Chapter 6

Monocilia on Chicken Embryonic Endocardium in Low Shear Stress Areas

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Abstract

During cardiovascular development, fluid shear stress patterns change dramatically due to extensive remodelling. This biomechanical force has been shown to drive gene expression in endothelial cells and, consequently, is considered to play a role in cardiovascular development. The mechanism by which endothelial cells sense shear stress is still unidentified. In this study, we postulate that primary cilia function as fluid shear stress sensors of endothelial cells. Such a function already has been attributed to primary cilia on epithelial cells of the adult kidney and of Hensen’s node in the embryo where they transduce mechanical signals into an intracellular Ca\(^2+\) signalling response. Recently, primary cilia were observed on human umbilical vein endothelial cells. These primary cilia disassembled when subjected to high shear stress levels. Whereas endocardial-endothelial cells have been reported to be more shear responsive than endothelial cells, cilia are not detected, thus far, on endocardial cells. In the present study, we use field emission scanning electron microscopy to show shear stress-related regional differences in cell protrusions within the cardiovascular system of the developing chicken. Furthermore, we identify one of these cell protrusions as a monocilium with monoclonal antibodies against acetylated and detyrosinated alpha-tubulin. The distribution pattern of the monocilia was compared with the chicken embryonic expression pattern of the high shear stress marker Krüppel-like factor-2. We demonstrate the presence of monocilia on endocardial-endothelial cells in areas of low shear stress and postulate that they are immotile primary cilia, which function as fluid shear stress sensors.

Introduction

Various types of hemodynamic forces such as hydrostatic pressure, stretch, cyclic strain due to the pulsatile nature of blood flow, and fluid shear stress act upon the vessel wall and can modulate the endothelial structure and function (reviewed by Lehoux and Tedgui\(^1\)). Fluid shear stress is the frictional force of blood along the vessel wall acting in parallel to the direction of the flow. It has been stated to drive gene expression in endothelial cells \textit{ex vivo} (reviewed by Resnick \textit{et al.}\(^2\)) and \textit{in vivo}\(^3\). Consequently, fluid shear stress is believed to play a substantial role in remodelling of the heart and vasculature. The presence of shear stress-related gene expression patterns in early cardiovascular development\(^4\) raises the question of how endothelial cells sense fluid shear stress. In the literature, many possible sensors, such as cell-matrix and cell-cell junction molecules, membrane structures, the endothelial
cytoskeleton (reviewed by Resnick et al.2), and the glycocalyx4,5, have been postulated. However, a general mechanism of fluid shear stress sensing remains to be elucidated. Recently, primary cilia have been shown to function as fluid shear stress sensors of cultured adult kidney epithelial cells6 and of Hensen’s node epithelial cells in the early embryo7,8. A cilium is a rod-like structure that contains a microtubule bundle as a core. The primary cilium is considered to be a non-motile structure, as it lacks axonemal dyneins and has a 9+0 configuration of microtubule doublets, lacking the inner microtubule doublet seen in motile cilia (reviewed by Praetorius and Spring9). Microtubules consist of two subunits: alpha- and beta-tubulin. Two post-translational modifications of the alpha-tubulin subunit, i.e., detyrosinated and acetylated alpha-tubulin that represent stability isoforms, co-localise in the primary cilium. Primary cilia are found solitary on the surface of most cells in the vertebrate body. They function as chemoreceptors, mechanosensors, and photoreceptors8.

We postulate that primary cilia function as fluid shear stress sensors of endothelial cells. Few reports of cilia on endothelial cells are present. Bystrevskaya et al.10 described primary cilia either entirely immersed in the cytoplasm or protruding from the abluminal cell surface in aortas of 22-24 week human fetuses. Briffeuil et al.11 observed primary cilia on senescent bovine aortic endothelial cells in static culture, protruding from the cell surface. Furthermore, primary cilia were detected on the corneal endothelium12.

Recently, primary cilia were identified on human umbilical vein endothelial cells (HUVEC). When HUVEC were exposed to a laminar shear stress of 15 dyne/cm², a shear stress level to which normally only arterial endothelial cells are subjected, all primary cilia disassembled13. These data suggest that primary cilia cannot endure high levels of shear stress. Primary cilia have not been described on the endocardial cells, being the endocardial-endothelial cells of the heart, despite that there is a striking contrast in shear responsiveness between fetal endothelial and fetal endocardial cells. Fetal arterial endothelial cells are less reactive to fluid shear stress than fetal endocardial cells, both in vivo3,14 and ex vivo (Hierck, unpublished data 2005).

We examined the endocardial surface of the developing chicken heart with field emission scanning electron microscopy (FESEM) and found regional- and time-dependent differences in cell protrusions. Sections of chicken embryos were stained for acetylated and detyrosinated alpha-tubulin. We demonstrate monocilia on endothelial and endocardial cells and show a noticeable relationship between the presence of monocilia and fluid shear stress distribution patterns.
Materials and Methods

FESEM
Fertilised White Leghorn eggs (*Gallus domesticus*) were incubated at 37°C and 60% relative humidity. Embryos were staged according to Hamburger and Hamilton\(^{15}\). All experiments were performed according to institutional guidelines. Distinct stages between HH17-37 (n=13) were prepared for FESEM, essentially as has been described previously\(^{16}\). Overview field emission electron micrographs of the chicken hearts are in the order of a x 45 to x 140 magnification, the micrographs of the cell surface are in the order of a x 5,000 to x 20,000 magnification.

Immunofluorescence
Embryos of stage HH24, 28 and 30 (n=3 for each stage) were used to demonstrate the presence of cilia. The hearts were perfusion fixed, using gravitational force (76 mm Hg), with 4% paraformaldehyde (PFA) in 0.1 mol/L PHEM buffer (60 mmol/L Pipes, 25 mmol/L Hepes, 10 mmol/L ethyleneglycoltetraacetic acid, 2 mmol/L MgCl\(_2\), pH 6.97). The embryos were subsequently fixed overnight in 4% PFA in 0.1 mol/L PHEM buffer, after which they were dehydrated in graded ethanol and embedded in paraffin. Specimens were sectioned transversely at 5 μm.

After deparaffination, the sections were treated for 12 minutes with 0.01 mol/L Citric acid buffer of pH 6.0 at 97°C for antigen retrieval. Routine immunofluorescence was performed. Acetylated alpha-tubulin was detected with a monoclonal antibody (clone 6-11B-1, Sigma-Aldrich Chemie)\(^{17}\) diluted 1:2,000 in PBS with 0.05% Tween-20 and 1% ovalbumin after an overnight incubation at room temperature. In alternate sections, detyrosinated alpha-tubulin was detected with a monoclonal antibody (clone 1D5, Synaptic systems, Germany)\(^{18}\) diluted 1:2 in PBS with 1% ovalbumin after an overnight incubation. After rinsing in PBS, the sections were incubated for one hour with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (diluted 1:50, DAKO, Denmark). After rinsing in PBS, administration of 2μg/ml propidium iodide (SERVA, Heidelberg, Germany) for 5 min to stain the nuclei, and subsequent washing in PBS, the sections were mounted with prolong gold antifading reagent (Molecular probes, Eugene, OR; Invitrogen, Carlsbad, USA). All slides were first examined with a Leica IRBE microscope. Suitable areas were selected for detailed confocal laser scanning microscopy (Bio-Rad, MRC 1024ES; Richmond, CA). Each data set collected on the confocal microscope was processed with Image J (http://rsb.info.nih.gov/ij/index.html).
Monocilia in Low Shear Stress Areas

Statistical Analysis
The amount of monocilia detected with immunofluorescence at HH24, 28, and 30 in a low shear stress area, i.e., the atrium, and a high shear stress area, i.e., the narrow part of the AV canal, was quantified. An independent-samples t-test was performed (SPSS; SPSS Inc.) on the mean values of the number of monocilia in a cohort of 30 endocardial cells (n=4).

Radioactive In Situ Hybridisation
In situ hybridisation with a 35S-labelled chicken-specific riboprobe from a KLF2 fragment was performed on sections of a 4% PFA-fixed HH22 chicken embryo as described by Groenendijk et al.3. KLF2 mRNA expression was visualised by darkfield imaging.

Results

Relevant Geometry Related to Shear Stress
The early embryonic chicken heart is a nearly straight tube that develops into a four-chambered pump through extensive growth and remodelling. This process generates changes in luminal diameter and accordingly in blood flow velocity and shear stress. An increase in lumen diameter results in a lower flow velocity and concomitant shear stress. Recently, shear stress levels in chicken embryonic hearts were found to be in the same range as adult arterial shear stress levels. A maximum of 50 dyne/cm² is reported for the outflow tract (OFT) of a Hamburger and Hamilton stage (HH) 15 chicken embryo compared with a maximum of 70 dyne/cm² in adult human vascular network19,20. According to the two-fluid model, blood flow velocity and concomitant shear stress are higher near the inner curvature compared with the outer curvature, due to the low Reynolds number in the embryonic heart21. At HH17 and HH18, the cardinal veins drain into the common atrium through the sinus venosus. The heart lumen is wide, and shear stress is expected to be moderate. However, the tube is still in the process of looping, and cushion development is in progress, resulting in local changes in shear stress patterns and levels. Atrial and ventricular septation commence at HH19 and HH20, respectively, altering the atrial and ventricular lumen diameter and, consequently, shear stress. The ventricular trabeculations become more prominent over time. The tips of the trabeculations are most probably subjected to higher flow velocities in comparison to the deep sinuses between the trabeculations. The lumen of the OFT is wide and the OFT cushions are not at their full expanse, yet. Therefore, shear stress is expected to be moderate. From HH25 onward, the atrioventricular (AV) and OFT cushions become more pronounced and their influence on shear stress increases. The
cushions cover the narrowest passages of the heart and, therefore, most likely are exposed to high levels of shear stress, which is exemplified by shear stress-related gene expression patterning. At HH25, one can distinguish a left and right atrium and ventricle. Ventricular septation is completed at HH33. At this stage, the cushions are developing into clear valve leaflets, which are fully developed at HH37.

The pharyngeal arch artery (PAA) system, the connection between the OFT of the heart and the aorta, is subjected to extensive remodelling. This results in broad variations in shear stress patterns and levels, which remain to be elucidated.

**FESEM**

To identify an ultrastructural fluid shear stress sensor on the surface of endothelial cells of the developing cardiovascular tissue, we prepared chicken specimens of distinct stages between HH17 and HH37 for FESEM and investigated the endothelial cell surface of specific areas. Note that all cell surface protrusions in this section are termed microvilli, as it is not possible to distinguish between specialised types of protrusions at the ultrastructural level with FESEM.

Microvilli of approximately 1-3 μm in length are present on the surface of the atrial endocardial cells (Fig. 6.1A) and on the atrial septum (Fig. 6.1B). The endocardial cells covering the AV cushions/valves have merely some asperities (Fig. 6.1C), while several short microvilli, of approximately 0.5 μm in length, are present on the transition of the AV cushion to the OFT cushion (Fig. 6.1D). The endocardial cells of the inner curvature are very smooth (Fig. 6.1E). In contrast, on the surface of the ventricular trabeculations, many microvilli are present (Fig. 6.2). In the young stages examined, a few cells demonstrate a short microvillus of approximately 0.5 μm (Fig. 6.2A); over time, the microvilli increase in number and length (Fig. 6.2B-D) and reach a length of approximately 2.0-2.5 μm (Fig. 6.2D). At all stages, the surface of the OFT cushion is smooth except for a few cells with a single microvillus of approximately 2-3 μm in length on the proximal part of the OFT (Fig. 6.1F).

*Figure 6.1.* Field emission scanning electron micrographs of the endocardial cell surface of the atrium of a stage 19 chicken heart (A), the atrial septum of a stage 28 chicken heart (B), an AV cushion of a stage 25 chicken heart (C), the transition of an AV cushion to an OFT cushion of a stage 25 chicken heart (D), the inner curvature of a stage 18 chicken heart (E), and the proximal OFT of a stage 17 chicken heart (F). Note the short microvilli on the smooth surface of the atrial endocardial cell and the microvilli on the atrial septum protruding into the lumen. The surface of the AV cushion is smooth in comparison to the surface of the transition of the AV cushion to the OFT cushion. The surface of the inner curvature is very smooth, whereas a microvillus of approximately 3μm is present on the surface of the proximal OFT. The inserts show an overview of the chicken heart at the specific stages. The boxed areas in the inserts are enlarged. A, atrium; AVC, atrioventricular canal; V, Ventricle; OFT, outflow tract. Scale bars = 1μm.
Figure 6.2. Field emission scanning electron micrographs of the endocardial cell surface of the ventricular trabeculations of a stage 17 (A), stage 25 (B), stage 26 (C), and stage 33 (D) chicken heart. The microvilli on the endocardial cells covering the trabeculations increase in number and length over time. The short microvilli in A are depicted by arrowheads. The inserts show an overview of the chicken heart at the specific stages. The boxed areas in the inserts are enlarged. A, atrium; AVC, atrioventricular canal; V, Ventricle; OFT, outflow tract; Ao, aorta; PA, pulmonary arteries. Scale bars = 2μm.

The OFT cushions eventually develop into the aortic and pulmonary valve leaflets. The surface of the aortic valve leaflets is irregular but without microvilli (not shown). An obvious difference exists between the surface of the ventricular and arterial side of the pulmonary valve leaflets (Fig. 6.3A,B). The cells on the arterial side contain several microvilli 0.5- to 1.0-μm long (Fig. 6.3A). In contrast, some irregularities but no microvilli appear on the ventricular side of the valves (Fig. 6.3B). Up- and downstream of these valves, several microvilli, with a maximum length of 1μm, are present (Fig. 6.3C). Going further downstream, the microvilli on the endothelial surface of the pulmonary arteries and the aorta are shorter (not shown). The surface of the bifurcation of the pulmonary arteries is irregular but no microvilli are present (Fig. 6.3D).
With FESEM, we clearly demonstrate an abundant amount of luminal cell protrusions on the endothelium of the developing chicken cardiovasculature. Because it is not feasible to differentiate between cilia and other specialised protrusions with FESEM, an additional approach was required to confirm the presence of cilia among the cell protrusions.

**Figure 6.3.** Field emission scanning electron micrographs of the cell surface of the arterial (A) and ventricular side (B) of the pulmonary valve leaflet of a stage 37 chicken heart and of the cell surface downstream of the pulmonary valve leaflets (C) and of the bifurcation of the pulmonary arteries (D) of a stage 33 chicken heart. Note that the arterial surface of the pulmonary valve leaflet has short microvilli, whereas only irregularities are present on the ventricular surface. Microvilli are present on the endothelial surface of the pulmonary arteries downstream of the pulmonary valve leaflets, while the surface of the bifurcation of the pulmonary arteries is without microvilli. The arrowheads in A and C depict obvious microvilli. The insert in A,B shows an overview of the pulmonary valve leaflets of the stage 37 chicken heart. The insert in C,D shows an overview of the pulmonary arteries of the stage 33 chicken heart. The boxed areas in the inserts are enlarged. PA, pulmonary artery; RV, right ventricle; PV, pulmonary valves; Ao, aorta. Scale bars = 2μm.
Immunofluorescence

We previously observed that the endothelium and endocardium are shear responsive at HH24-30, as we found mRNA expression of the high shear stress marker Krüppel-like factor-2 (\textit{KLF2} or \textit{LKLF}) in areas of expected high shear stress at these stages. To demonstrate the existence of cilia, we stained sections of these stages for acetylated and detyrosinated alpha-tubulin. Detyrosinated and acetylated alpha-tubulin are both present in the microtubule organising centre (Fig. 6.4A,D), which comprises two centrioles adjacent to the nucleus, as well as in cilia (Fig. 6.4A,D). In addition, acetylated alpha-tubulin, but not the detyrosinated isoform, is found in the microtubular cytoskeleton and in the mitotic spindle (Fig. 6.4E). A prevalence of one cilium per cell of approximately $5 \mu m$ in length is shown in Figures 6.4-6.6. Besides on the endothelium and endocardium where monocilia are either immersed in the cytoplasm or protruding from the luminal cell surface, monocilia are present on endocardial cushion mesenchymal cells, but also on chondrocytes and the adjacent mesenchyme (not shown). Furthermore, monocilia are detected at the luminal surface of the parietal layer of Bowman’s capsule, the segmental ducts of the pronephros (Fig. 6.4B,C), the mesothelium, the epicardium, and the pericardium (not shown). Below, the distribution of monocilia on the endocardium and endothelium of the heart and pharyngeal arch artery (PAA) system, respectively, following the blood from inflow to outflow, is described.

Occasionally monocilia are present in the veins, such as the cardinal veins (Fig. 6.5A). Going downstream into the right/common atrium, the monociliary density increases noticeably. Monocilia are located on the endothelial cells covering the myocardial wall of particularly the left sinus venosus horn. The sinoatrial valves have a striking pattern of monocilia (Fig. 6.6). At all stages examined, monocilia are positioned immediately downstream (Fig. 6.6C) and upstream (Fig. 6.6D) of the sinoatrial valves, although monocilia are considerably shorter on the upstream side. In contrast, the incidence of monocilia on the valves is lowest on the narrowest part of the sinus venosus, where \textit{KLF2} expression is present (Fig. 6.6B). The sinoatrial valves are joined to the wall of the atrium, where numerous monocilia are present (Fig. 6.5B,C,G-I). Several monocilia are present on the endocardial cells covering the most upstream and downstream parts of the AV cushions. Interestingly, no monocilia are detected on the part of the AV cushions lining the narrowest passage of the AV canal. Deep in the ventricular intertrabecular sinuses, many cells with a monocilium are present (Fig. 6.5D-F). On the tips of the trabeculations, less monocilia are found. On the upstream slope of the OFT cushion, which covers the wide proximal lumen of the OFT, several monocilia are present. Further downstream, the volume of the OFT cushions increases, narrowing the
lumen, and the number of monocilia decreases. Where the blood passage in the OFT is narrowest, the surface of the OFT cushions is free of monocilia. In the OFT, some small variations exist in cilium distribution in the HH28 embryos, most likely due to slight differences in developmental stages. In two of the embryos, the upstream slope of the OFT cushion is free of monocilia (not shown). The distribution of monocilia throughout the heart is schematically depicted in Figure 6.7. To determine whether the difference in amount of monocilia detected in different regions of the heart is significant, we quantified the number

![Figure 6.4](image-url)  
**Figure 6.4.** Confocal laser scanning microscopic (CLSM) images of the atrial wall of a HH24 chicken embryo stained for detyrosinated alpha-tubulin (A), monocilia on epithelial cells of a segmental duct of the pronephros stained for detyrosinated (B) and acetylated alpha-tubulin (C), the endocardium covering the proximal outflow tract of a HH30 chicken embryo stained for acetylated alpha-tubulin (D), a mitotic endocardial cell of the atrium of a HH28 chicken embryo stained for acetylated alpha-tubulin (E). A,D,E: Note that both detyrosinated (A) and acetylated (D) alpha-tubulin are present in the microtubule organizing centre (arrowhead) and in a monocilium (arrow), but only acetylated alpha-tubulin is present in the microtubules of the cytoskeleton (E). A,B: The monocilia on the segmental duct of the pronephros (B) accumulate more detyrosinated alpha-tubulin than the monocilia on the atrial endocardium (A). No such difference in acetylated alpha-tubulin accumulation is present. Acetylated and detyrosinated alpha-tubulin (green), nuclei (red; propidium iodide). Scale bars = 5µm.
of endocardial cells with a monocilium in a low shear stress area, i.e., the atrium, and a high shear stress area, i.e., the narrow part of the AV canal. The mean values of the number of monocilia in the high and low shear stress area were compared for all stages examined. The values are non-overlapping and p-values are <0.001. Therefore, it is concluded that there is a

**Figure 6.5.** CLSM images of monocilia on an endothelial cell of the right cardinal vein (A), an endocardial cell of the atrium (B), the atrial septum (C), endocardial cells of the ventricle (D-F), and on endocardial cells of the atrium (G-I). A-F are stained with a monoclonal antibody directed against acetylated alpha-tubulin, G-I are stained with a monoclonal antibody directed against detyrosinated alpha-tubulin. A-D,G: a HH28 chicken embryo. E-F: a HH30 chicken embryo. H,J: a HH24 chicken embryo. Acetylated and detyrosinated alpha-tubulin (green), nuclei (red). Scale bars = 5 µm.
significant difference in monocilia content (Table 6.1). The incidence of monocilia in the atria is approximately one on every two endocardial cells. Given an average cell diameter of 10 µm and a section thickness of 5 µm, we estimate that all interphase endocardial cells of the atria contain a monocilium.

Figure 6.6. CLSM images (A,C,D) and a darkfield image of an in situ hybridisation for KLF2 (B) of the sinus venosus of a HH22 (B) and HH24 chicken embryo (A,C,D). An overview of the sinus venosus is shown in (A), and a higher magnification of the downstream and upstream part of the future right sinoatrial valve are shown in (C) and (D), respectively. Primary cilia are present upstream and downstream of the future right sinoatrial valve (arrowheads), but not where KLF2 is expressed (arrows). (A,C,D) acetylated alpha-tubulin (green), nuclei (red). RA, right atrium; CV, cardinal vein. Scale bars (A,B) = 20 µm, (C,D) = 10 µm.
Figure 6.7. Schematic drawing showing the prevalence of monocilia (black line) throughout the embryonic heart. Monocilia are present on the myocardial wall of the sinus venosus, upstream and downstream of the sinoatrial valves, on the atrium, on the most upstream and most downstream parts of the AV cushions, deep in the ventricular intertrabecular sinuses, the most proximal part of the OFT, and on the proximal part of the PAA system. SV, sinus venosus; A, atrium; V, ventricle; OFT, outflow tract.

Table 6.1. Monocilia content in a low and high shear stress area of the heart

<table>
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<th>HH24</th>
<th>HH28</th>
<th>HH30</th>
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<tbody>
<tr>
<td>Atrium (low shear stress area)</td>
<td>12.8 ± 1.3</td>
<td>11.8 ± 1.5</td>
<td>12.8 ± 1.3</td>
</tr>
<tr>
<td>AV-canal (high shear stress area)</td>
<td>2.0 ± 1.6</td>
<td>2.0 ± 1.6</td>
<td>2.0 ± 1.6</td>
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*Quantification of the number of monocilia in a low shear stress area (atrium) and a high shear stress area (AV canal) of the heart at HH24, 28, and 30. The mean values of the number of monocilia in a cohort of 30 endocardial cells (n=4) are depicted. p-values are <0.001. AV, atrioventricular; HH, Hamburger and Hamilton stage.

A stage-dependent distribution of monocilia is seen in the PAA system. In the aortic sac at HH24, some monocilia are present, but going further downstream into the PAA system the occurrence of monocilia decreases. At HH28 and HH30, several cells with a monocilium are present in the proximal part of the PAA system, but the prevalence decreases when going further downstream. Strikingly, the highest incidence of monocilia is seen in the proximal part of mainly the left sixth PAA. During remodelling of the left fourth PAA and ensuing decrease in vascular diameter, we do not encounter differences in monocilia distribution between the left and right fourth PAA. In none of the stages examined were monocilia present at the connection of the PAA system to the descending aorta or in the aorta itself.
Discussion

In the present study, we demonstrate a regional- and time-dependent distribution of microvilli on the endothelium of the developing chicken cardiovasculature. With monoclonal antibodies directed against acetylated and detyrosinated alpha-tubulin, we detected monocilia on various cell types, including endothelial and endocardial cells. The prevalence of monocilia is consistent with the distribution of microvilli in the cardiovasculature. Therefore, we conclude that the monocilium is among the microvilli detected with FESEM. The other microvilli are presumably true microvilli that contain actin filaments. Reports on endothelial cilia describe them as non-motile primary cilia10-13. Moreover, motile monocilia, propelling fluid, are not to be expected in the flow dominated cardiovascular system. Consequently, we consider the monocilia that are present on the endothelium and endocardium to be non-motile primary cilia.

To demonstrate a relationship between primary cilia distribution and fluid shear stress, we compared the distribution of primary cilia to the chicken embryonic expression pattern of the high shear stress marker Krüppel-like factor-2 (KLF2 or LKLF). KLF2 expression is confined to areas of high shear stress3,22. We discovered an inverse correlation between KLF2 expression and the distribution of primary cilia. Primary cilia are present in areas where KLF2 is not expressed and, consequently, shear stress is expected to be low. This finding is consistent with the observation of Iomini et al.13 that primary cilia on HUVEC disassemble under high levels of shear stress. The inverse correlation is most obvious at the sinus venosus and the ventricular trabeculations, where shear stress is high on the tips of the sinoatrial valves and the ventricular trabeculations, which are the only areas where primary cilia are not present. The highest incidence of primary cilia is present on the endocardial cells of the atrium where shear stress is expected to be low. Specific parts of the AV cushions are subjected to high shear stress levels and, consequently, these endocardial cells exhibit significantly less monocilia. Several primary cilia are present on the most upstream and downstream parts of the AV cushion where the lumen is wider and shear stress levels are assumed to be lower. In the OFT, shear stress is high distally, where primary cilia are absent. On the proximal part of the OFT cushions, where the lumen is wider, are primary cilia present. The variations in primary cilia distribution in the OFT at HH28 are probably due to differences in OFT cushion morphology due to slight differences in developmental stages. Evidently, this area is susceptible to small variations in blood flow and, consequently, shear stress, indicating that HH28 is a key stage in OFT remodelling. The atrial septum is the only area of the heart where the presence of cilia is not inversely
correlated to high shear stress. Both KLF2 expression and primary cilia are present there. Considering that they exclude each other in the rest of the heart, we assume that micro flow patterns vary greatly in this geometrically complex area, as the septum has multiple perforations.

Shear stress-related gene expression in the PAA system is very complex due to intricate flow patterns. This complexity is also seen in the distribution pattern of primary cilia throughout the PAA system. The high prevalence of primary cilia in the proximal part of the sixth PAA pair, which is also seen in further embryonic development of the PAA system, can be explained by the fact that most of the blood in the embryonic circulation goes to the brain instead of the lungs. Moreover, regional differences in endothelial response to shear stress have been described (Hierck, unpublished data 2005) and, in part, could contribute to the complex distribution pattern of primary cilia in the PAA system.

It is not feasible to distinguish between specialised types of protrusions with FESEM. One of the protrusions, the primary cilium, is revealed by immunofluorescence, but the prevalence and length of the other protrusions appear to be shear stress related as well. The cell surface of high shear stress areas of the heart, such as the inner curvature, AV and OFT cushions, is smooth. Microvilli are present on the endocardial cell surface of the low shear stress areas, such as the atrium and ventricular trabeculations. The time-dependent increase in amount and length of microvilli on the ventricular trabeculations could suggest that shear stress levels decrease due to an increase in ventricular diameter. The localisation of the solitary microvilli in the proximal part of the OFT correlate with our immunofluorescence data and, therefore, most likely represent primary cilia. Remarkably, the ventricular side of the pulmonary valves subjected to high flow velocity and concomitant shear stress contains shorter microvilli than the arterial side subjected to a lower shear stress level. The difference in endothelial phenotype between the ventricular and arterial side of the valve could be due to endothelial heterogeneity and/or differences in shear stress level.

It is not surprising that only few reports of cilia on endothelial cells exist, as we find cilia mainly on endocardial cells and only occasionally on endothelial cells. In addition, consistent with the observations of Bystrevskaya and colleagues, we regularly observe primary cilia immersed in the cytoplasm, which are, consequently, invisible for surface scanning techniques. The presence of primary cilia inside the cell is most likely a result of cytoplasmic assembly and disassembly of the primary cilia due to the high rate of cell division in the developing heart, because cells that are about to enter mitosis disassemble their cilium. We detected two isoforms of alpha-tubulin in primary cilia, i.e., acetylated and detyrosinated alpha-tubulin. The most stable alpha-tubulin isoform, detyrosinated
alpha-tubulin\(^{28}\), is present in endothelial and endocardial cilia, but in small amounts compared with the primary cilia of the segmental ducts of the pronephros. This finding suggests that endothelial and endocardial primary cilia are less stable and that primary cilium-based shear stress sensing by these cells is a dynamic process.

Recently, primary cilia have been shown to function as fluid shear stress sensors on cultured kidney epithelial cells where they transduce mechanical signals into an intracellular Ca\(^{2+}\) signalling response. This flow response includes regulation of a key intracellular signal transduction pathway, i.e., the Wnt signalling pathway\(^{29}\). Experimental mechanical bending of the primary cilium in cultured kidney epithelial cells causes a Ca\(^{2+}\)-influx through mechanically sensitive channels\(^{6}\), like the polycystin complex. The genes \(Pkd1\) and \(Pkd2\) encode for Polycystin-1 (PC1) and polycystin-2 (PC2), respectively. These polycystins co-localise in the primary cilium of mouse kidney epithelial cells\(^{30}\). In early mouse development, primary cilia encountered on Hensen’s node\(^{31}\) play a role in breaking the symmetry of the body axis\(^{32-34}\). Two populations of primary cilia are present on the node, which both contain PC2. Motile cilia are present in the center of the node generating directed nodal flow and non-motile primary cilia sensing the nodal flow are located at the periphery. When the primary cilium is bent by nodal flow, a Ca\(^{2+}\)-influx is generated. In this way an asymmetric Ca\(^{2+}\) signal at the left border of the node is initiated, triggering left-sided gene expression\(^{7,8}\). Recently, we described distinct expression of genes involved in left-right patterning in spontaneous mutants of the freshwater snail \textit{Lymnea stagnalis}, indicating a high level of conservation of this shear stress-induced regulatory mechanism among distant species\(^{35}\). The shear stress-induced rise in intracellular Ca\(^{2+}\), seen in epithelial cells of the kidney and Hensen’s node, is also seen in endothelial cells\(^{36}\). Both PC1 and PC2 were detected in human fetal endocardial cells and in endothelial cells of human fetal vessels from different tissues\(^{37}\). Considering these data, a comparable mechanism of shear stress sensing can be expected on endothelial and endocardial cells.

Although not all endothelial and endocardial cells possess a primary cilium, they are all shear stress responsive. The potential endothelial fluid shear stress sensors described previously are all directly or indirectly linked by the cytoskeleton (reviewed by Helmke and Davies\(^{38}\)). Primary cilium-based mechanosensing by kidney epithelial cells is very much dependent on an intact cytoskeleton and adhesion to the extracellular matrix\(^{39}\). The basal body of the cilium is connected to the cortical actin cytoskeleton\(^{40}\). Therefore, we postulate that the cytoskeleton functions as a central shear stress transducer. Endothelial and endocardial cells in low shear stress areas use primary cilia to sense changes in shear stress, which then transmit the low shear forces they are subjected to, to the cytoskeleton, which
generates a shear stress response. Therefore, the primary cilium is an essential component of the endothelial biosensor for shear stress, especially in regions of low shear stress.

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