Primary cilia on endothelial cells : component of the shear stress sensor localized to athero-prone flow areas

Heiden, K. van der

Chapter 9

The Effect of Connexin 37 Deletion in *Apoe*$^{-/-}$ Mice on Endothelial Ciliation

Kim Van der Heiden[1]; Brenda R. Kwak[2]; Robert E. Poelmann[1]; Simone van de Pas[1]; Beerend P. Hierck[1].

[1]Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands; [2]Foundation for Medical Research, Division of Cardiology, Geneva University Hospitals, Geneva, Switzerland.

Abstract

Atherogenesis occurs at flow-determined sites. The shear stress sensing endothelium plays an essential role in this process. Previously, we showed that primary cilia are a component of the endothelial shear stress sensor and demonstrated a shear-related distribution of ciliated endothelial cells in the cardiovascular system. Primary cilia are located at atherosclerotic predilection sites in wild-type mice and on and around atherosclerotic lesions in apolipoprotein-E-deficient (*Apoe*$^{-/-}$) mice, suggesting a role for primary cilia in the onset and progression of atherosclerosis. Not all endothelial cells in these areas are ciliated. Ciliated cells may propagate the flow-induced signal to neighboring (non-ciliated) endothelial cells by intercellular communication through gap junctions, rendering shear stress sensing a coordinated event within a layer of endothelial cells. One of the gap junction structural proteins (connexins) present in endothelial cells is connexin (Cx)37. Deletion of the Cx37 gene (*Gja4*, also known as *Cx37*) results in accelerated atherosclerosis in *Apoe*$^{-/-}$ mice. We hypothesize that a reduction in gap junctional proteins leads to an increase in shear stress sensing primary cilia to maintain proper shear stress sensing in the cell layer and that this increase might be involved in the acceleration of atherosclerosis in *Gja4*$^{-/-}$*Apoe*$^{-/-}$ mice. In this study, we investigated the effect of a reduction of gap junctional proteins on ciliation of a mouse endothelial cell line (clone bEnd.3) and the effect of Cx37 deficiency on endothelial ciliation in the aortic arch of *Apoe*$^{-/-}$ mice. We show that, both *in vitro* and *in vivo*, the prevalence of endothelial primary cilia is identical in the Cx-deprived group compared to the control group. In conclusion, these data demonstrate that a decrease in gap junctional proteins does not affect endothelial ciliation and that the accelerated atherosclerosis in *Gja4*$^{-/-}$*Apoe*$^{-/-}$ mice compared to *Gja4*$^{+/+}$*Apoe*$^{-/-}$ mice is not due to alterations in the prevalence of endothelial primary cilia.

Introduction

Atherosclerosis develops at sites of low and oscillatory (multi- or bidirectional) blood flow, termed athero-prone flow[1]. Blood flow-induced shear stress is sensed and translated into a biological response by the endothelium[2]. The endothelium, therefore, plays a crucial role in atherogenesis. Previously, we showed that the primary cilium is part of the endothelial shear stress sensor[3,4] and related the presence of primary cilia on endothelial cells to atherosclerotic predilection sites[5], suggesting a link between the endothelial shear stress sensing apparatus and atherogenesis. A primary cilium is a rod-like, non-motile, solitary, cellular protrusion that functions as a shear stress sensor of many flow-exposed cells[6-11]. Primary cilia contain microtubules and are connected to the microtubular cytoskeleton of the cell at their basal body, as this is part of the microtubule organizing center (MTOC) of the cell[12,13]. Primary cilia have been shown to respond to shear stress through a protein complex located in the ciliary membrane, i.e., the polycystin (PC) complex. Activation of this complex by flow results in a Ca^{2+} transient[6,11,14] that generates a shear stress response (reviewed by Torres and Harris[15]). However, not all endothelial cells carry a primary cilium, indicating that the primary cilium is not the sole sensor. We demonstrated that the endothelial shear stress response depends on the microtubular cytoskeleton. The microtubular cytoskeleton is connected to the other cytoskeletal elements[16] and together they link all shear stress-responsive components of the endothelial cell, e.g., integrins and the vascular endothelial growth factor receptor 2/vascular endothelial-cadherin/platelet endothelial cell adhesion molecule-1 (VEGFR2/VE-cadherin/PECAM-1) complex (for

excellent reviews see Helmke and Davies[17], Resnick *et al.*[2], Lehoux and Tedgui[18], Li *et al.*[19]). The cytoskeleton undergoes a conformational change upon shear stress[17,20,21] and transduces the force throughout the cell to all these shear-responsive cell components[22]. Primary cilia sensitize the endothelial cells for shear stress[3], probably by amplifying cytoskeletal deformation. In conclusion, endothelial primary cilia play a double role in endothelial shear stress sensing[23]. (I) In an immediate response to ciliary bending PCs mediate a Ca^{2+} transient[11]. (II) The cilium amplifies the shear-induced conformational change of the cytoskeleton, which leads to a prolonged effect on gene expression[3].

Even in the highly ciliated areas not all endothelial cells are ciliated. Ciliated cells have been shown to propagate the shear-induced rise in intracellular Ca^{2+} to neighboring cells[6,11], indicating that shear stress sensing is not confined to a single cell but it is a synchronized process within a layer of endothelial cells. Functionally blocking gap junctions results in an abrogation of Ca^{2+} wave propagation, suggesting that gap junctions are responsible for this form of intercellular communication[6]. Gap junctions consist of two hemichannels or connexons, which in turn consist of six connexins. The connexin (Cx) family contains several members which can form homomeric and heteromeric channels with different properties. Three connexins have been described in endothelial cells, i.e., Cx37, Cx40, and Cx43 [24]. Polymorphisms in Cx37 have been associated with a higher risk for atherosclerosis in man (reviewed by Chanson and Kwak[25]). Furthermore, absence of Cx37 accelerates atherosclerosis in apolipoprotein-E-deficient (*Apoe*$^{-/-}$) mice[26]. We hypothesize that a reduction in connexin expression, which may lead to a diminished propagation of the shear-induced signal throughout the endothelial cell layer, will cause an increase in main shear stress sensors, i.e., primary cilia, to maintain proper shear stress sensing of the cell layer. Considering the previously established correlation between ciliated endothelial cells and atherosclerosis[5], this increase in primary cilia could be involved in the accelerated atherosclerosis in Cx37-deficient *Apoe*$^{-/-}$ mice[26].

In this study we test this hypothesis by analyzing endothelial ciliation in an endothelial cell line with reduced Cx levels. In addition, the effect of deleting the mouse Cx37 gene (*Gja4*, also known as *Cx37*) on endothelial ciliation in *Apoe*$^{-/-}$ mice was investigated.

Materials and Methods

bEnd.3 cells

PymT-transformed mouse endothelial cells (clone bEnd.3) express Cx37, Cx40, and Cx43. bEnd.3 cells transfected with p3243H7Et (bEnd.3/3243H7), an expression vector that encodes a chimeric polypeptide that exhibits dominant negative inhibitory activity on gap junction channels, have reduced expression levels of Cx37 and Cx43[27]. bEnd.3 and bEnd.3/3243H7 cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mmol/L L-Glutamine (Invitrogen), 1x antibiotic/antimyotic solution (Invitrogen). Confluent cells on gelatin-coated glass coverslips were subjected to an oscillatory flow of 0.5 ± 2.0 dyne/cm^2 at a frequency of 2Hz for 5 hrs at 37°C and 5% CO_2. To this aid, a parallel plate flow chamber with a 2 cm^2 flow area (height of 0.25 mm; Glycotech) was connected to a peristaltic pump (Masterflex) and an osci-flow™ controller (Flex cell™ international corporation). The flow was recorded with a TS410 Transonic flowmeter (Transonic Systems Inc.). Signals were digitized with a Powerlab (AD Instruments) recorder to calculate the mean, minimum, and maximum shear stress. Directly after flow exposure cells were used for primary cilia quantification (static control n = 4, flow-exposed

n = 2). The number of ciliated and non-ciliated cells of a minimum number of 200 cells was quantified for each coverslip.

Animals

Cx37-deficient (*Gja4*$^{-/-}$) and *Apoe*$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, USA), both on a C57BL/6 background, were interbred. *Gja4*$^{+/-}$*Apoe*$^{+/-}$ offspring was subsequently bred to obtain *Gja4*$^{+/+}$*Apoe*$^{-/-}$ (n = 4) and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ (n = 4) female mice. Mice were placed on a Clinton/Cybulsky high fat diet (Research Diets #D12108) at 13 weeks of age for 10 weeks. Mice were then sacrificed and perfusion fixed via the left ventricle with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). The aortic arch was removed and subsequently fixed overnight in 2% PFA in PBS, after which it was dehydrated in graded ethanol and embedded in paraffin. Specimens were sectioned at 5 µm and mounted serially. The Geneva University Animal Care and Use Committee and the local veterinary office, in accordance with Swiss guidelines and regulations, approved all mouse experiments.

Immunofluorescence

Primary cilia were visualized with a monoclonal antibody directed against acetylated α-tubulin (clone 6-11B-1, Sigma-Aldrich Chemie)[28]. Fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (DAKO) was used as secondary antibody. After deparaffination, the sections were stained and examined as described previously[4]. Fluorescence was detected by a Leica IRBE microscope equipped with a BD CARVII Confocal Imager (Imsol). Each data set collected on the microscope was processed with Image Pro 6.2 (MediaCybergenic).

Statistical analysis

The number of primary cilia in a delineated region of the aortic arch was quantified. The segment between the branching point of the brachiocephalic artery and the branching point of the left subclavian artery, excluding the three branch points, was analyzed. The number of cilia was quantified in every fifth tissue section and multiplied by 5 to estimate the absolute number of cilia in the delineated area. Subsequently, the medial plus the intimal volume of the vessel wall was estimated with the Cavalieri technique[29], a point counting method using a grid. The number of cilia normalized for a distinct volume of the vessel wall, i.e., 0.5 mm^3, was determined and an independent-samples *t*-test was performed (SPSS; SPSS Inc.). The aortic arch of *Gja4*$^{+/+}$*Apoe*$^{-/-}$ mice was compared to *Gja4*$^{-/-}$*Apoe*$^{-/-}$ mice (Table 1). Data are presented as mean ± standard error of the mean.

Results

bEnd.3

To determine whether a reduction in connexin expression affects endothelial ciliation, bEnd.3 and bEnd.3/3243H7 cells were analyzed. bEnd.3/3243H7 cells have reduced expression levels of Cx37 and Cx43 compared to bEnd.3 cells. Acetylated α-tubulin is present in both cell types (Fig. 1a,c). Acetylation of the cytoskeletal microtubules is irregular and the MTOC is not discernible. Primary cilia are not observed under static conditions. In a previous *in vitro* study, we demonstrated that oscillatory shear stress induces endothelial ciliation (Chapter 5). Therefore, we subjected both cell lines to

oscillatory flow with a maximum of 2.5 dyne/cm^2 and analyzed endothelial ciliation. Even after a 5 hr exposure to oscillatory flow primary cilia are not present (Fig. 1B,D). Staining for acetylated α-tubulin is identical between cell types and flow conditions and the organization of the cytoskeletal microtubules is not affected by the reduced expression of Cx37 and Cx43.

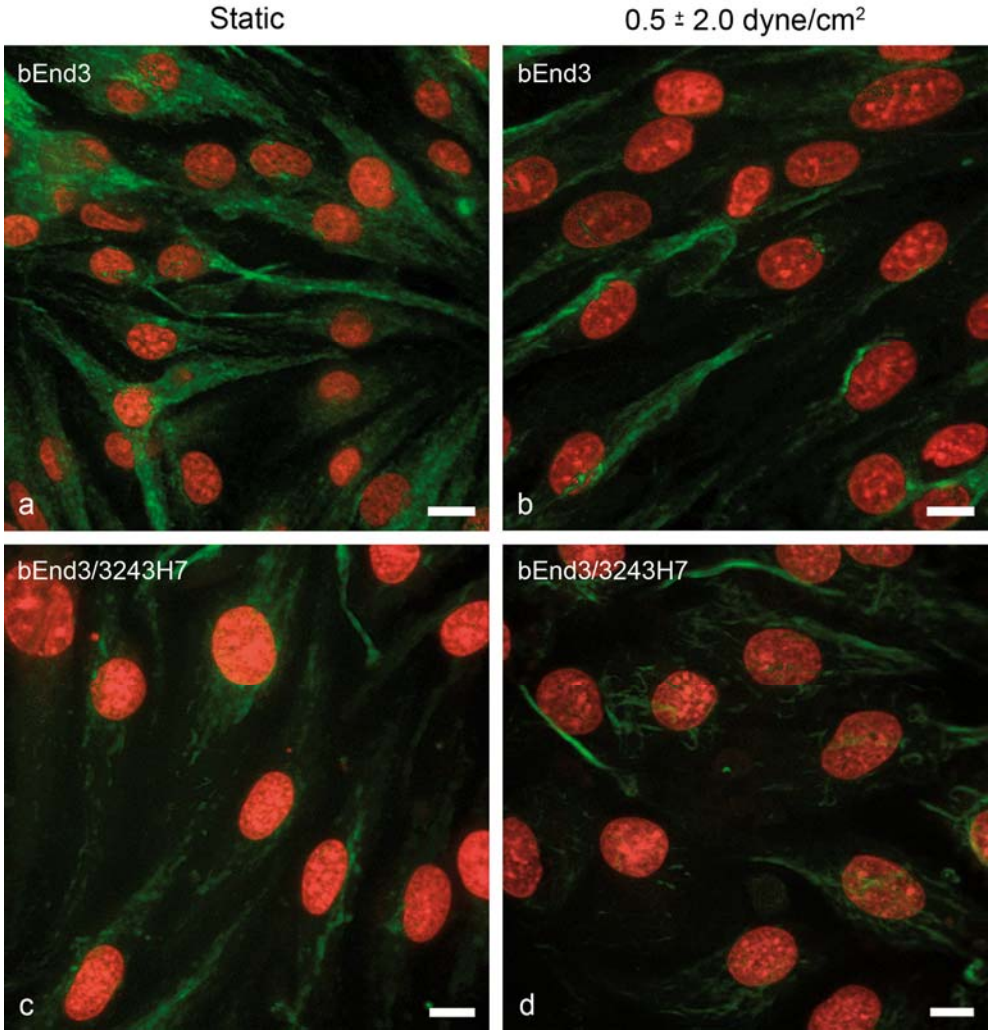

Figure 1. Confocal images of cultured bEnd.3 cells (a,b) and bEnd.3/3243H7 cells (c,d) under static (a,c) or flowed (b,d) conditions. Note that no primary cilia are detected. Acetylated α-tubulin (green), nuclei (red; 7-AAD). Scale bar = 10 μm.

Effect of Cx37 deficiency on the prevalence of primary cilia in *Apoe*$^{-/-}$ mice

Previously, we showed that endothelial cells in the aortic arch of wild-type and *Apoe*$^{-/-}$ mice are ciliated[5]. To investigate whether a reduction in connexin expression affects endothelial ciliation *in vivo* we analyzed the effect of Cx37 deficiency on the distribution pattern and the number of endothelial primary cilia in the *Apoe*$^{-/-}$ mice. The endothelial cell surface of the aortic arch of *Gja4*$^{+/+}$*Apoe*$^{-/-}$ and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ adult mice was analyzed for primary cilia by immunofluorescent staining for acetylated α-tubulin. Acetylated α-tubulin is found in the primary cilium, MTOC, and microtubular cytoskeleton. Primary cilia protruding from the luminal cell surface were found in both strains and measured 1-2 μm in length (Fig. 2). In the *Gja4*$^{+/+}$*Apoe*$^{-/-}$ (Fig. 2a,c,e) and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ (Fig. 2b,d,f) aortic arch ciliated endothelial cells are located upstream and downstream of the atherosclerotic lesions that are present in the inner curvature of the aortic arch (Fig. 2a-d) and on the upstream side of the branch points of the aorta with the brachiocephalic artery (Fig. 2e), the left common carotid artery, and the left subclavian artery (Fig. 2f). Occasionally a ciliated endothelial cell is found in the descending aorta, while the endothelial cells in the outer curvature of the aortic arch and on the downstream side of the aortic branch points are devoid of primary cilia. The primary cilia distribution patterns of the *Gja4*$^{+/+}$*Apoe*$^{-/-}$ and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ aortic arch are identical.

To quantify potential differences in the amount of primary cilia between *Gja4*$^{+/+}$*Apoe*$^{-/-}$ and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ mice, the number of ciliated endothelial cells in a delineated region of the aortic arch was determined, i.e., the segment between the branching point of the brachiocephalic artery and the branching point of the left subclavian artery. This number was normalized for the estimated vessel wall volume (Table 1), which was similar between *Gja4*$^{+/+}$*Apoe*$^{-/-}$ and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ mice (not shown). No differences in the prevalence of primary cilia between the *Gja4*$^{+/+}$*Apoe*$^{-/-}$ and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ mice are present.

Table 1. Number of cilia in the aortic arch of *Gja4*$^{+/+}$*Apoe*$^{-/-}$ and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ mice

	Aortic arch
Gja4$^{+/+}$*Apoe*$^{-/-}$	437.28 ± 90.92
Gja4$^{-/-}$*Apoe*$^{-/-}$	453.16 ± 72.30

Quantification of the absolute number of cilia per 0.5 mm^3 vessel wall volume in the aortic arch of *Gja4*$^{+/+}$*Apoe*$^{-/-}$ (n = 4) and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ (n = 4) mice. There are no significant differences in the number of primary cilia between *Gja4*$^{+/+}$*Apoe*$^{-/-}$ and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ mice (P = 0.896).

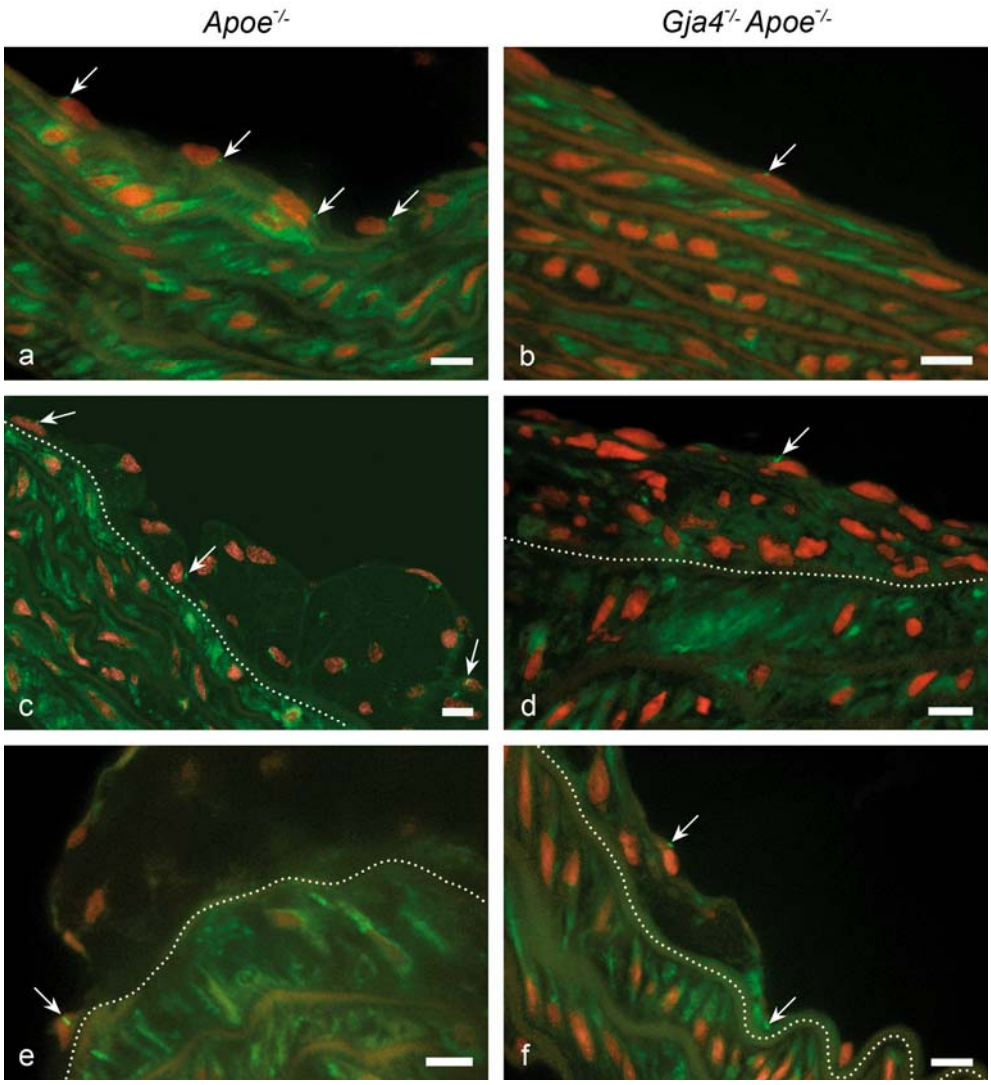

Figure 2. Primary cilia on the endothelial cell layer in the inner curvature of an aortic arch (a-d), overlying an atherosclerotic lesion in C and D, and at the shoulder of a lesion in the brachiocephalic artery (Fig. 1e) and in the left subclavian artery (Fig. 1f) of an *Gja4*$^{+/+}$*Apoe*$^{-/-}$ (a,c,e) and *Gja4*$^{-/-}$*Apoe*$^{-/-}$ (b,d,f) mouse. Primary cilia are indicated by arrows. The dashed lines designate the internal elastic lamella. Acetylated α-tubulin (green), nuclei (red; propidium iodide). Scale bar = 10 μm.

Discussion

In the present study, we analyzed the effect of reduced Cx expression in an endothelial cell layer on the ciliation of these cells. The endothelial response to shear stress is dependent on an intact microtubular cytoskeleton[3], which undergoes a conformational change upon exposure to shear stress[17,20,21]. The primary cilium, which is only present on endothelial cells subjected to low and oscillatory shear stress[4,5], is connected to the microtubular cytoskeleton at its basal body and sensitizes endothelial cells for shear stress[3], probably through the amplification of shear-induced cytoskeletal deformation. The primary cilium plays an additional role in endothelial shear stress sensing through the PC complex located in the ciliary membrane which mediates a Ca^{2+} transient[11]. Ciliated cells have been shown to propagate the Ca^{2+} signal to neighboring cells via gap junctions[6,11], rendering shear stress sensing by the endothelial cell layer a synchronized process rather than a single cell phenomenon. Ciliation of contiguous cells is, therefore, unnecessary and indeed only a subset of endothelial cells in areas of low and oscillatory shear stress carries a primary cilium[4,5]. We hypothesized that a reduction in gap junctional proteins, which may result in a diminished propagation of the shear-induced signal, would cause an increase in main shear stress sensors, i.e., primary cilia, to maintain proper shear stress sensing in the endothelial cell layer.

To test this hypothesis, we analyzed ciliation of bEnd.3 and bEnd.3/3243H7 cells. Endothelial gap junctions are formed by homomeric or heteromeric channels of Cx37, Cx40, and Cx43 [24]. bEnd.3 cells express all endothelial connexins, while bEnd.3/3243H7 cells have reduced levels of Cx37 and Cx43 [27]. However, both cell types are non-ciliated, even after exposure to oscillatory shear stress, which has been shown to induce endothelial ciliation (Chapter 5). We stained for acetylated α-tubulin, which is a post-translational modified form of the α-tubulin subunit of microtubules generally present in primary cilia and the centrioles of the MTOC[28]. The MTOC, which is present in all cells, is not discernible in bEnd.3 and bEnd.3/3243H7 cells, suggesting that microtubules without acetylated α-tubulin are present in the MTOC. As a consequence, we can not rule out the presence of non-acetylated α-tubulin containing primary cilia. More likely, however, is that the bEnd.3 cell line is not ciliated, as it has been described that the ability of endothelial cells in culture to present a primary cilium is lost after multiple passages[30].

Therefore, we turned to an *in vivo* model in which we previously demonstrated the occurrence of ciliated endothelial cells. Endothelial primary cilia are present at atherosclerotic predilection sites in wild-type mice and at the shoulders of atherosclerotic lesions in *Apoe*$^{-/-}$ mice[5], suggesting a correlation between endothelial primary cilia and atherosclerosis. Diminished connexin expression has been associated to atherosclerosis as well. In the absence of Cx37, atherosclerosis is accelerated in *Apoe*$^{-/-}$ mice. Cx37 deficiency affects the activity of hemichannels on leukocytes[26]. Cx37 hemichannels are normally present in monocytes and macrophages and Cx37 deficiency leads to an increased recruitment of monocytes and macrophages into the vessel wall and consequently accelerates atherosclerosis. However, if alterations in intercellular communication affect endothelial ciliation, primary cilia might be involved in the accelerated atherosclerosis. Therefore, we analyzed endothelial ciliation of the aortic arch of *Gja4*$^{+/+}$*Apoe*$^{-/-}$ and *Gja4*$^{-/-}$ *Apoe*$^{-/-}$ mice. Consistent with our previous study[5], primary cilia protruding from the endothelial cell are located upstream and downstream of the atherosclerotic lesions in the inner curvature of the aortic arch and on the upstream side of the branch points of the aorta. However, the distribution pattern and number of primary cilia in *Gja4*$^{+/+}$*Apoe*$^{-/-}$ and *Gja4*$^{-/-}$

Apoe$^{-/-}$ mice is similar. A possible explanation is that Cx37 deficiency does not affect the propagation of the shear-induced Ca^{2+} signal in the endothelial cell layer as the other connexins, i.e., Cx40 and Cx43, are still present in *Gja4*$^{-/-}$*Apoe*$^{-/-}$ mice and sustain intercellular communication. Furthermore, communication might be maintained through transduction of the shear-induced conformational change in the cytoskeleton to neighboring cells via cytoskeletal linkage at the cell-cell junctions. Although we can not eliminate the possibility that an absence of trans-endothelial communication affects ciliation, due to the redundancy of Cx40 and Cx43 in our model, we can conclude that the accelerated atherosclerosis in *Gja4*$^{-/-}$*Apoe*$^{-/-}$ mice compared to *Gja4*$^{+/+}$*Apoe*$^{-/-}$ mice is not due to alterations in the prevalence of primary cilia.

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