Images of Tg737\textsuperscript{orpk/orpk}-LacZ and Tg737\textsuperscript{orpk/orpk}-Klf4 cells under static conditions and under 0.5 Pa shear stress. Arrows indicate the direction of flow. Scale bars: 25 μm. D, Q-PCR showing relative mRNA expression of CD31, αSMA, Pai1, Snail1, and Ncad in Tg737\textsuperscript{orpk/orpk}-LacZ and Tg737\textsuperscript{orpk/orpk}-Klf4 cells under 0.5 Pa shear stress. Expression is normalized to GAPDH and relative to static shams, as represented by the dashed line. E, Western blot analysis and quantification of CD31 protein in Tg737\textsuperscript{orpk/orpk}-LacZ and Tg737\textsuperscript{orpk/orpk}-Klf4 cells under static conditions and 0.5 Pa shear stress.](http://circres.ahajournals.org/doi/10.1161/CIRCRESAHA.111.243681)

![Figure 5: Rescue of Ift88 leads to functional rescue of the cilia and prevents shear stress–induced EndoMT in Tg737\textsuperscript{orpk/orpk} ECs. A and B, Q-PCR and Western blot analysis, respectively, showing Ift88 levels in WT and Tg737\textsuperscript{orpk/orpk} stably transfected with pEGFP-N1 (Tg737\textsuperscript{orpk/orpk}-eGFP, sham) and Ift88-mCherry/Ift88* (Tg737\textsuperscript{orpk/orpk}-Ift88*) expression constructs. Transfection with Ift88* results in a 2-fold increase in mRNA level of Ift88* in Tg737\textsuperscript{orpk/orpk} ECs and normalizes protein levels to those of Ift88 in WT ECs. C, Confocal images with optical cross section of Tg737\textsuperscript{orpk/orpk} cells, immunostained for acetylated tubulin, showing the presence of cilia in rescued Tg737\textsuperscript{orpk/orpk}-Ift88* ECs but not in nonrescued cells (inset). Arrowheads point toward primary cilia and scale bar represents 10 μm. D, Images of Tg737\textsuperscript{orpk/orpk}-Ift88* cells under static conditions and under 0.5 Pa shear stress. Arrows indicate the direction of flow and scale bars represent 25 μm. E, Q-PCR showing relative mRNA expression of CD31, αSMA, Pai1, Snail1, Ncad, Klf2, and Klf4 in Tg737\textsuperscript{orpk/orpk}-Ift88* cells under 0.5 Pa shear stress.](http://circres.ahajournals.org/doi/10.1161/CIRCRESAHA.111.243681)
markers, including αSMA, in vascular smooth muscle cells and is rapidly induced following vascular injury. Smooth muscle differentiation also largely depends on TGFβ signaling and probably involves a similar transition mechanism.

To ensure that the differences in response to shear stress and the process of EndoMT were specific to the lack of cilia on ECs, Ift88 was stably overexpressed in Tg737<sup>orpk/orpk</sup> cells. This resulted in the reappearance of primary cilia in a normal distribution pattern of one per cell. Tg737<sup>orpk/orpk</sup> ECs responded to shear stress in a manner similar to the WT ECs, reflected by the induction of Klf2, lack of downregulation of Klf4, and the retention of an endothelial phenotype under flow. This identifies the primary cilium as a necessary element to retain endothelial quiescence under low flow conditions. Together, this confirms the central role of primary cilia in defining a functional response to fluid flow in ECs, as is illustrated in Figure 7. In short, ciliated ECs retain their cobblestone morphology under shear stress, whereas nonciliated ECs undergo flow mediated EndoMT. This transition is preceded by the activation of TGFβ/ALK5 signaling and downregulation of transcription factor Klf4. Shear stress–induced EndoMT in nonciliated cells can be prevented in vitro by interfering with TGFβ signaling, by rescuing the cilium, or by induction of Klf4 expression.

Our in vivo data confirm the relation between the absence of primary cilia and activation of TGFβ signaling. In the hearts of Tg737<sup>orpk/orpk</sup> embryos, ECs that would normally be ciliated now showed increased Smad2 phosphorylation and αSMA expression, indicating activation of TGFβ signaling. This is supported by increased production of extracellular matrix, resulting in more cardiac jelly, as observed in the Tg737<sup>orpk/orpk</sup> embryos. However, these embryos did not show concomitant excessive EndoMT in the low-flow areas. Apparently, other factors, most probably locally secreted by the myocardium, inhibit the final transition into mesenchymal cells in these embryos. This is in line with previous studies that suggest an EndoMT-stimulatory environment, specifically in the atrioventricular canal and outflow tract of the embryonic heart, resulting from paracrine signaling. As a consequence of the high levels of shear stress, the ECs in these areas are already nonciliated during cushion development were identified. Whether cilia and altered flow–induced EndoMT play a role under pathological conditions in adult mutant mice, such as atherosclerosis or heart failure–related cardiac fibrosis, will need to be determined in future studies.

Appreciation for ciliary function in development and normal human physiology has led to reanalysis of a number of human syndromes that have previously been associated merely on the basis of similar clinical features. Several ciliopathies are characterized by gross cardiac anomalies, probably related to the failed orientation of heart looping along the left–right axis, corresponding to the high incidence of situs inversus in these patients. Here, we provide data to suggest that ECs might contribute to the phenotypes

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**Figure 6. Lack of cilia primes ECs for shear-induced activation of TGFβ signaling, in vivo.** Transverse sections through the hearts of WT (A, C, E, G, and I) and Tg737<sup>orpk/orpk</sup> (B, D, F, H, and J) mouse embryos of stage E11.5 comparing the expression of αSMA and phosphorylated Smad2 (pSmad2). **Boxed areas in A and B** are magnified in **C through J** in these or adjacent sections. αSMA-positive cells are observed in the endothelium of the ventricular septum and trabeculations of Tg737<sup>orpk/orpk</sup> embryos (compare C with D). The ECs of Tg737<sup>orpk/orpk</sup> embryos have higher expression of pSmad2 compared to WT littermates (compare E with F). The subendothelial space of the ventricular septum and trabeculations, representing cardiac jelly, is increased in the mutants (compare C and E with D and F). The atria of WT animals are lined by ECs negative for αSMA and pSmad2, whereas these cells stain positive for both markers in the Tg737<sup>orpk/orpk</sup> embryos (compared G and I with H and J). A indicates atrium; AVC, atrioventricular canal; V, ventricle; VS, ventricular septum. Scale bars: 100 µm (A and B); 50 µm (C through J).
observed in these syndromes; in which case, their defects in the cardiovascular system could be masked by the gross anomalies of disturbed left-right asymmetry. Studies have shown mice with mutations leading to complete loss of cilia or defective mechanosensation to have 100% penetrance of intracardiac defects, next to the prominent left–right patterning abnormalities. In contrast, only ~40% of mice with structurally normal, but immotile, cilia have intracardiac defects that are mostly associated with abnormal left–right development. These observations lead to the belief that cilia have a broader role in heart development than through their function in node cilia alone. Furthermore, endothelial dysfunction and increased carotid intima–media thickness has been reported with increased frequency in adult polycystic kidney disease patients, pointing toward a role for cilia in vascular function.

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Disclosures
None.

References


31. What Is Known?

- Primary cilia, which are rod-like extensions from the cell, serve on endothelial cells (ECs) to translate information about blood flow into cellular responses.
- The presence or absence of cilia on ECs is locally regulated by the blood flow profile, ie, ciliated cells are present in areas of low and disturbed blood flow.
- Endothelial-to-mesenchymal transition (EndoMT) is a process by which ECs that are exposed to high blood flow, eg, on the developing heart valves in an embryo, lose their endothelial characteristics and become mesenchymal or stromal cells.

32. What New Information Does This Article Contribute?

- Fluid-induced EndoMT is inhibited by the presence of a primary cilium.
- Induction of EndoMT by fluid flow in nonciliated cells depends on activation of the transforming growth factor (Tgfβ), and on regulation through transcription factor Krüppel-like factor (Klf4).
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SUPPLEMENTAL MATERIAL

Lack of primary cilia primes shear-induced Endothelial-to-Mesenchymal Transition

Surname first author: Egorova
Short title: Role of primary cilia in shear induced EndoMT

Anastasia D. Egorova¹ MSc, Padmini P.S.J. Khedoe¹ MSc, Marie-José T.H. Goumans² PhD, Bradley K. Yoder³ PhD, Surya M. Nauli⁴ PhD, Peter ten Dijke² PhD, Robert E. Poelmann¹ PhD, Beerend P. Hierck¹ PhD

¹Departments of Anatomy and Embryology, Leiden University Medical Center, The Netherlands; ²Molecular Cell Biology and Center for Biomedical Genetics, Leiden University Medical Center, The Netherlands; ³Department of Cell Biology, University of Alabama at Birmingham, Birmingham, Alabama, USA; ⁴Pharmacology and Medicine, College of Pharmacy and Medicine, University of Toledo, Ohio, OH, USA

Corresponding author:
Beerend P. Hierck, PhD
Dept. of Anatomy and Embryology, Leiden University Medical Center
P.O. Box 9600, Postzone S-1-P, 2300 RC Leiden, The Netherlands
tel: +31 71 5269309
fax: +31 71 5268289
email: B.P.Hierck@LUMC.nl

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METHODS

Cell culture and supplements
Generation of mouse embryonic wild type EC (WT) and mouse embryonic EC with a mutation in the Tg737 gene (IFT88Tg737RPW or Tg737orpk/orpk) from the Oak Ridge Polycystic Kidney mouse was previously described. Cells were passaged twice a week and maintained on 1% w/v gelatin (Merck, Darmstadt, Germany) in advanced DMEM medium (Invitrogen, Breda, the Netherlands) supplemented with 4.5 g/L D-glucose (Invitrogen), 110 mg/L sodium pyruvate (Invitrogen), non-essential amino acids (Invitrogen), 2% (v/v) heat inactivated Fetal Calf Serum (Sigma-Aldrich Chemie, Steinheim, Germany), 0.5% (v/v) antibiotic/antimyotic solution (Invitrogen), 1% (v/v) insulin, transferin, selenium supplement (Invitrogen), and 2mM L-glutamine (Invitrogen). For some of the experiments, the culture medium was supplemented with Tgfβ3 (1ng/ml), Tgfβ neutralizing antibody (α-Tgfβ, 2G7 IgG2b, 10ug/ml)2, Alk5 kinase inhibitor SB431542 (10μmol/L in DMSO; Tocris)3, or DMSO (Sigma).

Shear stress exposure
For the shear stress experiments, WT and Tg737orpk/orpk EC were seeded on fixed 1% (w/v) gelatin coated coverslips and grown to confluence. EC were subjected to 0.5 and 2.5 Pa shear stress for 2 or 24 hours at 37°C and 5% CO2 in a re-circulation parallel plate flow system as previously reported. Responses of shear-exposed cells were compared to those of static cultures. To block Alk5 kinase activity during exposure to shear stress, cells were pre-incubated with SB431542 for 1 hour and then exposed to shear stress in medium supplemented with the compound at the same concentration. 0.1% (v/v) DMSO (Sigma) served as a sham for the SB431542 experiments. To neutralize functional Tgfβ either present in the medium or produced by the cells during exposure to shear stress, cells were pre-incubated with a pan-Tgfβ neutralizing antibody for 1 hour prior to shear exposure in the presence of antibody in equal concentration. Static controls were treated identically with the omission of flow. Directly following incubation or exposure to flow, cells were fixed for immunofluorescence analysis (n=3) or lysed for RNA (n=4) or protein (n=3) isolation. Hoffman modulation contrast images were taken using Nikon Eclipse Ti inverted microscope system (20x and 40x objectives).

Constructs and transfection
The Ift88*/Tg737 expression construct encodes the Ift88 protein (90 kDa) and a 28kDa tag (mCherry, denoted by an asterisk (*)). Tg737orpk/orpk EC were transfected with Lipofectamine (Invitrogen) according to the manufacturer’s protocol. Stable cell lines were generated by drug selection using 500 μg/ml G418 (Invitrogen) to obtain Tg737orpk/orpk-Ift88*. Expression from the Ift88* construct was evaluated using Q-PCR and Western Blot analysis and compared to Tg737orpk/orpk cells which were stably transfected with pEGFP-N1 (Clontech R&D, Palo Alto, USA), which served as control.

Immunocytochemistry
Cells were fixed with 4% paraformaldehyde in 0.1mol/L phosphate buffer (pH7.4) for 10 min at room temperature. Permeabilized cells were incubated with antibody against acetylated α-tubulin (6-11B-1, 1:2000, Sigma) and αSMA (1A4, 1:1000, Sigma) for 3 hours at 37°C, followed by incubation with FITC-labeled rabbit-anti-mouse antiserum (1:100, DAKO). For the CD31
staining, cells were incubated overnight with PECAM-1 antibody (M-20, 1:100, Santa Cruz), followed by incubation with biotin-labeled swine-anti-rabbit antiserum (1:100, DAKO) and FITC conjugated avidin (1:100, Vector labs). DAPI (1:1000, Invitrogen) was used for nuclear counterstaining and cells were mounted in ProLong Gold (Molecular Probes). Confocal scanning was performed using a Leica SP5 confocal scanning laser microscope (63x and 100x oil immersion objectives). ImageJ and Photoshop 10.0 were used for processing the data.

**Q-PCR**
Total RNA was isolated (RNeasy, Qiagen) and was treated with DNase-I (Qiagen) according to the manufacturer’s protocol. IScript cDNA synthesis kit (Bio-Rad) was used to reverse transcribe 500ng of RNA into cDNA. Real-time Q-PCR was performed using iQ SYBR Green Supermix (Bio-rad) in a Mx3000 real-time thermocycler (Stratagene) as described. The reaction mixture consisted of the following: 1x PCR Master Mix, 1µl cDNA template, and 10 pmol of each specific primer. Gene specific primers are listed in Online Table I. The PCR program consisted of a hot start activation step, followed by 50 cycles of 30 seconds at 95°C, 60 seconds annealing at 58°C, and 30 seconds extension at 72°C. Dissociation analysis was performed in all reactions to exclude the presence of primer-dimers and confirm the amplification of unique targets. No-template controls were used as negative controls. Relative expression levels were normalized to the housekeeping gene GAPDH to compensate for the differences in RNA input.

**Western Blotting**
Western blot analysis was performed as described. Protein samples were separated on a 8% SDS polyacrylamide gel, transferred onto a nitrocellulose membrane (Hybond P; Amersham Pharmacia Biotech), and incubated overnight at 4°C with antibody against CD31 (M-20, 1:500, Santa Cruz), Klf4 (ab34814, 1:700, Abcam), Ift88 (Polaris B1700, 1:5000), phospho-Smad2 (138D4, 1:1000, Cell Signaling) and GAPDH (6C5, 1:5000, Millipore). The blots were then incubated with peroxidase-conjugated anti-rabbit (CD31, Klf4, Ift88, phospho-Smad2) and anti-mouse (GAPDH) secondary antibody (1:1000; GE Healthcare), and developed using the ECL Plus Western blotting detection system (Thermo Scientific). Signals were quantified using densitometry and normalized to GAPDH. Western Blots shown in the Figures are representative of 3 independent experiments.

**Mice**
The Oak Ridge Polycystic Kidney (Tg737 orpk/orpk) animals were generated by a transgene insertion mutagenesis as described previously and lines were maintained as heterozygous crosses on an inbred FVB/N genetic background. Animals were treated and maintained in accordance with the Institutional Animal Care and Use Committee regulations at the University of Alabama at Birmingham. Genotyping was performed as described previously. E11.5 embryos were fixed overnight in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH7.4) and routinely processed for paraffin immunohistochemical analysis. Specimens were sectioned transversely through the heart at 10µm.

**Immunohistochemistry**
After deparaffination and rehydration of the sections, microwave antigen retrieval was applied by heating the slides for 12 min to 98°C in citric acid buffer (0.01M in aqua-dest, pH6.0). Inhibition of endogenous peroxidase was performed after which the sections were incubated overnight with anti-phospho-Smad2 antibodies (138D4, 1:100 in PBS, Cell Signaling) and anti-αSMA antibodies (1A4, 1:3000 in 1%BSA/PBST, Sigma-Aldrich Chemie). Slides were rinsed in PBS and incubated with the secondary antibodies for 1 hour at room temperature: for phospho-Smad2 with biotin conjugated goat-anti-rabbit secondary antibodies (1:200 in PBS/1.5% NGS, Vector Laboratories) and for αSMA with horseradish peroxidase conjugated rabbit-anti-mouse antibodies.
(1:250 in 1%BSA/PBST, DAKO). Subsequently phospho-Smad2 stained slides were incubated with ABC-reagent (Vector Laboratories) for 45 min and all the slides were visualized with 400ug/ml 3-3’di-aminobenzidin tetrahydrochloride (DAB, Sigma-Aldrich Chemie). The slides were then dehydrated and mounted with Entellan (Merck). All the slides were processed simultaneously and Tg737<sup>orpk/orpk</sup> embryos (n=3) were compared with wild type littermates (n=4).

**Statistical Analysis**
For comparison of the means, independent experiments were performed and analyzed using SPSS 14.0 (SPSS Inc.). All results are expressed as mean ± SEM. Independent t-tests, including Levene’s analyses for equality of variances, were used to analyze differences between groups. Values of P < 0.05 and a power ≥ 0.80 were considered statistically significant and are marked by an asterisk (*) in the figures.
FIGURES AND FIGURE LEGENDS

Online Figure I. Tgfβ induced EndoMT in ciliated WT and non-ciliated Tg737<sup>orpk/orpk</sup> EC.
(A) Morphology of WT and Tg737<sup>orpk/orpk</sup> cells which were stimulated with Tgfβ ligand to induce EndoMT under static conditions. Note the transition from cobble-stone to fibroblast-like morphology upon stimulation with Tgfβ. Scale bars: 25 μm. (B) Relative mRNA expression of CD31, αSMA, Pai1, Snai1, and Ncad in WT EC upon stimulation with Tgfβ ligand. Expression is normalized to GAPDH and relative to shams, as represented by the dashed line.
Online Figure II. WT EC looses their cilia and undergo EndoMT after exposure to high flow.

(A) Morphology of WT (left and middle column) and Tg737<sup>orpk/orpk</sup> cells (right column) under static conditions (top row) and after exposure to 0.5 and 2.5 Pa shear stress (middle and lower rows, respectively). The central column shows immunostaining for acetylated α-tubulin. Arrowheads indicate cilia. Note the presence of primary cilia at 0.5 Pa shear and the non-ciliated phenotype of WT cells after exposure to 2.5 Pa shear stress. Scale bars: 25 μm.

(B) Relative mRNA expression of CD31, αSMA, Pai1, Snai1, and Ncad in WT cells under 0.5 and 2.5 Pa shear. Expression is normalized to GAPDH and relative to static shams, as represented by the dashed line.
Online Figure III. Exposure to shear stress results in enhanced phospho-Smad2 expression in Tg737<sup>orpk/orpk</sup> cells.
Western Blot analysis and quantification of P*Smad2 protein levels in WT and Tg737<sup>orpk/orpk</sup> cells under static conditions and after exposure to 2 hours of 0.5 Pa shear stress. Western Blot shown is representative of 3 independent experiments.
**TABLES**

**Online Table I. List of Q-PCR primers used.**

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Gene ID</th>
<th>Oligonucleotide sequence</th>
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| *muActa2 (αSMA)*| 11475   | F: 5'-CATCATGCGTCTGGACTTG-3'  
R: 5'-ATCTCAGCTCAGCAGTAG-3' |
| *muCD31*        | 18613   | F: 5'-CTCCAACAGAGCCACGAGTA-3'  
R: 5'-GACCACTCCTCAATGACAAACA-3' |
| *muGAPDH*       | 14433   | F: 5'-TTGATGGCCAACAAATCTCCAC-3'  
R: 5'-CGTCCCTAGACAAAATGTT-3' |
| *muIf88*        | 21821   | F: 5'-GCAATGGAACGTTGAAAGG-3'  
R: 5'-AAGACGGCTTCGATCACAGG-3' |
| *muKlf2*        | 16598   | F: 5'-ATTGCAACTGCGAAGGATG-3'  
R: 5'-GTGACGACTGAAAGGTTCTG-3' |
| *muKlf4*        | 16600   | F: 5'-CAGGCCAGAACACCTTACCA-3'  
R: 5'-TGTTGTGTTGCGTAAGTGC-3' |
| *muCdh2 (Ncad)*| 12558   | F: 5'-AATCCACCTTATGGCCCTTTCTC-3'  
R: 5'-AGGATTTGGGGCAAAAATAAG-3' |
| *muSerpine1 (Pai1)* | 18787   | F: 5'-GCCAAACAAGAGCCTACAC-3'  
R: 5'-ACCCTTCTCCAGAGACACAG-3' |
| *muSnai1*      | 20613   | F: 5'-CTTGTGTCAGGATCGCC-3'  
R: 5'-CACGTGGAACAGGAGAAT-3' |
REFERENCE LIST


