Endothelial Primary Cilia in Areas of Disturbed Flow are at the Base of Atherosclerosis

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Abstract

Atherosclerosis develops in the arterial system at sites of low as well as low and oscillating shear stress. Previously, we demonstrated a shear-related distribution of ciliated endothelial cells in the embryonic cardiovascular system and postulated that the primary cilium is a component of the shear stress sensor, functioning as a signal amplifier. This shear-related distribution is reminiscent of the atherosclerotic predilection sites. Thus, we determined whether a link exists between location and frequency of endothelial primary cilia and atherogenesis. We analyzed endothelial ciliation of the adult aortic arch and common carotid arteries of wild-type C57BL/6 and apolipoprotein-E-deficient mice. Primary cilia are located at the atherosclerotic predilection sites, where flow is disturbed, in wild-type mice and they occur on and around atherosclerotic lesions in apolipoprotein-E-deficient mice, which have significantly more primary cilia in the aortic arch than wild-type mice. In addition, common carotid arteries were challenged for shear stress by application of a restrictive cast, resulting in the presence of primary cilia only at sites of induced low and disturbed shear. In conclusion, these data relate the presence of endothelial primary cilia to regions of atherogenesis, where they increase in number under hyperlipidemia-induced lesion formation. Experimentally-induced flow disturbance leads to induction of primary cilia, and subsequently to atherogenesis, which suggests a role for primary cilia in endothelial activation and dysfunction.

Introduction

Atherosclerosis develops at flow-determined sites, i.e., at arterial bifurcations, branch points, and the inner curvature of arched arteries. The geometry of these athero-prone areas invokes low and disturbed blood flow regions and concomitant low and oscillatory (multi-or bidirectional) wall shear stress. In contrast, high and unidirectional wall shear stress is athero-protective. This myriad of mechanical signals is sensed and translated into a biological response by the endothelium, which therefore plays an essential role in the initiation of atherosclerosis at low and oscillatory shear regions.

Both at the cellular and molecular level, the endothelial response is velocity pattern and shear level-dependent. When exposed to high, unidirectional shear stress endothelial cells align and elongate in the direction of flow, while endothelial cells exposed to low and oscillatory shear have no preferred alignment. Moreover, disturbed flow, compared with undisturbed flow, differentially affects expression of vasoactive substances, such as endothelial nitric oxide synthase (eNOS) and leukocyte adhesion molecules, and alters the oxidative and inflammatory state of the endothelium. This renders it susceptible to proatherogenic stimuli. It is conceivable that the endothelial shear stress sensing apparatus directs the differential response to various velocity patterns and could even be involved in pathologic alterations of endothelial cells and their signals into the vessel wall. Therefore, a correlation between atherogenesis and the mechanism of shear stress sensing is likely. Previously, we postulated that the primary cilium is an integral component of the endothelial shear stress sensor in areas of low and oscillating shear in the embryonic cardiovascular system. Because the shear-related distribution of primary cilia in the embryonic cardiovascular system is reminiscent of the atherosclerotic predilection sites we determined if a correlation exists between primary cilia occurrence and atherogenesis.

The primary cilium is a rod-like, non-motile structure with a 9+0 configuration of microtubule doublets as a core, which protrudes from the luminal cell surface. The basal
body of the primary cilium is linked to the microtubule organizing center from which the cytoskeletal microtubules spread throughout the cell. The microtubular system is in turn connected to other components of the cytoskeleton. Bending of the primary cilium, therefore, results in transduction of mechanical force throughout the cell, including the cell-cell and cell-matrix junctions that are connected to the cytoskeleton. Primary cilia have been shown to function as fluid shear stress sensors of cultured adult kidney epithelial cells, of cells in Hensen’s node, the organizing center of the early embryo, and of cholangiocytes, where they transduce mechanical signals into an intracellular Ca\textsuperscript{2+} transient. Since cilia are also present on endothelial cells and a similar shear-induced rise in intracellular Ca\textsuperscript{2+} is seen, a comparable role in endothelial shear stress sensing is conceivable. Already early in development, a vascular shear-dependent primary cilium distribution is present coinciding with shear-related gene expression. Embryonic and adult models in which shear stress is altered in blood vessels also show responsiveness of these genes to shear stress alterations. Endothelial primary cilia are present in areas of low and oscillatory shear.

In this study, the correlation between patterns of shear stress, the frequency of primary cilia, and sites of atherogenesis is investigated. The most suitable mouse model for atherosclerosis, with lesions similar to those in humans, is the apolipoprotein-E-deficient (ApoE\textsuperscript{-/-}) mouse, which is hyperlipidemic and develops atherosclerosis spontaneously, i.e., not diet-induced. We analyzed the athero-prone and athero-protected areas in the aortic arch and common carotid arteries (CCAs) of adult wild-type and ApoE\textsuperscript{-/-} mice for endothelial ciliation. In addition, the presence of primary cilia in areas exposed to various velocity profiles, experimentally induced by in vivo application of a flow-modifying cast around the CCA, was investigated.

Materials and Methods

Animals
C57BL/6 (n = 5, 2 males, 3 females) and ApoE\textsuperscript{-/-} (n = 6, 4 males, 2 females) mice in C57BL/6 background were acquired from The Jackson Laboratory (Bar Harbor, USA). After weaning the animals received a chow-diet for 22 weeks. At 25 weeks of age a restrictive cast was unilaterally placed around the CCAs, termed challenged CCAs (3 C57BL/6, 3 ApoE\textsuperscript{-/-}). Unchallenged CCAs served as controls (4 C57BL/6, 5 ApoE\textsuperscript{-/-}). In one of the unchallenged ApoE\textsuperscript{-/-} CCAs an atherosclerotic lesion was present, which was therefore excluded from statistical analysis. Gender differences in the presence and patterning of primary cilia were not observed (not shown), which confirms data that regional hemodynamic effects dominate gender effects in endothelial cells. Therefore, males and females were treated collectively in the analysis. Animal care and experiments complied with institutional and national guidelines.

In vivo manipulation of flow profiles
A restrictive cast was placed to change the patterns of shear stress. Cast placement results in a low shear stress region upstream of the cast, a high shear region in the cast area, and a low, oscillatory shear region downstream of the cast. For surgery, mice were anesthetized with isoflurane.
Chapter 8

Tissue harvesting and preparation
Mice were sacrificed 2 days after surgery by exposure to an overdose of isoflurane. Perfusion fixation of the vasculature was performed with 4% paraformaldehyde (PFA) in 0.1 mol/L PHEM buffer (60 mmol/L Pipes, 25 mmol/L Hepes, 10 mmol/L EGTA, 2 mmol/L MgCl₂ pH 6.97) via the left atrium. The aortic arch and CCAs, including the bifurcation of the CCA, were removed and subsequently fixed overnight, after which they were dehydrated in graded ethanol and embedded in paraffin. Specimens were sectioned at 5 μm and mounted serially.

Immunofluorescence
Primary cilia were detected with a monoclonal antibody directed against acetylated α-tubulin (clone 6-11B-1, Sigma-Aldrich Chemie)²². Alternate sections were stained with a monoclonal antibody directed against detyrosinated α-tubulin (clone 1D5, Synaptic systems)²³. Fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (DAKO, Denmark) was used as secondary antibody. After deparaffination, the sections were stained and examined as described previously⁶. In addition, routine staining with hematoxylin and eosin (HE) was performed.

Statistical analysis
The number of primary cilia in delineated regions of the aortic arches, the unchallenged CCAs, and the challenged CCAs was quantified. In the aortic arches the segment between the aortic root and the branching point of the left subclavian artery, excluding the aortic valves and branch points, was analyzed. The unchallenged CCAs were divided into two regions: 0.25 mm of the disturbed shear stress area, i.e., the bifurcation of the CCA into the internal and external carotid artery, including the internal carotid artery sinus, and, 1.25 mm more proximal, 0.25 mm of the undisturbed shear stress area of the CCA. Similarly, two regions of the challenged CCAs were analyzed: 1 mm of the high, undisturbed, shear stress region in the cast and 0.25 mm of the low and oscillatory, disturbed, shear stress region directly distal to the cast.

The number of cilia was quantified in every fifth tissue section and multiplied by five to estimate the absolute number of cilia in the delineated areas. Subsequently, the medial plus the intimal volume of the vessel wall was estimated with the Cavalieri technique²⁴, a point counting method using a grid. The number of cilia normalized for a distinct volume of the vessel wall, i.e., 0.005 mm³ for CCAs and 0.5 mm³ for aortic arches was determined and an independent-samples t-test was performed (SPSS; SPSS Inc.).

The aortic arch of wild-type mice was compared with Apoe⁻⁻ mice (Table 1). The disturbed shear stress and undisturbed shear stress area of the unchallenged CCA were compared with each other and between wild-type and Apoe⁻⁻ mice (Table 2). In the challenged CCAs the undisturbed shear stress area and the disturbed shear stress area were compared with each other and between wild-type and Apoe⁻⁻ mice (Table 3). Data are presented as mean ± standard error of the mean. P value < 0.05 and a power > 80% were considered significant.

3D-reconstruction
To visualize the relation between cilia and an atherosclerotic lesion in the unchallenged CCA of an Apoe⁻⁻ mouse, micrographs were reconstructed three-dimensionally with Amira.
Ciliated Endothelial Cells in the Adult Mouse

Results

Primary cilia distribution
To demonstrate a link between primary cilia occurrence and atherogenesis we analyzed the endothelial cell surface of athero-prone and athero-protected areas of the aortic arch and CCAs of adult wild-type and Apoe−/− mice for primary cilia by immunofluorescent staining for acetylated and detyrosinated α-tubulin. Acetylated α-tubulin is found in the primary cilium, microtubule organizing center, and microtubular cytoskeleton, whereas detyrosinated α-tubulin is not present in the murine endothelium (not shown). Primary cilia protruding from the luminal cell surface are 1-2 μm in length (Fig. 1). In highly ciliated areas approximately one in four cells presents a primary cilium. We hypothesize that the mechanical signal will be transferred from the ciliated cell to neighboring cells. The distribution of primary cilia in the wild-type (Fig. 1) and Apoe−/− (Fig. 2) aortic arch and CCAs is described below.

The distribution pattern of primary cilia throughout the wild-type aortic arch is striking. Ciliated endothelial cells are located on the aortic valve leaflets (Fig. 1A,B) with a higher incidence on the arterial side of the leaflets than on the ventricular side (Fig. 1A). Directly downstream of the valves primary cilia occur as well. In the inner curvature of the aortic arch many ciliated endothelial cells are present (Fig. 1C,D,3A). Moreover, primary cilia are present at the upstream side of the branch points of the aorta with the brachiocephalic artery, the left CCA, and the left subclavian artery. The number of cilia decreases with each successive branch point. Occasionally a cilium is found in the descending aorta, while the endothelial cells in the outer curvature of the aortic arch are devoid of primary cilia. The distribution of primary cilia throughout the wild-type aortic arch is schematically depicted in Figure 3A.

In the CCAs and their bifurcations into the external and internal carotid arteries a high prevalence of ciliated endothelial cells is present in the sinus area of the internal carotid arteries (Fig. 1E,F). Furthermore, primary cilia are found only occasionally on the flow divider of the bifurcations and in the CCAs itself (not shown).

In the aortic arch of the Apoe−/− mice atherosclerotic lesions developed downstream of the aortic valves, in the inner curvature of the aortic arch, and at the upstream side of the branch points in the aorta, mainly in that of the brachiocephalic artery. Primary cilia are present upstream and downstream of the lesions both on the, presumably, unaffected endothelium and on the endothelium overlying the lesions (Fig. 2). In addition, in one of the examined brachiocephalic arteries the endothelial cells covering the atherosclerotic lesion present a cilium where facing the downstream part of the branch point (Fig. 2C and 3B). The outer curvature of the Apoe−/− aortic arch lacks primary cilia, in contrast to the descending aorta where a cilium is seen sporadically. Fig. 3B schematically shows the distribution of atherosclerotic lesions and primary cilia in the Apoe−/− aortic arch.

In one of the unchallenged Apoe−/− CCAs an atherosclerotic lesion developed spontaneously (Fig. 4). The lesion almost completely obstructs the lumen of the superior thyroid artery and runs through the most proximal part of the external carotid artery. Primary cilia are present at the shoulder of this lesion (Fig. 4A-D). Two clusters of cilia can be observed (Fig. 4E and F). Lesion-free Apoe−/− CCAs have the same distribution of primary cilia as wild-type CCAs.
Figure 2. Primary cilia on the endothelial cell layer overlying an atherosclerotic lesion in the inner curvature of an aortic arch (A and B) and in the brachiocephalic artery (C and D) of an Apoe<sup>−/−</sup> mouse. Primary cilia are indicated by arrows. The dashed lines designate the internal elastic lamella, which is below the level of the picture in C and D. The inserts show the adjacent section, stained with hematoxylin and eosin, at lower magnification. The boxed areas in the inserts indicate the parts shown in the confocal images. Acetylated α-tubulin (green), nuclei (red). Scale bar = 4 μm. Magnification A-C = 100x, D = 200x, insert A and B = 4x, insert C and D = 2x.

Figure 1. Primary cilia on the endothelial cell layer of the aortic valve leaflet (A and B), the inner curvature of the aortic arch (C and D), and the internal carotid artery sinus (E and F) of a wild-type C57BL/6 mouse. More primary cilia are present on the arterial surface (top of the leaflet in A) of the aortic valve leaflet. Arrows depict primary cilia. Acetylated α-tubulin (green), nuclei (red; propidium iodide). Scale bar A = 6 μm, B-F = 2 μm. Magnification A = 100x, B-D = 200x, E = 400x, F = 300x.
Figure 3. Schematic drawing showing the distribution of primary cilia throughout the wild-type (A) and Apoe<sup>−/−</sup> (B) aortic arch. Primary cilia are shown in green, atherosclerotic lesions are shown in red. Primary cilia length does not represent in vivo length, which is 1-2 μm. The asterisk in B depicts primary cilia on the atherosclerotic lesion in the brachiocephalic artery where the lesion faces the downstream part of the branch point. AV, aortic valve; AA, ascending aorta; BA, brachiocephalic artery; LCCA, left common carotid artery; LSA, left subclavian artery; DA, descending aorta.

Figure 4. Primary cilia on endothelial cells overlying and at the shoulder of an atherosclerotic lesion in the bifurcation of an Apoe<sup>−/−</sup> common carotid artery (A-D) and a 3D-reconstruction of this bifurcation (E and F). A-D: Primary cilia are indicated by arrows. The dashed lines depict the internal elastic lamella. The inserts in A and C show alternate sections, stained with hematoxylin and eosin, at lower magnification. The boxed areas in the inserts are enlarged. The boxed areas in A and C are enlarged in B and D, respectively. Acetylated α-tubulin (green), nuclei (red). E and F: luminal reconstruction of the bifurcation with the atherosclerotic lesion (red) and primary cilia (green) visualized. In the frontal view of the complete reconstruction (E) the locations of the sections in A and C are depicted. The clusters of primary cilia on the luminal cell surface are visible in the cross section through the reconstruction (F). STA, superior thyroid artery; ECA, external carotid artery; LA, Lingual artery; ICA, internal carotid artery; CCA, common carotid artery. Scale bar = 4 μm. Magnification A and C = 100x, B and D = 200x, insert A and C = 10x.
Effect of shear stress alteration on primary cilia distribution
To determine if the occurrence of primary cilia is flow-induced, we experimentally-altered shear stress levels and patterns in vivo by changing the geometry of the CCA by cast placement. Upstream and downstream of the cast, where shear stress is low and low and oscillatory, respectively, primary cilia are present. However, the cast region itself, which represents a high shear area, is completely devoid of cilia. A similar distribution was observed in wild-type and Apoe<sup>−/−</sup> challenged CCAs.

Statistical analysis
To quantify the difference in the amount of primary cilia per vessel wall volume between disturbed and undisturbed shear stress areas and between wild-type and Apoe<sup>−/−</sup> mice, the number of cilia per vessel wall volume in specific regions of the aortic arches and CCAs was determined (Tables 1-3). Apoe<sup>−/−</sup> aortic arches contain more cilia compared with wild-type arches. In both unchallenged and challenged CCAs more cilia are present in the disturbed shear region than in the undisturbed shear region. However, there are differences in these disturbed flow areas between wild-type and Apoe<sup>−/−</sup> mice. In the disturbed shear region of the unchallenged CCAs more cilia are present in the wild-type mice than in the Apoe<sup>−/−</sup> mice, while in the disturbed shear region of the challenged CCAs more cilia occur in the Apoe<sup>−/−</sup> than in the wild-type mice. No differences are observed in the undisturbed shear areas between wild-type and Apoe<sup>−/−</sup> mice.

Table 1. Number of cilia in the aortic arch of wild-type and Apoe<sup>−/−</sup> mice

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<th>Wild-type</th>
<th>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<td>Aortic arch</td>
<td>234.4 ± 39.7</td>
<td>416.2 ± 29.5*</td>
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Quantification of the absolute number of cilia per 0.5 mm<sup>3</sup> vessel wall volume in the aortic arch of wild-type (n = 5) and Apoe<sup>−/−</sup> (n = 6) mice. *P = 0.007 vs. wild-type aortic arch

Table 2. Number of cilia in naturally occurring disturbed and undisturbed shear stress areas of wild-type and Apoe<sup>−/−</sup> mice

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<th>Undisturbed</th>
<th>Disturbed</th>
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<td>Wild-type</td>
<td>13.4 ± 5.1</td>
<td>56.8 ± 6.4*</td>
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<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>2.3 ± 1.4</td>
<td>30.5 ± 4.0#</td>
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Quantification of the absolute number of cilia per 0.005 mm<sup>3</sup> vessel wall volume in the disturbed and undisturbed shear stress area of unchallenged CCAs of wild-type and Apoe<sup>−/−</sup> mice (n = 4). *P = 0.002 vs. undisturbed shear stress area of wild-type CCA. #P = 0.001 vs. undisturbed shear stress area of Apoe<sup>−/−</sup> CCA. †P = 0.013 vs. Apoe<sup>−/−</sup> disturbed shear stress area.

Table 3. Number of cilia in experimentally-induced disturbed and undisturbed shear stress areas of wild-type and Apoe<sup>−/−</sup> mice

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<th>Undisturbed</th>
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<td>Wild-type</td>
<td>0.7 ± 0.4</td>
<td>19.0 ± 1.9*</td>
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<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.0 ± 0.0</td>
<td>38.6 ± 2.4†</td>
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Quantification of the absolute number of cilia per 0.005 mm<sup>3</sup> vessel wall volume in the disturbed and undisturbed shear stress area of challenged CCAs of wild-type and Apoe<sup>−/−</sup> mice (n = 3). *P = 0.009 vs. undisturbed shear stress area of wild-type CCA. †P = 0.004 vs. undisturbed shear stress area of Apoe<sup>−/−</sup> CCA. ‡P = 0.003 vs. wild-type disturbed shear stress area.
Discussion

In the present study, we detected primary cilia on endothelial cells of the aortic arch and CCAs of C57BL/6 wild-type and \textit{Apoe}^{-/-} mice. This is the first study to demonstrate primary cilia on mouse endothelial cells, \textit{in vivo}. Endothelial primary cilia were previously detected on cultured human umbilical vein endothelial cells \cite{12} and on endothelial cells of the chicken embryonic heart \cite{6}. Differences between mouse and chicken endothelial primary cilia are reviewed in the Appendix. Primary cilia have been shown to function as shear stress sensors of various cell types \cite{10,12-14}. The mechanism by which endothelial primary cilia function in mechanosensation and whether they are involved in intracellular signaling via Hedgehog and/or Wnt \cite{25} is a subject of ongoing investigation. The first indications of cilia on atherosclerotic lesions were described in the 1980s \cite{26,27}. However, functional roles were not attributed to primary cilia until they were associated to human disorders like Bardet-Biedl syndrome and polycystic kidney disease (PKD) \cite{25}. Dysfunction of renal cilia coincides with PKD. In addition, these patients have cardiovascular abnormalities \cite{28}. Recently, a link between PKD and atherogenesis was demonstrated as patients display endothelial dysfunction and increased carotid intima-media thickness \cite{29}. It is tempting to postulate that PKD patients do not only have dysfunctional renal cilia, but also have dysfunctional endothelial cilia that contribute to the formation of cardiovascular abnormalities.

In mice, endothelial cells with primary cilia are located on and downstream of the aortic valve leaflets, in the inner curvature of the aortic arch, on the upstream side of the branch points of the aorta, and in the sinus of the internal carotid artery. In all these areas flow is disturbed to various extents \cite{30-34}. This correlation is unknown in other areas due to lack of flow data. Disturbed flow is characterized by regions of separation, recirculation, and temporal and spatial gradients of shear stress and is termed oscillatory shear stress. In the unchallenged CCAs significantly more primary cilia are present in the disturbed than in the undisturbed shear stress area. In order to confirm the naturally occurring correlation between the presence of primary cilia and disturbed shear we experimentally challenged CCAs for different shear stress patterns and levels by means of a restrictive cast. Placement of this cast elevates the number of primary cilia in low and oscillatory shear regions, within two days. Interestingly, these are the exact areas where atherosclerotic lesions will develop subsequently when the cast is placed in a diet-induced \textit{Apoe}^{-/-} model for atherosclerosis \cite{21}.

The distribution pattern of primary cilia in \textit{Apoe}^{-/-} mice is similar to that in wild-type mice. However, the number of endothelial cilia in the aortic arch and in the experimentally-induced disturbed shear stress area of \textit{Apoe}^{-/-} mice is significantly higher compared with the aortic arch and experimentally-induced disturbed shear region of wild-type mice. In contrast, in the naturally occurring disturbed flow region of the unchallenged CCAs, preceding lesion formation, less cilia are present in \textit{Apoe}^{-/-} mice than in wild-type mice. Differences between wild-type and \textit{Apoe}^{-/-} mice could be due to variations in several factors, including blood viscosity, flow velocity \cite{5}, and hyperlipidemia, affecting shear stress levels and patterns or ciliation directly. Therefore, it appears that \textit{Apoe}^{-/-} genotype-related differences manifest itself in a decrease in cilia number before lesion formation, while additional flow disturbance by either lumen obstructing atherosclerotic lesion or placement of a restrictive cast increases cilia number when compared to wild-type mice, which is suggestive of an altered shear responsiveness of \textit{Apoe}^{-/-} endothelial cells. Primary cilia are located upstream, downstream, and at the shoulders of atherosclerotic lesions.
These regions are known for a high prevalence of rupture and inflammation. Although high shear has been demonstrated for the upstream side of atherosclerotic lesions, the occurrence of cilia in this region suggests that the local velocity pattern is disturbed. In the Apoe$^{-/-}$ mice atherosclerotic lesions were present in the aortic arch. In the specific occasion of a lesion in the CCA two clusters of primary cilia at the shoulder of the lesion are observed that imply flow disturbance in these regions.

In the above-mentioned syndromes primary cilia are dysfunctional. Whether the endothelial primary cilia in Apoe$^{-/-}$ mice are dysfunctional remains to be elucidated. Nevertheless, at the athero-prone areas endothelial cells, which are ciliated (this study), are primed for atherosclerosis, as the oscillatory shear in these regions increases inflammatory gene expression profiles. Moreover, it affects the barrier function of the endothelium by disruption of the glycocalyx, increasing the permeability of endothelial cells. Additionally, reactive oxygen species can be formed in endothelial cells exposed to oscillatory shear stress due to uncoupling of the eNOS enzyme. These factors can, in the presence of a cardiovascular risk factor, lead to atherosclerosis. How they are connected is unclear but it is apparent that they are all flow-induced. Our data indicate that a correlation exists between the presence of flow sensing primary cilia on endothelial cells at atherosclerotic predilection sites in wild-type and on atherosclerotic lesions in Apoe$^{-/-}$ mice and the onset and development of atherosclerosis, respectively.

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References

8. Yang J, Adamian M, Li TS. Rootletin interacts with C-Nap1 and may function as a physical linker between the pair of centrioles/basal bodies in cells. *Mol Biol Cell.* 2006;17:1033-1040.
Appendix

Results

Differences between mouse and chicken endothelial primary cilia

Beside the corresponding shear stress-dependent primary cilia distribution, differences between mouse and chicken endothelial primary cilia exist. Chicken endothelial cilia are approximately 5 μm in length, while mouse endothelial cilia are approximately 1-2 μm. Furthermore, only acetylated α-tubulin, not detyrosinated α-tubulin, is detected in mouse endothelial cilia, while both are present in the chicken endothelial cilia. Acetylated and detyrosinated α-tubulin are stability isoforms of α-tubulin, the latter being the most stable. The presence of shorter, less stable endothelial primary cilia in mice compared with chickens could be due to inter-species variations. Another possibility is that the length of primary cilia is shear stress level-dependent. Vennemann et al. measured a maximum shear of 50 dyne/cm² in the outflow tract of a chicken embryo, which is comparable to that in the human adult vascular network. Levels of up to 142 dyne/cm² have been calculated for the CCA of adult mice. The relatively high shear stress level in mice could be responsible for the short, less stable cilia.

References
