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Endothelial Primary Cilia Are Dispensible for Development but Restrict Atherosclerosis in Mouse

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Endothelial Primary Cilia Are Dispensible for
Development but Restrict Atherosclerosis in the Mouse

by
Colin Jelinek Dinsmore

DISSERTATION

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DOCTOR OF PHILOSOPHY

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of the

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Acknowledgments

It is said it takes a village to raise a child. A thesis is no different (and, depending on how one calculates, the thesis is likely more expensive). I am fortunate enough to have the very best of villages supporting me, without which this work would never have come to fruition.

First and foremost I need to thank my family. My parents Richard and Joanie were unwaveringly supportive throughout my graduate career. They did not bat an eye when I decided to move to San Francisco and pursue biology as a career and have since taken a keen interest in my research, themselves. It was always reassuring to know they have my back, whether in the form of an encouraging word or an invitation to raid my dad's frequent flyer miles to come home at Christmas. My sister Mary, an excellent biologist herself, has been so much fun to talk to over the years about science, graduate school, students, teaching, funding, and so on. Academic science is such a peculiar world I am lucky to have someone else in the family who understands this strange thing we're both a part of. I was really excited and flattered she flew out to San Francisco for my thesis talk and I look forward to reciprocating for her defense in a few years.

My girlfriend Anju was my lifeline to normality and deserves acknowledgement for the many ways the enriched my life and kept me sane. The world of science can be very insular and have distorting effects on how one views time, regular work-hours, and days of the week. Anju was really understanding of when I needed to put in long hours but also a keen sense of when I was spinning my wheels and needed some time away. It was a boon to take weekend trips together or simply go home at a regular hour and have dinner with her.

Beyond and more important than the therapeutic influence she had on my scheduling, Anju is a great person. She's fun and funny and insightful and clever and I cannot thank her enough for being in my life.

Many thanks and a great deal of credit are due to my Tetrad classmates. They were an excellent group of compatriots and could always be relied upon to help tackle problem sets, edit proposals, provide feedback on practice talks, share reagents and protocols, and occasionally share a beer or two. I look forward to the day we're all famous scientists and I get to introduce them at seminars.

My thesis committee, Didier Stainier, Wallace Marshall, and Orion Weiner, did their best to keep me on the straight and narrow during my graduate career. They were largely successful. My committee was always supportive of my project during committee meetings and did an excellent job of suggesting insightful experiments and controls while maintaining a big-picture view that clarified what was novel and interesting about my research. Beyond our committee meetings, they were tremendous in myriad ways from being game for collaborations to playing pick-up soccer on the quad.

I had a number of collaborators that we're wonderful to work with. Ann Zovein, Ben Barres, Rong Wang, Mark Ginsberg, and Per Vogelstrand all shared mice and expertise that were essential to my project. Gillian Griffiths and Maike Heffner initiated a collaboration with the Reiter lab to study the hedgehog pathway in T-cells. I was proud to be included in this project and they were a joy to work with.

The Reiter lab was and continues to be a wonderful assemblage of expertise and personalities. I could rely on them for technical and emotional support from Day 1 in the lab until Day... well, it's perhaps best not to count. Sharing my time in the lab with them has

made me better at designing and executing experiments. Learning about their work, meanwhile, gave me a broader appreciation for the amazing variety of questions and techniques that abound in biology. I am excited to stay in touch with them in the future and read about their amazing discoveries.

I would also like to acknowledge the organizers of the Developmental Biology Journal Club, which included my labmates Bill and Laura, as well as Jane, Erin, Philipp, David, and Stephanie from the Schneider and Stainier labs. This gave me the chance to hear about new research and, via the symposium we organized, meet numerous scientists, an opportunity that enhanced my thinking about my project and about science more generally. It was a ton of work to manage the journal club and organize a symposium every year, but they made it a lot of fun and handled the chaos with aplomb. I would have gone nuts without their talent and calm.

Lastly, there are not enough words for my advisor, Jeremy Reiter. I was tempted to paraphrase Douglas Adams and simply write, "So long and thanks for all the mice," but Jeremy deserves more than my urge to be glib. Jeremy is a brilliant scientist and it has been a pleasure to do my thesis work in his lab. I can go through the list of standard scientist / advisor qualities and check them all off: Jeremy is really smart and asks insightful questions, he writes well and is generous with resources, and he's always willing to talk about projects and lend his insight. All of these virtues are important and in each case I've learned a lot from Jeremy. What I find unique about him, though, might instead be considered his intangibles. Things that do not show up on a stat sheet. Dynamism maybe comes closest. Jeremy is really excited about science. All kinds of science. I would be hard-pressed to name an instance in which I could describe him as glum, even when writing

grants, doing mini-preps (yes he still clones – a lot), or scheduling meetings. Over the course of a graduate student's career there are lots of ups and downs and Jeremy's ability to remain optimistic was just as important as his encyclopedic knowledge of mouse genetics. He can infect those around him with an excitement that is also purposeful, rather than simple blind optimism. Jeremy's ability to inspire as well as his merits as a scientist combined to make him a really effective advisor. Jeremy, thanks for making me a better scientist. And for all the mice.

Abstract

Primary cilia are membrane-bound microtubule-based structures present on most mammalian cells and are important for intercellular signaling. Cilia are present on a subset of endothelial cells, which form the innermost layer of blood vessels, where they project into the vessel lumen and are implicated as mechanical sensors of blood flow. To test the *in vivo* role of endothelial cilia, I conditionally deleted *Ift88*, a gene required for ciliogenesis, in endothelial cells of mice. I found that endothelial primary cilia are dispensable for vascular development. Removing endothelial cilia increased atherosclerosis in $Apoe^{-/-}$ mice fed a western-type diet, indicating that cilia protect against atherosclerosis. Consistent with this result, $Apoe^{-/-}$ mice lacking endothelial cilia had increased inflammatory gene expression.

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Introduction

Primary cilia are membrane-bound microtubule-based signaling organelles that protrude from the apical surface of cells. They are constructed by a system of intraflagellar transport and extend from mother centrioles, which in this context are known as basal bodies. Primary cilia are immotile, occur at a frequency of one or zero per cell and can be found on most vertebrate cell types. Primary cilia play important roles in chemosensation in a wide variety of systems (1). They are best known for their obligate role in vertebrate Hedgehog (Hh) signaling, but also function in other intercellular signaling pathways, including PDGFRa and some GPCR signaling (1). In addition to chemosensation, cilia are thought to function in some contexts as mechanosensors. For example, kidney epithelial cells may use cilia to sense urine flow (2-5).

Endothelial cells (ECs) line the lumen of blood vessels and are ciliated both *in vitro* and *in vivo* but the function of these cilia has not been fully explored. The presence of primary cilia on ECs suggests they could respond to Hh signals. The Hh pathway is important for cardiovascular development but whether or not Hh acts directly on ECs remains controversial. $Smo^{-/-}$ mice, which are incapable of transducing Hh signals, have defects in cardiac morphogenesis (6) and dorsal aorta formation (7). Hh ligands can alter EC behavior *in vitro* arguing that ECs respond directly to Hh signals (8, 9). In addition, in adult mice the Hh pathway regulates vessel permeability in the brain (10). However, the best-characterized role for Hh in vertebrate cardiovascular development is indirectly via regulation of Notch and VEGF activity (11-13) and the Hh pathway is not required in ECs for vascular development (14) (data not shown).

If not Hh signaling, what function might EC cilia might have? EC cilia possess the polycystic kidney disease-associated proteins Pkd1 and Pkd2 (15, 16). Pkd1 is a transmembrane protein that may function as a mechanosensor which couples to and regulates Pkd2, a TRP-family calcium channel (17). Cultured mouse aortic endothelial cells flux calcium and synthesize nitric oxide in response to the onset of flow (15). Critically, this response is dependent on both Pkd proteins and primary cilia (15). It has therefore been proposed that EC cilia are mechanosensors whose primary purpose is to sense blood flow (18) and a recent report described how cilia and Pkd proteins sense blood flow to regulate vascular morphogenesis in zebrafish (19).

ECs and local flow conditions control atherosclerosis, a slowly progressive inflammation and narrowing of arteries that causes most myocardial infarctions, peripheral vascular disease and strokes. Cardiovascular disease is the leading cause of mortality among Americans. Atherosclerosis is promoted by hypercholesterolemia, though the disease is affected by additional factors such as diabetes, smoking and genetics. Hypercholesterolemia leads to EC activation and accumulation of oxidized low-density-lipoprotein in the sub-endothelial space of the vessel wall causing a complex inflammatory response involving ECs, macrophages, T cells, dendritic cells, smooth muscle cells and likely other cell types. Early lesions grow into larger plaques that protrude from the vessel wall and this growth involves infiltration of blood cells – especially macrophages – and local proliferation of smooth muscle cells. As plaques grow, they can diminish blood flow and cause ischemic heart disease, including myocardial infarction, downstream of the occlusion. Plaques can also rupture and the resulting thrombi can cause strokes (20, 21).

Atherosclerosis does not occur uniformly throughout the vasculature. Plaques occur most commonly at areas of high curvature and branch points, where blood flow is more turbulent (22, 23). Elegant studies *in vitro* and *in vivo* have shown that disturbed, non-laminar flow is sufficient to drive inflammatory EC gene expression (24) and atherosclerosis (25), respectively. Intriguingly, primary cilia are not found on all ECs, but occur most commonly at these atherosclerosis-prone locations (26) (Figure 1). The more frequent occurrence of cilia on ECs in areas of turbulent flow is consistent with the *in vitro* observation that laminar flow disassembles EC cilia(27). Furthermore, primary cilia are found in association with atherosclerotic plaques in mice (26) and humans (28-30). Finally, autosomal dominant polycystic kidney disease (ADPKD) patients, who have heterozygous mutations in *PKD1* or *PKD2*, suffer from cardiovascular anomalies such as hypertension, left-ventricular hypertrophy, increased carotid intima-media thickness and perhaps most importantly clinically, aneurysm (31). Some of these effects can be linked to dysregulation of the renin-angiotensin system caused by kidney cysts (31). However, many patients exhibit endothelial dysfunction and increased carotid intima-media thickness, both warning signs for atherosclerosis, even before signs of renal dysfunction or hypertension (31).

These clinical findings raise the possibility that impaired PKD1/2 function in tissues other than kidneys could promote atherosclerosis and that EC cilia could affect vascular function. To date, the role of endothelial cilia has not been explicitly tested in a mammal. In this study, I use a genetic approach to examine the *in vivo* role of endothelial primary cilia during development and in the adult. I assess both intrinsic vascular properties such as angiogenesis and polarity as well as the development of a clinically relevant cardiovascular disease: atherosclerosis.

Endothelial Primary Cilia Are Dispensable for Vascular Development

In order to characterize the distribution of cilia within the aorta, I stained whole-mount mouse aortas for the ciliary marker Arl13b. ECs were identified by co-staining for Pecam1 (CD31). Cilia projected from the apical surface of a subset of ECs into the lumen of the vessel. The frequency of ciliated cells depended on location within the vasculature. Cilia were more common on the lesser curvature (more caudal and ventral side) of the aortic arch and less common on the greater curvature (more rostral and dorsal side) (Figure 1A and 1B). The distribution of ciliated cells was more unifom in the descending thoracic aorta, where blood flow is less turbulent. These findings are concordant with a previous study that found that cilia are enriched in curved and branched regions of the aorta (26).
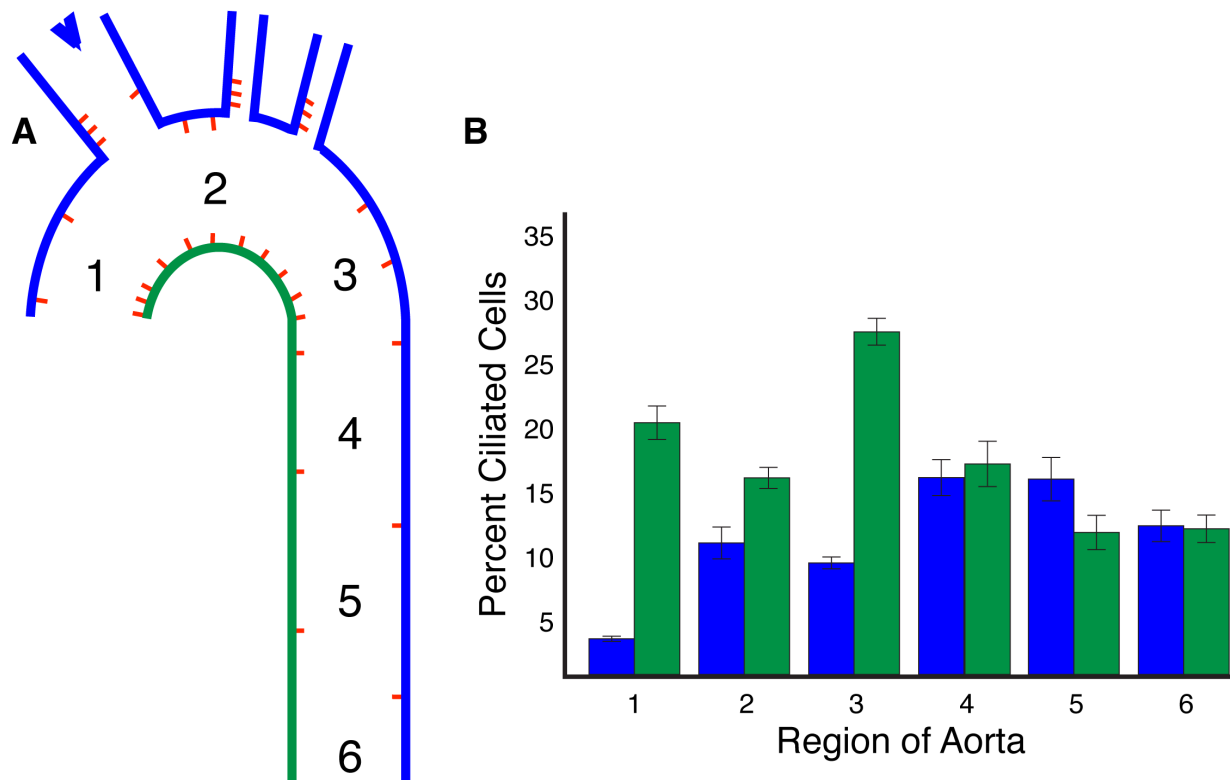
Figure 1. Endothelial primary cilia are asymmetrically distributed in the adult mouse aorta. Primary cilia are enriched at branch points and the greater curvature of the aorta, schematized in (A) and quantified in (B). The color in (A) indicates which side of the aorta is quantified in (B). Numbers indicate in (A) which region of the aorta is quantified in (B). Error bars are +/- 1 S.E.M.

In order to study the role of endothelial cilia *in vivo*, I used a conditional loss of function approach. I developed mice lacking EC cilia by deleting *Intraflagellar transport 88* (*Ift88)*, a gene essential for ciliogenesis and cilia maintenance, specifically in ECs using *Tek:Cre*. *Tek:Cre* is expressed by E7.5 in angioblasts (32) and induces recombination in ECs and the hematopoietic lineage. In order to confirm that *Tek:Cre* was expressed where and when I expected, I crossed this mouse with a $ROSA^{mT/mG}$ reporter mouse in which all cells express a membrane targeted tdTomato red fluorescent protein, but in the presence of Cre protein lose tdTomato expression and instead express membrane targeted EGFP. The *Tek:Cre* transgene recombined the $ROSA^{mT/mG}$ reporter allele by gestational day E7.5, indicating that *Tek:Cre* activity is early and concomitant with the differentiation of angioblasts (Figure 2A). Reporter expression was retained throughout development (Figure 2B-D) indicating *Tek:Cre* labeling is robust rather than mosaic within the endothelial lineage.
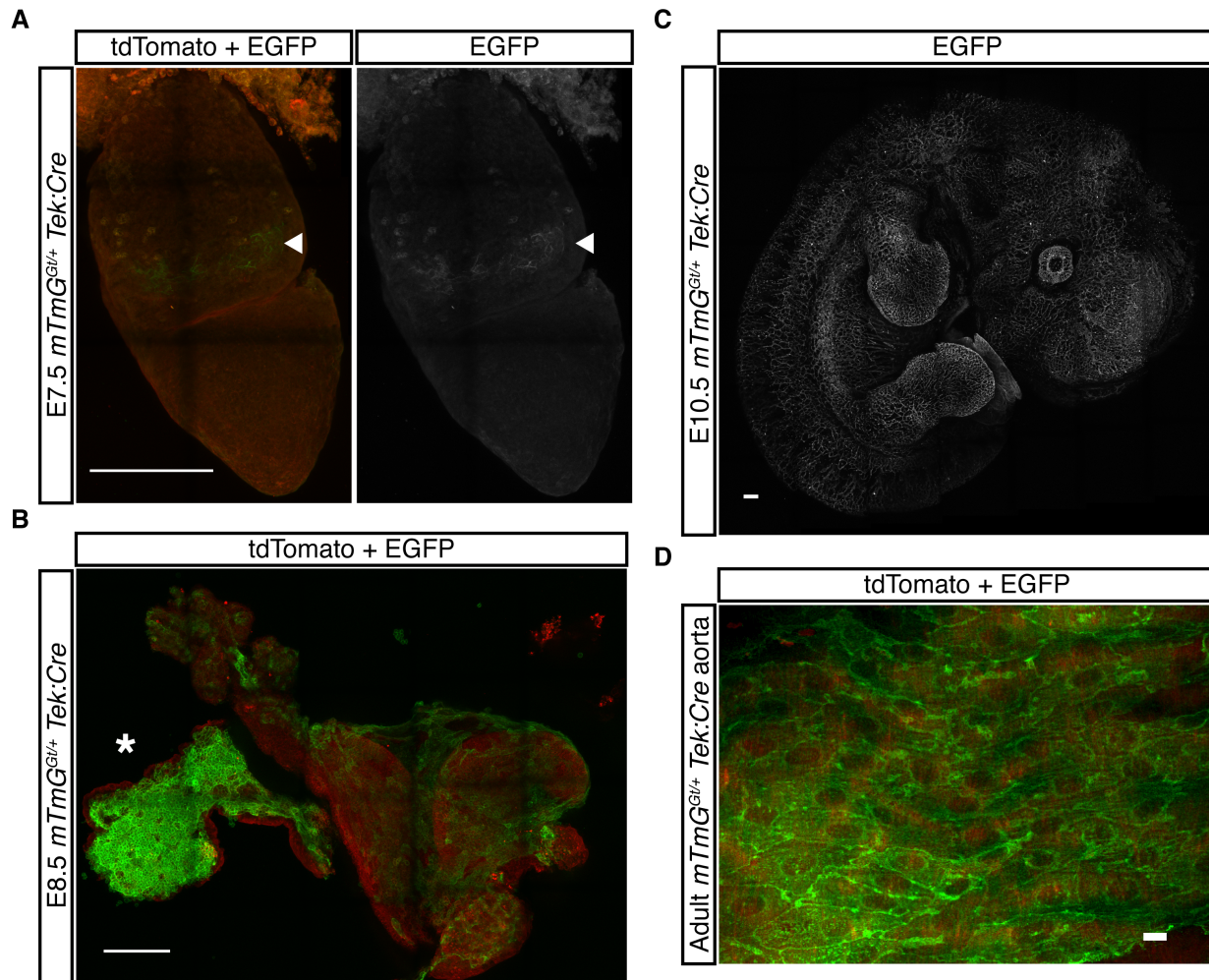
Figure 2. *Tek:Cre* robustly marks the endothelial lineage. *Tek:Cre* activates EGFP expression in *Rosa26^{mTmG}* reporter mice be embryonic day 7.5 (A). This expression is robust in throughout development within the vasculature shown here at embryonic days 8.5 (B) and 11.5 (C). Expression is retained in adult aortic endothelial cells (D).

Ift88$^{C/-}$ Tek:Cre mice were viable, fertile and born in normal ratios (data not shown). This indicated that loss of EC cilia was dispensable for development in mouse. I confirmed absence of cilia by immunofluorescent staining in adult aortas (Figure 3A). To confirm that EC cilia are dispensable for vascular development, I deleted a different gene essential for ciliogenesis, *Kif3a,* in ECs (Figure 3B). *Kif3a$^{C/-}$ Tek:Cre* mice also develop normally and completely lack EC cilia, confirming that EC cilia are not essential for formation of the vasculature.
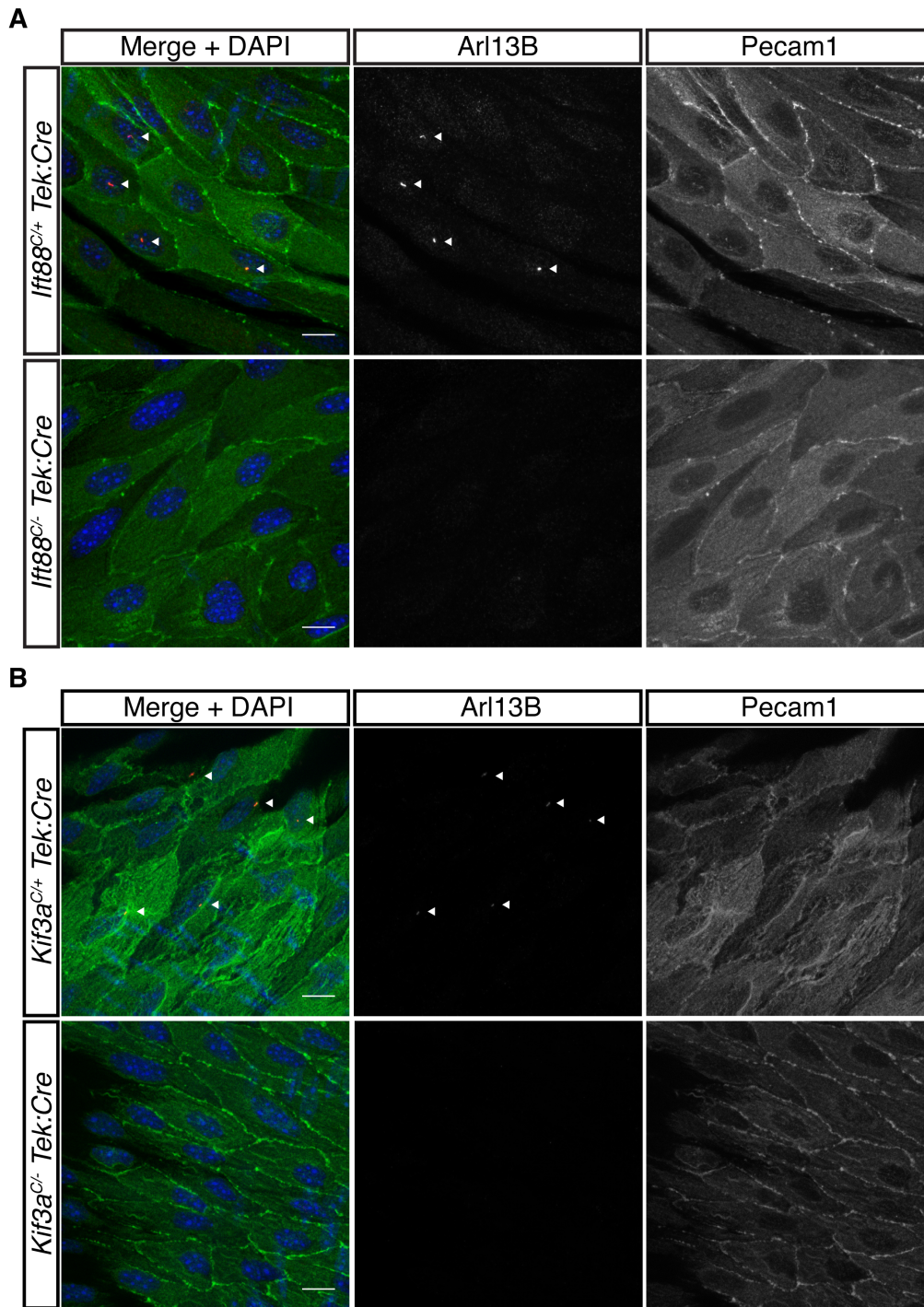
Figure 3. Endothelial cilia (A and B, top row) are efficiently removed by *Tek:Cre* mediated deletion of *Ift88* (A, bottom row) or *Kif3a* (B, bottom row). Cilia are marked by staining with Arl13B (middle panels, red in merge) and endothelial cells are marked by Pecam1 (right panels, green in merge). Arrowheads highlight cilia. Scale bar is 10μm.

Most vertebrate Hh signaling depends on primary cilia and the absence of a developmental phenotype in mice lacking EC cilia suggests that EC Hh signaling is not required for vascular development. To explicitly test whether EC Hh signaling is required for vascular development, I generated $Smo^{C/C}$ *Tek:Cre* mice which lack Smoothened, a critical component of Hh signaling (6), in ECs. $Smo^{C/C}$ *Tek:Cre* mice were also viable and did not display any discernable vascular phenotype (data not shown) indicating Hh signaling, like primary cilia, is not required in mouse ECs during development.

Although EC primary cilia are clearly not required during mouse development, they could have a more subtle effect on vascular processes, such as angiogenesis or cellular polarity. Angiogenesis, the growth of new blood vessels from existing blood vessels, is regulated by a variety of intercellular signaling pathways (33, 34) as well physical forces such as blood flow (35, 36). Primary cilia, as mediators of intercellular signaling and putative blood flow sensors could be involved in this process. A recent report in zebrafish suggested this was the case (19). Angiogenesis is required for development(37-39) and mice lacking EC cilia survive, indicating EC cilia are not strictly required for this process. However, there are many examples of mutations in which angiogenesis is still functional enough to give rise to a living organism, but is nonetheless defective in some way. For instance the *Fz4 Tek:Cre* conditional mouse mutant, which deletes a Wnt pathway receptor in ECs, is viable but has marked angiogenesis defects (40).

To explore possible defects in angiogenesis, I examined the postnatal growth of the retinal vasculature. This is a well-established assay for angiogenesis (41) and one affected in the *Fz4* conditional mutant discussed above. I quantified the outgrowth of retinal vasculature in postnatal day 5 (the date of birth is P0) mice, but did not find a significant difference between

control and *Ift88$^{C/-}$ Tek:Cre* mice (Figure 4A-D). These results indicate that, unlike in the zebrafish (19), mouse endothelial primary cilia and Hh signaling are dispensable for development.
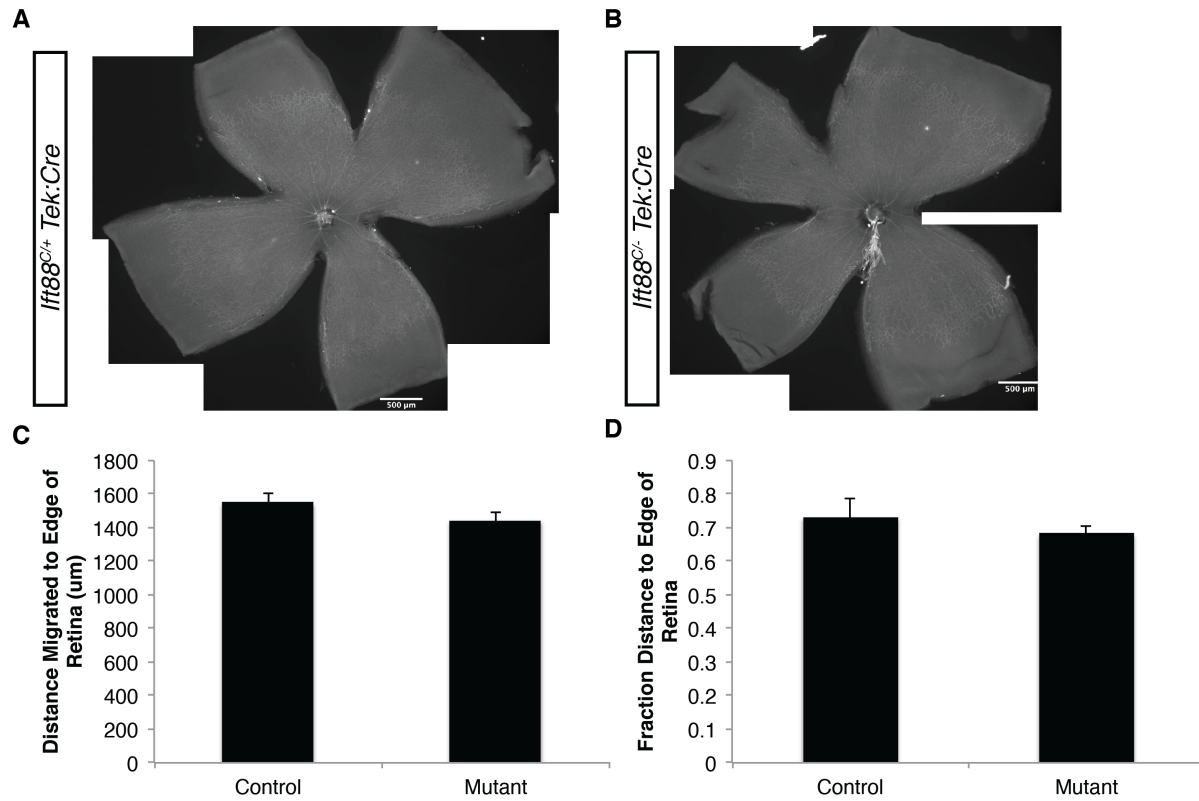
Figure 4. Retina angiogenesis is unaffected in mice lacking EC cilia. The vasculature in P5 retinas from control (A) and mutant (B) mice was visualized by staining for Pecam1. The raw distance migrated from the center of the retina to the edge (C) and, to correct for size differences between mice, the fractional distance to the edge of the retina (D) were quantified. There was no statistically significant difference in either metric. n=4 mice for each group. The average value of 8 measurements (4 retina leaflets measured per eye on both eyes) was used for each mouse. Error bars are +1 S.E.M.

Because ECs polarize along the direction of flow and EC cilia have been proposed to sense fluid flow, I surmised that EC polarity may be disturbed in *Kif3a$^{C/-}$ Tek:Cre* mice lacking endothelial cilia. The Golgi apparatus and centrosome are normally polarized upstream of the nucleus (towards the heart) in aortic ECs (42, 43). The centrosome anchors the primary cilium and cilia are thus ideally placed to affect the polarization of this organelle, as well as the tightly-opposed Golgi. I first determined whether the Golgi and centrosome were closely associated with one another in aortas with and without EC cilia by staining for the Golgi marker GM130 and centrosome marker Pericentrin. In both sets of mice the centrosome was found near the midpoint of the Golgi as viewed *en face* from the lumenal side of the vessel (data not shown), thus one serves as a good proxy for the other. Next, I quantified the angle between the Golgi and nucleus relative to the axis of the tissue in multiple locations within aortas of mutant and control mice (Figure 5A-D). Interestingly, there was neither a difference in the angle of polarization between mutant and control mice, nor was there a difference in the variance (Figure 5D). Thus, cilia are not required for either the direction or tightness of EC polarization in the direction of blood flow *in vivo*.
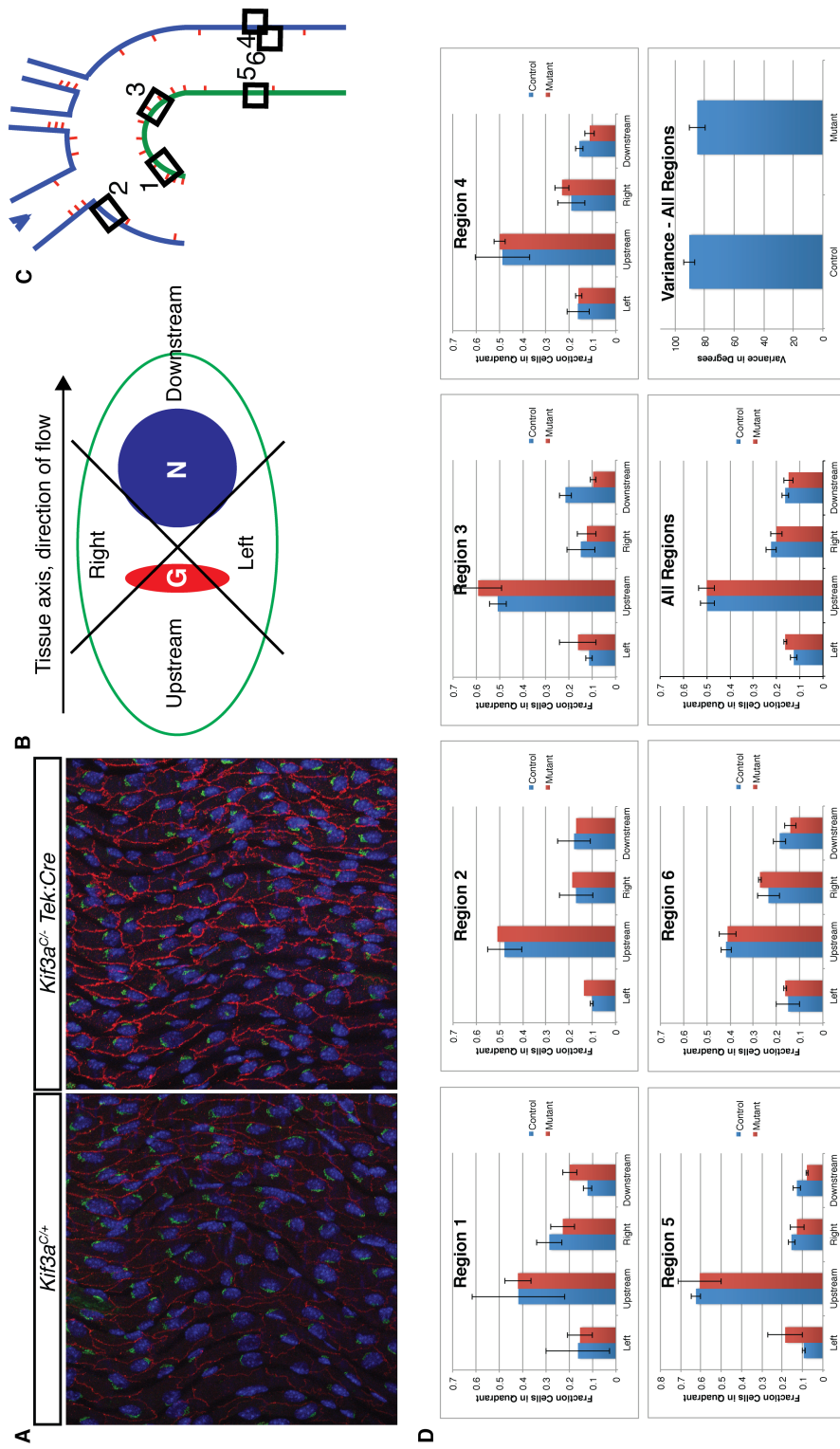
Figure 5. Aortic EC polarity is unaffected in mice lacking EC cilia. Control and mutant mice were stained for GM130 (green) to mark the Golgi, CD144 (red) to mark ECs and DAPI (blue) to label nuclei (A). The angle between the Golgi, nucleus and tissue axis was measured and binned as 'left', 'right', 'upstream' or 'downstream' (B) at multiple locations in the aorta (C). There was no difference in Golgi distribution at any single region or overall, nor was there a difference in the variance of distribution. Error bars are +/- 1 S.E.M. except for variance where they are 95% C.I. (D).

Collectively these results indicate that primary cilia and the hedgehog pathway are dispensable for development in the mouse. In addition, several key facets of endothelial biology, namely angiogenesis and planar polarity, are unaffected by the loss of primary cilia. It remains possible that loss of cilia sensitizes endothelial cells in some way to further genetic or environmental challenge, but in the context of the developing mouse loss of cilia alone yields endothelial cells and vasculature indistinguishable from their wild type counterparts.

Endothelial Primary Cilia Protect Against the Development of Atherosclerosis

Given the close correlation between the distribution of cilia and the atherosclerosis-prone

areas of the aorta (near vessel branchpoints and the lesser curvature of the aortic arch), I

investigated whether loss of EC cilia influences the development of atherosclerotic plaques.

Apoe$^{-/-}$ mice lack Apolipoprotein E, have increased circulating LDL, VLDL and cholesterol

levels and develop atherosclerosis (44). I fed experimental (*Ift88$^{C/-}$ Tek:Cre Apoe$^{-/-}$*) and control

(pooled *Ift88$^{C/+}$ Tek:Cre Apoe$^{-/-}$*, *Ift88$^{C/+}$ Apoe$^{-/-}$*, *Ift88$^{C/-}$ Apoe$^{-/-}$*) mice a high-fat, high-cholesterol

"Western" diet in order to accelerate the development of atherosclerosis. After eight weeks of

Western diet, I quantified the percent surface area of the thoracic aorta covered by

atherosclerotic plaques as visualized by Oil Red O staining. The experimental approach is

summarized in Figure 6. Experimental mice lacking endothelial Ift88 and cilia displayed an

approximately 58% increase in females and 70% increase in males in atherosclerotic lesional

surface area over control mice (Figure 7). Loss of cilia did not change the distribution of plaque

formation or induce ectopic plaques, but increased the size of plaques at atherosclerosis-prone

sites including the branch points of the great arteries and the inner curvature of the aorta.
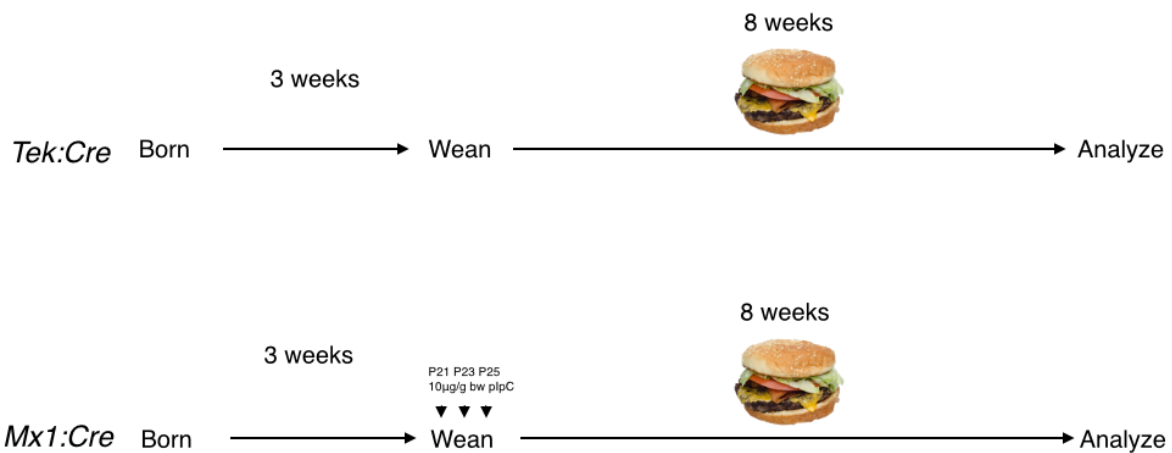
Figure 6. Experimental outline for assessing atherosclerosis. *Tek:Cre* and *Mx1:Cre* mice were both weaned at three weeks of age. At this point recombination was induced in *Mx1:Cre* mice by three intraperitoneal injections every other day of 10μg/g body weight pIpC dissolved in PBS. Both mouse lines were then placed on a high-fat high-cholesterol "Western" diet, here represented as a cheeseburger, for 8 weeks and then analyzed.
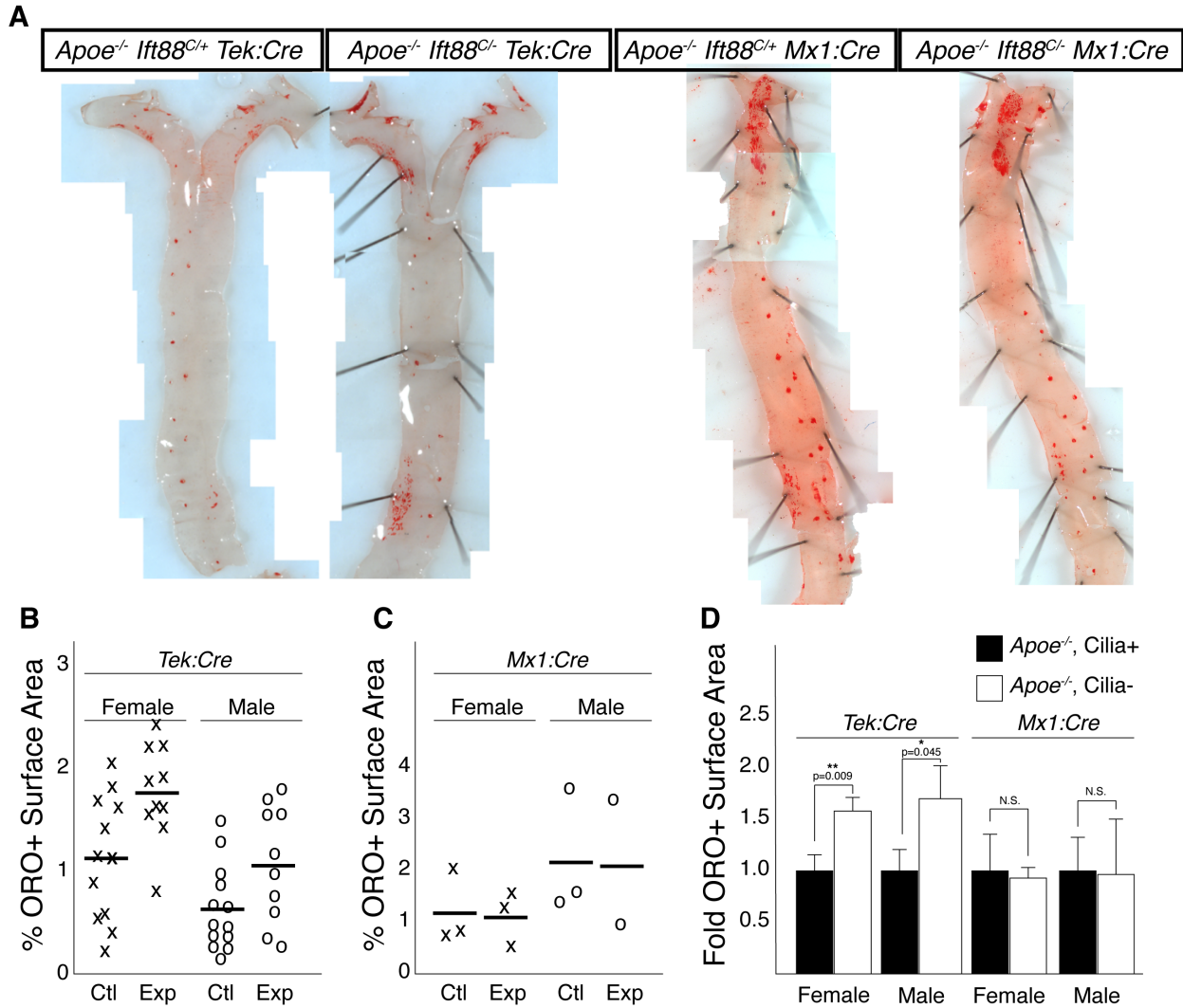
Figure 7. Loss of EC cilia accelerates atherosclerosis. Loss of EC cilia by *Tek:Cre* increases atherosclerosis in Apoe-/- mice on a Western diet as visualized by Oil Red O staining (A, left panels) whereas loss of cilia genes in blood by *Mx1:Cre* has no effect (A, right panels). Individual data points for *Tek:Cre* and *Mx1:Cre* are plotted in (B) and (C), respectively. Black bars represent the mean. Normalized data and statistics are in (D). Error bars are +/- 1 S.E.M. Statistics calculated with Student's t-test.

Because *Tek:Cre* induces recombination in the angioblast, the common precursor of ECs and the hematopoietic lineage (32), the increased atherosclerosis could be attributable to either loss of Ift88 in the ECs, the blood, or both. Although blood cells are not known to possess primary cilia, non-ciliary roles for IFT proteins have been described in immune cells (45). To distinguish whether ciliogenic genes are required in the blood lineages or the endothelium to inhibit atherosclerosis, I removed Ift88 from blood postnatally using the inducible *Mx1:Cre* (Figure 6). The *Mx1:Cre* recombined in blood as efficiently as *Tek:Cre* but to a much lesser degree in whole tail tissue reflecting far weaker vascular recombination (Figure 8). Experimental (*Ift88$^{C/-}$ Mx1:Cre Apoe$^{-/-}$*) and pooled control (*Ift88$^{C/+}$ Mx1:Cre Apoe$^{-/-}$*, *Ift88$^{C/+}$ Apoe$^{-/-}$*, *Ift88$^{C/-}$ Apoe$^{-/-}$*) mice displayed indistinguishable amounts of atherosclerosis (Figure 7). Thus, loss of Ift88 in blood cells did not alter atherosclerosis. To confirm that ciliogenic genes do not function in the blood to affect atherosclerosis, I generated *Kif3a$^{c/-}$ Mx1:Cre* mice to remove Kif3a from blood cells. As with Ift88, loss of Kif3a from the blood cells did not affect atherosclerosis (data not shown). Thus, the anti-atherosclerotic effect of ciliogenic genes is due to their role in ECs, not blood.
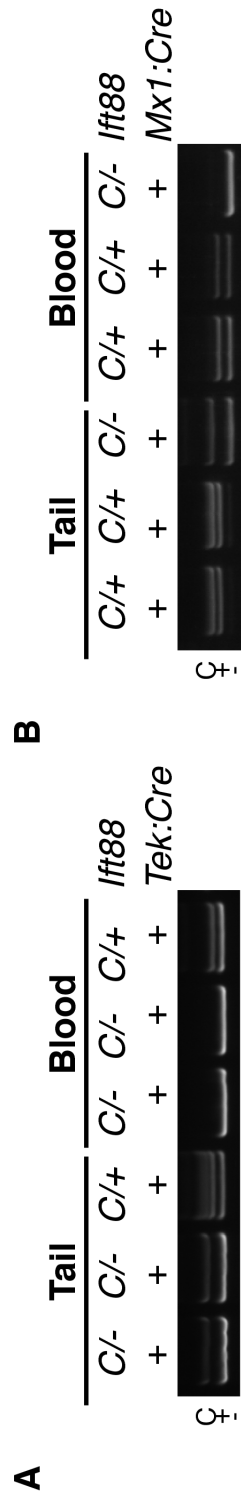
Figure 8. Tail but not blood recombination differs between *Tek:Cre* and *Mx1:Cre* mice. Tail and peripheral blood DNA was isolated from adult *Ift88* mice of the indicated genotypes carrying either *Tek:Cre* (A) or *Mx1:Cre* (B). Genotyping for *Ift88* indicated total loss of the conditional allele in blood from both Cre lines, however only *Tek:Cre* had noticeable recombination in tail DNA, reflecting vascular recombination in the *Tek:Cre* but not *Mx1:Cre* lines. In each panel the same 3 mice were assayed for blood and tail recombination and are presented in the same order. 'C' indications conditional allele, '+' indicates the wild type allele and '-' indicates the recombined null allele. The listed genotypes are the germ-line genotypes of each mouse.

To investigate how EC cilia limit atherosclerosis, I analyzed whole aortas for gene expression changes by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Control genes used to normalize expression were unchanged between control and experimental groups (Figure 9). As expected, removal of *Ift88* from ECs was detected as a decrease in the aortic expression of *Ift88* (Figure 9). Because the cDNA was transcribed from total aortic RNA derived from multiple cell types, the signal in EC cilia mutants for *Ift88* is non-zero, despite evidence of complete recombination by lineage tracing and immunofluorescent staining for cilia (Figures 2 and 3).
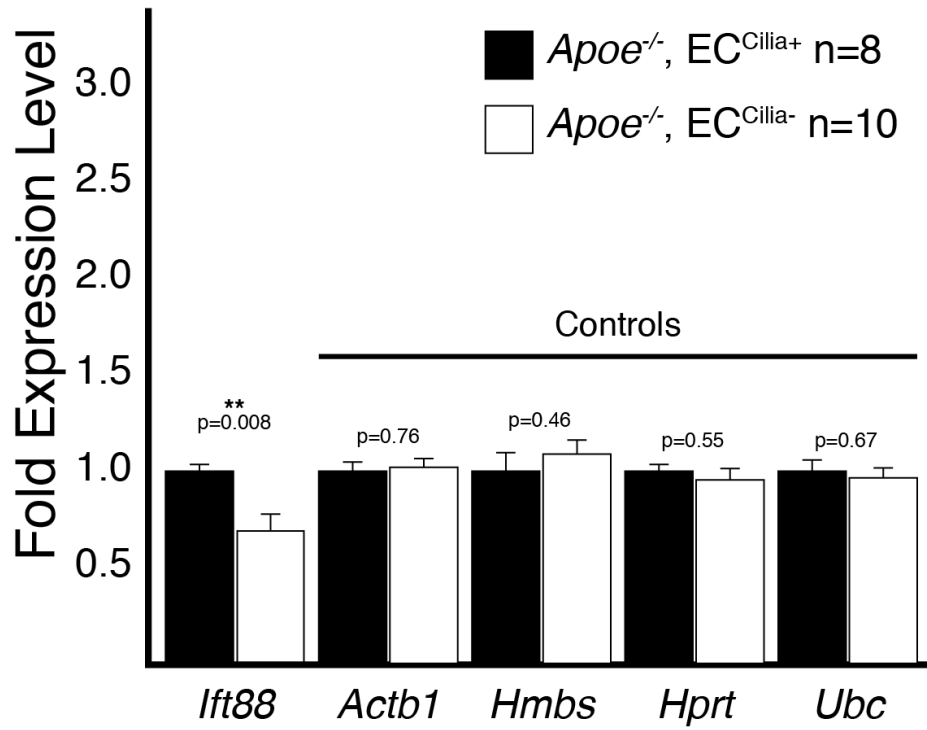
Figure 9. *Ift88* expression is reduced but control genes are unchanged by EC cilia loss in *Apoe⁻ᐟ⁻* mice. p-values calculated by Student's t-test. Error bars are +1 S.E.M. Each control gene has been normalized to the other three control genes.

As cilia are required for Hh signaling, I first tested whether the Hh pathway was perturbed. Expression of Hh pathway signaling components, including readouts of pathway activation *Ptc1, Ptc2* and *Gli1*, was not altered by removal of endothelial cilia (Figure 10). Therefore, alterations in Hh signaling do not correlate with changes in atherosclerosis, suggesting that the function of cilia in protecting against atherosclerosis is independent of Hh signaling. Also, Hh signaling may not function in adult aortic ECs, similar to the absence of a role for Hh signaling in developing ECs.
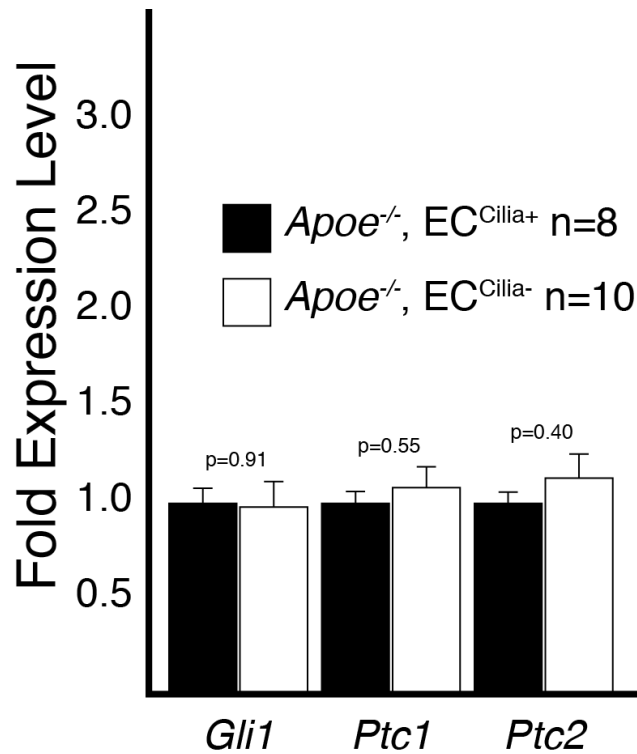
Figure 10. Hedgehog signaling is unaffected by loss of EC cilia in *Apoe*^-/-^ mice. Expression levels of three transcriptional readouts of the Hh pathway, *Gli1*, *Ptc1* and *Ptc2* are unchanged in *Apoe*^-/-^ mice without EC cilia. p-values calculated by Student's t-test. Error bars are +1 S.E.M.

Atherosclerosis is often viewed as a chronic inflammatory disease of the vessel wall, so I next investigated whether markers of inflammation were different between control and experimental mice. In contrast to the Hh pathway, removing EC cilia changed the aortic expression of several inflammatory genes. Loss of cilia upregulated proinflammatory cytokines *Il1b*, *Il6*, *Tgfb* and *Tnfa*, the NF-κB target *Tnfaip3*, as well as the inflammatory adhesion molecules *Vcam1* and *Sele* (Figure 11). I found an increase in the macrophage marker *Cd68* and lymphocyte marker *Nos2*, suggesting increased immune cell infiltration. I also noted an increase in expression of *Hmox1*, encoding the anti-inflammatory enzyme Heme Oxygenase-1. This seems counter-intuitive given the increased atherosclerosis in these mice, however this enzyme is known to be upregulated in response to a variety of stress pathways such as NF-κB (46) and its increase here may indicate a more advanced state of inflammation. These data further support an anti-inflammatory role for EC cilia.

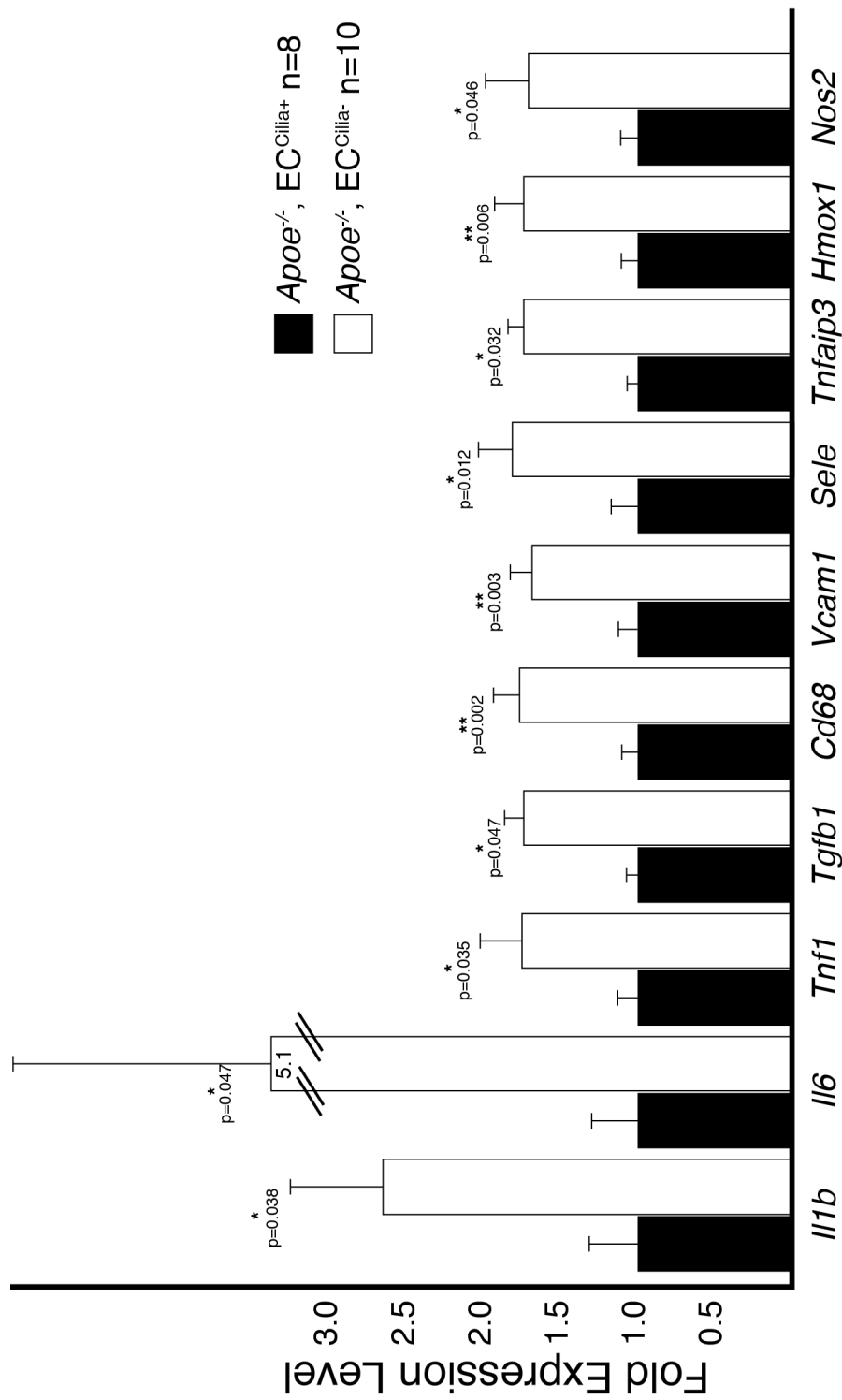**Figure 11.** Loss of EC cilia enhances inflammation in *Apoe⁻/⁻* mice. Inflammatory cytokines *Il1b, Il6, Tnf1* and *Tgfb1*, the adhesion molecules *Vcam1 and Sele*, the lymphocyte marker *Nos2* and macrophage marker *Cd68*, the Nf-κb responsive genes *Tnfaip3* and *Hmox1* were upregulated in *Apoe⁻/⁻* mice lacking EC cilia by RT-qPCR. p-values calculated by Student's t-test. Error bars are +1 S.E.M.

Because cilia were affecting inflammation and this effect was unlikely to be via hedgehog signaling, I was interested in other pathways that could function via cilia and explain this result. I used two approaches to implicate signaling molecules in EC cilia biology. I stained for several signaling molecules and cilia in adult mouse aortas to test whether they localized to cilia in ECs *in vivo*. I also expressed EGFP-tagged signaling molecules known to function in ECs in mIMCD-3 cells, an easily transfected ciliated mouse kidney cell line (endothelial cell lines did not readily transfect), to test cilia localization *in vitro*. I first tested whether two G-protein coupled receptors (GPCRs), AT1 and AT2, localized to cilia. These are the two receptors for Angiotensin II, a potent pro-inflammatory molecule (47). Neither receptor localized to cilia *in vivo* by immunofluorescent staining or when expressed in cells (data not shown).

Next, I was interested in testing whether purinergic P_1Y and P_2Y receptors, GPCRs that respond to extracellular adenosine and nucleotide, respectively, localize to cilia. These receptors' function has been linked to cilia in the kidney (48) and one receptor, P_2Y_{12}, localizes to cilia in the bile duct (49). Several of these receptors are implicated in EC function and cardiovascular disease (50). Intriguingly, many of these receptors localized to cilia when expressed in cells (data not shown). None of the commercial antibodies tested thus far recognized overexpressed protein by immunofluorescence making it impossible to determine the *in vivo* localization pattern.

Finally, I tested whether Pkd2, implicated in ciliary flow sensation and activation of nitric oxide signaling in ECs, localized to cilia both in cells and *in vivo* in adult mouse aortas. Pkd2 fused to GFP robustly localized to mIMCD-3 cilia (data not shown). Pkd2 also consistently localized to endothelial primary cilia *in vivo* (Figure 12). This confirms what has been observed in cultured endothelial cells (15) and *in vivo* (16). In addition to ciliary localization, substantial signal was detected on other membranous structures in the cell, indicating that Pkd2 could have

roles outside cilia. In order to explore the role of Pkd2 in relation to cilia in endothelial cells, I attempted to generate *Pkd2$^{C/-}$ Tek:Cre* mice. Unlike EC cilia mutant mice, EC *Pkd2* mutant mice rarely survived to birth (51) (data not shown). This further supports a function for Pkd2 outside of cilia in endothelial cells in addition to its reported role in EC ciliary mechanosensation.

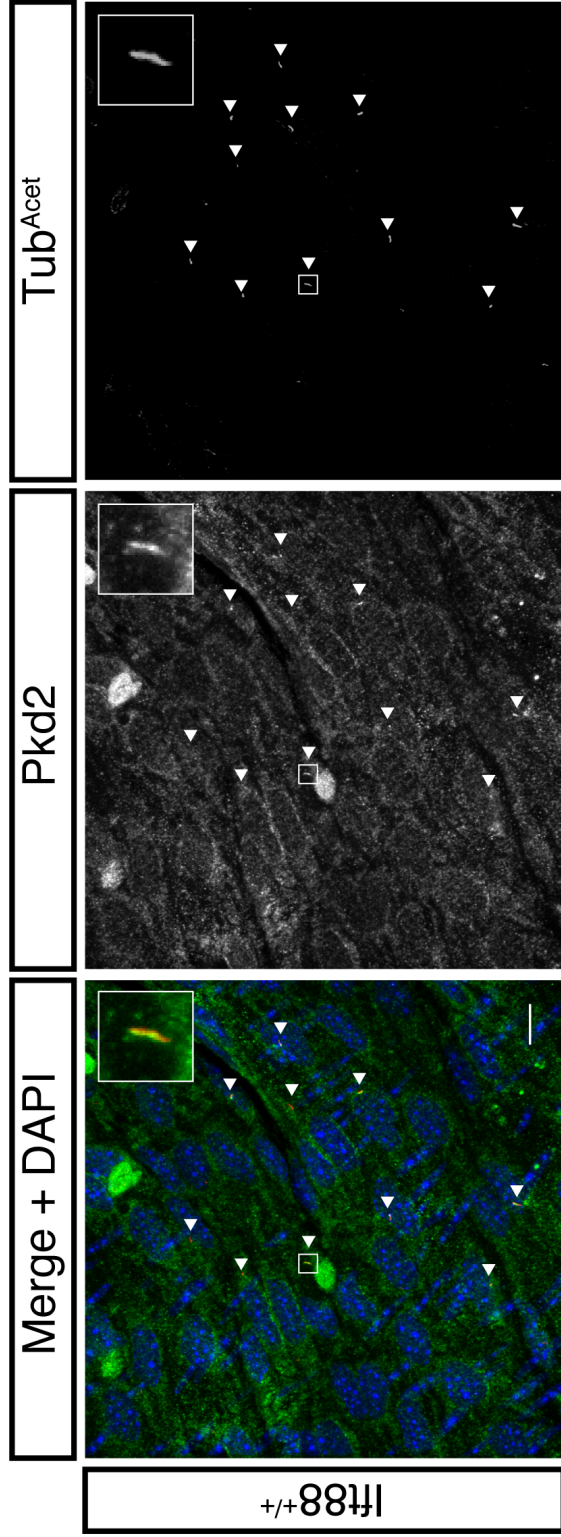**Figure 12**. Pkd2 localizes to EC cilia *in vivo*. Adult mouse aortas were stained for acetylated α-Tubulin (right panel, red in merge), Pkd2 (middle panel, green in merge) and DAPI. Pkd2 localized strongly to primary cilia but also to other membranous structures in endothelial cells, belying both ciliary and non-ciliary functions. Arrowheads point out cilia. Example cilium highlighted in inset. Scale bar is 10μm.

Conclusions and Future Directions

I have used mouse genetics to investigate the *in vivo* function of EC primary cilia. EC primary cilia are not required for vascular development or EC polarity, but do limit atherosclerosis and vascular inflammation.

I observed an asymmetric distribution of cilia within the vasculature. Cilia were enriched at vascular branch points and sites of high curvature, both of which are known sites of disturbed flow. This is consistent with an *in vitro* observation that high shear stress disassembles cilia on ECs (27). It is unclear from the static picture I see from staining fixed aortas whether individual cells are consistently ciliated or unciliated over time or, alternatively, whether the situation is more dynamic and cells sample their environment via cilia at different rates depending on their location and local flow conditions. There is little evidence for dynamic ciliary assembly and disassembly in non-dividing cells and understanding how cilia behave in relation to varying flow conditions would increase understanding of the regulation of ciliogenesis, which remains poorly understood. The mouse is not an ideal system to address this question, but *in vitro* studies or time-lapse microscopy of living zebrafish could answer this question.

In order to study the role of endothelial cilia in a mammal, I ablated cilia genetically in the mouse via *Tek:Cre* mediated deletion of *Ift88* and *Kif3a.* These mice had no obvious developmental defects, despite completely lacking endothelial cilia as assessed by immunofluorescent staining. This illustrates endothelial cilia are not necessary for vascular development in the mouse.

Because cilia are, with possible exceptions in blood cells (52), broadly required for mammalian hedgehog signaling, the fact that EC cilia-less mice survive suggests hedgehog

signaling is dispensable in the *Tek:Cre* lineage for development. Indeed, the observation that *Smo Tek:Cre* conditional mutant mice are also viable confirms that Hh pathway responsiveness is not required in angioblasts during development in mouse. Several studies show Hh ligands induce tube formation in cultured mouse ECs (7, 8). I propose that while Hh signals may be sufficient to induce tube formation *in vitro*, EC Hh signaling is not necessary for proper vascular development *in vivo*. My findings are consistent with the well-established idea that the main role of the Hh pathway in early vascular patterning is an indirect one: Hh is necessary for subsequent VEGF and Notch induction, but does not act on ECs directly (11). Coultas et al. found vascular phenotypes associated with hyperactive Hh pathway activity (by deletion of the receptor and negative regulator *Ptch1*) that could not be explained by changes in VEGF or Notch activity, but it was not determined if the phenotype was EC autonomous (53). My results differ, however, from what has been reported in zebrafish, where Williams et al. showed the Hh pathway is required in angioblasts for arterial specification (54).

A further difference between mouse and zebrafish may exist in whether EC cilia play a role in angiogenesis. Goetz et al. argued that primary cilia and Pkd2 were required in endothelial cells for proper angiogenesis (19) whereas my results in the mouse retina showed loss EC cilia did not affect angiogenesis in that system. It will be interesting to learn whether these conflicting results represent true differences in mouse and fish or simply differences in experimental approach. It could be that the defect seen in zebrafish is transient or dynamic and if the fish were allowed to develop further they would be completely viable. Conversely live imaging of mouse inter-somitic vessel growth might show a defect in EC cilia mutants similar to the zebrafish, though this experiment would be technically challenging. It remains safe to conclude that mammalian EC cilia and ciliary Hh signaling are unessential for vascular development.

One well-characterized response of ECs to fluid flow is polarization in the direction of flow. Primary cilia are important for mechanosensation in several systems, including ECs, and I assessed whether EC cilia participate in flow-induced polarization and found that aortic EC polarization was unaffected by removal of cilia *in vivo*. Thus, while cilia can respond to flow as evidenced in *in vitro* (15) and *ex vivo* (16) studies, they are not critical for polarization in response to flow *in vivo*.

ECs are known to have multiple means for mechanosensation and thus there may be redundant mechanisms at play. For example, Pecam1 is implicated in EC mechanosensation (55). However, *Pecam^{Gt/Gt} Kif3a^{C/-} Tek:Cre* mice are viable, suggesting that these two pathways do not have overlapping roles critical for vascular development (data not shown). It will be interesting to determine which EC responses to flow are affected by loss of primary cilia.

High shear stress is known to disassemble EC cilia *in vitro* (27) and this correlates with their distribution *in vivo*. This relationship may extend further such that cilia are only responsible for sensing and responding to a particular regime of flow, namely weak or low flow. Any EC behaviors caused by strong flow would thus be unaffected by loss of EC cilia. A recent report in zebrafish in fact argued that EC cilia were sensors for low flow (19).

I show that mice lacking primary cilia in the *Tek:Cre* lineage are more susceptible to atherosclerosis when on an *Apoe^{-/-}* background and placed on a Western diet. Consistent with this result, I find elevated expression of several known inflammatory cytokines and adhesion molecules in mutant aortas. A multitude of genes and signaling pathways are known to modulate atherosclerosis to varying degrees (21). For instance, *Cav1^{-/-} Apoe^{-/-}* mice, which lack Caveolin-1, an inhibitor of eNOS, exhibit a 33% reduction in atherosclerotic lesional area compared to *Apoe^{-/-}* control mice (21, 56). Conversely *Enos^{-/-} Apoe^{-/-}* mice that lack eNOS entirely show an

80% increase in atherosclerotic lesional surface area versus $Apoe^{-/-}$ controls (21, 57). The approximately 60-70% increase in atherosclerosis I observe in $Apoe^{-/-}$ mice without EC cilia indicates that primary cilia are a modest but significant regulator of atherosclerosis.

An anti-inflammatory role for cilia has previously been proposed by several groups. Nauli et al. showed EC cilia could activate nitric oxide synthesis in a *Pkd1* dependent fashion in response to shear stress (15), Hierck et al. showed that cultured ECs lacking cilia are less likely to activate the anti-inflammatory transcription factor Klf2 in response to shear stress (18) and Egorova et al. showed ECs lacking cilia were more likely to undergo endothelial-to-mesenchymal transition (58). Nitric oxide is atheroprotective (57) and, in ECs, is primarily made by endothelial nitric oxide synthase (eNOS). eNOS is regulated by multiple mechanisms including but not limited to activating phosphorylation by Akt and AMPK, inhibition by caveolin binding and activation via calmodulin binding (59). If Pkd mediated eNOS activation, presumably by calcium flux and calmodulin activation, is responsible for EC cilia's ability to restrict atherosclerosis then one would expect to see lower levels of activated eNOS protein in aortas before the onset of atherosclerosis and this difference should be dependent on both cilia and Pkd1 and Pkd2. I attempted to quantify differences in eNOS activation via phospho-specific Western blots but did not find clear differences between the mice with and without EC cilia (not shown). It is possible that because only a subset of cells are ciliated at any one time, any difference in eNOS activation in mutant animals is restricted to this subset of cells and may be difficult to detect in a bulk assay. I also attempted to take a genetic approach in order to study Pkd2 EC function in relation to cilia and vascular disease, however *Pkd2* also has roles in ECs outside of cilia that could complicate epistasis analysis. Indeed, $Pkd2^{C/-}$ *Tek:Cre* conditional

mutant mice rarely survive to birth, in stark contrast to mice lacking EC cilia (51) (data not shown).

While it seems likely but not proved that Pkd proteins are involved in EC cilia mechanosensation and their effect on the development of vascular disease, there may be other proteins that work with Pkd proteins at cilia or that could function in parallel to Pkd proteins at cilia. A good set of candidates for additional ciliary signaling proteins are the purinergic family of GPCRs. GPCRs generally are good candidates as several GPCRs have been to shown to localize to and function via cilia in a variety of tissues (60, 61). Purinergic receptors consist of the P_1Y receptors, which bind extracellular adenosine and the P_2Y receptors, which bind extracellular nucleotide. Some of these receptors have been implicated in cilium-dependent flow sensation in kidney cells (48) and one purinergic receptor has been shown to localize to cholangiocyte cilia in the bile duct (49). In addition, P_2Y_1, P_2Y_2, P_2Y_4, P_2Y_6 and all four adenosine receptors are expressed in endothelial cells and their function can modulate vascular disease and inflammation (50). Suggestively, most purinergic receptors tested localized to primary cilia when expressed in mIMCD-3 cells. It would be interesting if this localization were found *in vivo* in aortas, as well, though that analysis is currently hindered by the lack of adequate antibodies. Concentration of these receptors in cilia could sensitize ciliated cells to extracellular nucleotide. Nucleotide is known to induce intracellular calcium release and nitric oxide mediated vessel relaxation(50, 62, 63), effects that are often seen as anti-inflammatory. Alternatively, one group has suggested in the kidney that cilia- and Pkd-dependent flow sensation induces release of ATP that in turn activates purinergic receptors. If this model were true in the aorta, the purinergic receptors need not localize to cilia to still be an important step in the EC cilia response to flow. It will be interesting to explore which, if any, aspects of purinergic signaling are affected

to loss of EC cilia. This is also another opportunity to make use of mouse genetics as many purinergic receptor mutant mice exist and are viable and are thus amenable to epistasis analysis with EC cilia vis-à-vis atherosclerosis.

My data that EC primary cilia serve a primarily anti-inflammatory role stand in contrast to several recent studies reporting that cilia potentiate the cellular response to Il-1β, a pro-inflammatory cytokine (64-66). These studies were performed in chondrocytes and may indicate the cilia can have pro- or anti-inflammatory effects, depending on the cell type. Alternatively, EC cilia may similarly respond to Il-1β but simultaneously have anti-inflammatory properties. The net effect of removing cilia in my system is to enhance inflammation, but the opposite effect could be seen in other tissues or following different challenges. Indeed, loss of cilia can yield Hh pathway gain of function and loss of function phenotypes depending on the tissue examined. The role of cilia in inflammation may be similarly superficially inconsistent.

The significant effect EC cilia have on modulating atherosclerosis in the mouse raises the question of whether cilia function similarly in human disease. Primary cilia have been observed near atherosclerotic plaques not only in the mouse (26), but also in humans (28-30). Genome-wide association studies (GWAS) have not implicated cilia mutations in human populations as risk factors for cardiovascular disease (CVD). Mutations in genes affecting cilia, however, usually cause severe disease and patients rarely live to the point that atherosclerosis is a major concern. Thus these mutations are unlikely to show up in CVD GWAS. One potential insight comes from autosomal dominant polycystic kidney disease (ADPKD) patients who harbor mutations in one copy of either *PKD1* or *PKD2*. These patients suffer from cardiovascular complications including hypertension, left-ventricular hypertrophy, increased carotid intima-media thickness and most important clinically, aneurysm (31). Some of these effects can be

linked to dysregulation of the renin-angiotensin system caused by kidney cysts (31). However, many patients exhibit endothelial dysfunction and increased carotid intima-media thickness, both warning signs for atherosclerosis, even before signs of renal dysfunction or hypertension (31). This may suggest PKD1 or PKD2 dysfunction within endothelial cells could contribute to these clinical manifestations. While my results certainly do not prove this is the case, they are consistent with this interpretation and suggest that endothelial Pkd and cilia dysfunction contribute to vascular disease.

In conclusion, EC primary cilia are dispensable for mouse development but play an atheroprotective role in the adult animal. This effect is unlikely to be do to Hh signaling but could be mediated via polycystins and eNOS. These results suggest a novel role for EC cilia in adult mammalian tissue homeostasis and the development of cardiovascular disease, a leading cause of human mortality.

Methods

Mouse Lines

Tek:Cre (MGI: 5052345), *Mx1:Cre* (MGI: 2176073), *Pecam1* (MGI: 1888376), *Ift88* (MGI: 3710185), *Kif3a* (MGI: 2386464) and *Smo* (MGI: 2176256) mice have been described elsewhere and were gifts of Rong Wang, Ben Barres, Mark Ginsberg, Bradley Yoder, Larry Goldstein and Andrew McMahon, respectively. *Apoe* (MGI: 1857129), *Pkd2* (MGI: 4843125) and *mTmG* (MGI: 3716464) mice were purchased from Jackson Labs. All mouse protocols were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. Mice were maintained on a mixed background. Genotyping primers are listed in Table 2.

Conditional Cre Induction

Mx1:Cre mice were administered three doses of 10μg/g body weight polyinosinic-polycytidylic acid (pIpC, Sigma-Aldrich) by intraperitoneal injection at 48-hour intervals at approximately three weeks of age. pIpC was dissolved in PBS to a concentration of 10mg/ml.

Immunofluorescence

Mice were anesthetized with 2.5% w/v 2,2,2-Tribromoethanol (Sigma-Aldrich) in PBS and perfused via the left ventricle with 10ml of cold PBS containing 1U/ml heparin (Sigma-Aldrich) followed by 10ml 4% formaldehyde in PBS. Aortas were dissected and post-fixed for 15' in 4% formaldehyde, rinsed in PBS, cleaned of fat and connective tissue including the adventitia and opened longitudinally with microscissors. Samples were incubated in blocking buffer (D-PBS,

0.1% Triton-X100, 2% BSA, 1% donkey serum) for one hour. For samples stained with mouse

or rat primary antibodies, Mouse on Mouse (M.O.M.) IgG blocking reagent (Vector Labs) was

included in the blocking buffer. Samples were incubated with primary antibodies in blocking

buffer at 4 degrees overnight. The next day samples were rinsed 3x5' in D-PBS, stained with

appropriate AlexaFluor 488, 568, or 647 conjugated secondary antibodies (Life Technologies) at

1:1000 and Hoechst or DAPI in blocking buffer for one hour, rinsed 3x5' in D-PBS and mounted

in Gelvatol or ProLong Diamond (Life Technologies). All steps were at room temperature unless

otherwise noted.

Primary antibodies used were mouse anti-acetylated Tubulin (Sigma, 1:1000), rabbit anti-Arl13B

(gift from Tamara Caspary and ProteinTech Labs 17711-1-AP, 1:5000), rat anti-CD144 (BD

550548, 1:100), rat anti-CD31 (BD 550274, 1:100), rabbit anti-Pericentrin (Covance PRB432-C,

1:500), mouse anti-GM130 (BD 610822, 1:200), rabbit anti-Agtr1 (Abcam ab9391, 1:200),

rabbit anti-AT2 (Abcam ab19134, 1:200), rabbit anti-GFP (Abcam ab290, 1:2500), goat anti-

GFP (Rockland 600-101-215, 1:2000) and rabbit anti-Pkd2 (gift Feng Qian, 1:200). Stained

samples were imaged on a Leica SP-5 or SPE confocal microscope.

Cilia Quantification

Male and female wild type adult mouse aortas were stained for Arl13B, CD31, and Hoechst as

described above. Two fields in each region indicated in Figure 1 were imaged and the number of

cilia and number of endothelial cells in each field were quantified. The two fields were summed

so that at least 100 cells were counted per region per mouse. The percent of ciliated cells was

then averaged across the mice.

Polarity Analysis

Adult mouse aortas were stained for GM130 to mark the Golgi, CD144 to mark ECs and DAPI to mark the nucleus and imaged as described above. Images were oriented so that upstream (towards the heart) was up. The nucleus and Golgi of each cell in the field were manually traced in Metamorph and the angle between the center of mass of the organelles relative to the axis of the tissue was computed. n=3 mice for each group and at least 50 cells were measured per field.

Atherosclerosis Studies

Mice were crossed onto an *Apoe*$^{-/-}$ background. Following weaning at three weeks of age, mice were place on a Western type diet (TestDiet 5TJN; 39.9% calories from fat, 0.171% cholesterol) for 8 weeks before analysis.

Mice were perfused as for immunofluorescence, but were post-fixed in 10% formalin overnight at 4 degrees. Aortas were rinsed in PBS, cleaned of fat and connective tissue, then equilibrated twice in 60% isopropanol, stained 15 minutes in freshly prepared and filtered 0.3% Oil Red O (Sigma-Aldrich) in 60% isopropanol, destained 5 minutes in 60% isopropanol, then stored in dH20 until imaging. Samples were opened longitudinally, pinned onto a silicon dish with insect mounting pins and imaged using a Zeiss Discovery V12 Stereo dissecting scope. Individual images from the same aorta were stitched together using Photoshop (Adobe) and Oil Red O-positive area between the aortic sinus and celiac artery quantified using Fiji. For this the aorta area of interest was outlined and the area in pixels measured. Then the Oil Red O-positive area was measured using the RGB threshold function (threshold values of red: 120-255, green: 0-120,

blue: 0-80 were usually used, though they were occasionally modified slightly when variations in image brightness or white balance caused obvious errors in lesion selection) followed by the select and measure commands.

RT-qPCR

Aortas were prepared as for immunoblots, but were instead lysed in 200µl RLT buffer. RNA was prepared using the RNEasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using the iScript cDNA Synthesis Kit (BioRad) according to the manufacturer's instructions. cDNA was diluted 1:4 in dH20 and stored at -20 degrees. qPCR was performed using EXPRESS SYBR GreenER with premixed ROX (Invitrogen) and run on a 7900HT thermocycler (Applied Biosystems). 5µl total reaction volume, 0.4µl diluted cDNA and a concentration of 200nM for each primer was used. Expression levels were normalized to the geometric mean of four control genes (*Actb, Hmbs, Hprt and Ubc*), average normalized Ct values for control and experimental groups determined and relative expression levels determined by ΔΔCt. Significance determined by Student's t-test. Primers are listed in Table 1.

Immunoblots

Mice were perfused as above with the exception that the fixation step was omitted. Following dissection, aortas were quickly cleaned of excess fat in ice-cold PBS then ground in 0.2ml glass tissue grinders on ice in 120µl lysis buffer (50mM Tris-HCl pH7.4, 100mM NaCl, 0.1% SDS, 0.1% Sodium Deoxycholate, 1% NP-40, 0.1mM EDTA, 0.1mM EGTA, 1mM PMSF, 1x Protease Inhibitor Cocktail III (Calbiochem), 1x PhosStop Phosphatase Inhibitor Tablets (Roche)). The lysate was cleared 5' at 4 degrees in a tabletop centrifuge at maximum speed.

Protein concentration was determined by Bradford assay. Samples were boiled 5' in an equal volume of 2x sample buffer and stored at -20 degrees. Samples were run on 4-15% TGX Criterion gels (BioRad) in running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH8.3) at 300V for approximately 30 minutes until the dye front passed through the gel. Protein was next transferred to nitrocellulose membranes in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, pH8.3) for 30 minutes at 100V using a Criterion blotter with plate electrodes (BioRad). Membranes were blocked in 5% w/v milk in TBS 1 hour at room temperature. Blots were incubated with primary antibody overnight at 4 degrees, washed 3x5' TBST at room temperature, stained with AlexaFluor-800 conjugated secondary antibodies (Rockland, 1:2,500) 1 hour at room temperature, washed 3x5' TBST and imaged on a Licor Odyssey imager. Primary antibodies used were mouse anti-eNOS (BD 610296, 1:1200), mouse anti phospho-S1777-eNOS (BD 612392, 1:1000), mouse anti-α-Tubulin (Sigma T5158, 1:2500).

Cell Culture and Transfection

Mouse inner medullary collecting duct (mIMCD-3) cells were originally obtained from ATCC and thawed from liquid nitrogren-frozen stocks. They were cultured in 50:50 DMEM High Glucose:F12 medium (UCSF Cell Culture) supplemented with 15mM HEPES (UCSF Cell Culture), 1x Penicillin/Streptomycin (UCSF Cell Culture) and 10% FBS (Life Technologies). Cells were passaged 1:10 when near confluence using 0.25% Trypsin + EDTA (UCSF Cell Culture). For transfection, 125,000 cells were seeded onto #1.5 glass coverslips in 24-well plates and 0.5ml media the night prior to transfection. The next morning, the media was replaced as cells were transfected using 500ng DNA and JetPrime transfection reagent according to the manufacturer's instructions (Polyplus Transfection). After four hours, the media was replaced as

cells were allowed to recover for 8-24 hours, at which point they were at or near confluence. The media was then replaced with Opti-MEM (UCSF Cell Culture) supplemented with 1x Penicillin/Streptomycin to induce ciliation. After 24 hours the cells were fixed using 4% formaldehyde in PBS for 8' at room temperature. They were subsequently stained for GFP and cilia according to the protocol for aortas.

Cloning and DNA Constructs

Human A_1R-, A_2AR-, and rat A_3R-EGFP were gifts of TM Palmer. Human Pkd2-EGFP was a gift of Rachel Gallagher. Rat AT_1R- and AT_2R-EGFP were gifts of Guangyu Yu. A_2B and all P_2Y purinergic receptor constructs were made in the Clontech N-1 EGFP backbone, which results in C-terminal GFP fusions. Receptors were amplified by PCR from mouse genomic DNA using primers containing EcoRI and BamHI restriction sites and the ExpandHF PCR kit (Roche) according to manufacturer's instructions. PCR product was separated on 1% agarose gels, the correct sized band was gel-purified and then TA-cloned into pCR2.1 according to the manufacturer's instructions (Life Technologies). Subclones were digested, gel purified and ligated into digested and Antarctic Phosphatase (NEB) treated vector by standard methods. Clones were validated by sequencing.

Retina Vasculature Measurements

Eyes of euthanized P5 mice were removed and fixed 45' at room temperature in 4% formaldehyde in PBS. The lens and outer layer of the eye were removed, followed by the hyaloid vessels. Retinas were then cut with microscissors so they could lay flat and processed for immunofluorescence according to the same protocol for aortas.

Gene	Forward Primer	Reverse Primer	Source
Actb	TTCTTTGCAGCTCCTTCGTT	ATGGAGGGGAATACAGCCC	Primerdepot
Cd68	CCAATTCAGGGTGGAAGAAA	CTCGGGCTCTGATGTAGGTC	Fernandez-Hernando et al. (56)
Gli1	GGATGAAGAAGCAGTTGGGA	ATTGGATTGAACATGGCGTC	Primerdepot
Hmbs	TTCTGCAGACACCAGGGG	TCTTTGAGCCGTTTTCTTCC	Primerdepot
Hmox1	AAGCCGAGAATGCTGAGTTCA	GCCGTGTAGATATGGTACAAGG	Zhang et al. (67)
Hprt	TCCTCCTCAGACCGCTTTT	CATAACCTGGTTCATCATCGC	Primerdepot
Ift88	TTGCGAGGCTCTGCATTTGA	ACAACTGTTGGCAATACAGCTTT	Primerbank
Il1b	TGTGAAATGCCACCTTTTGA	GGTCAAAGGTTTGGAAGCAG	Primerdepot
Il6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC	Primerbank
Ptc1	CTCCTCATATTTGGGGCCTT	AATTCTCGACTCACTCGTCCA	Primerdepot
Ptc2	CTCCGAGTGGCTGTAATTGAG	CCCAGCTTCTCCTTGGTGTA	Primerdepot
Sele	ATGCCTCGCGCTTTCTCTC	GTAGTCCCGCTGACAGTATGC	Primerbank
Tgfb1	GGAGAGCCCTGGATACCAAC	CAACCCAGGTCCTTCCTAAA	Primerdepot
Tnf	CCACCACGCTCTTCTGTCTAC	AGGGTCTGGGCCATAGAACT	Primerdepot
Tnfaip3	AAGCTCGTGGCTCTGAAAAC	TTCCTCAGGACCAGGTCAGT	Primerdepot
Ubc	TCCAGAAAGAGTCCACCCTG	GACGTCCAAGGTGATGGTCT	Primerdepot
Vcam1	TTGGGAGCCTCAACGGTACT	GCAATCGTTTTGTATTCAGGGGA	Primerbank

Table 1. Primer pairs for RT-qPCR of aortic total cDNA. All primers are listed in a 5' – 3' orientation.

Gene	Forward Primers	Reverse Primers	Product Sizes
Apoe	GCCTAGCCGAGGGAGAGCCG	GCCGCCCCGACTGCATCT TGTGACTTGGGAGCTCTGCAGC	WT: 155 Mut: 245
Ift88	GCCTCCTGTTTCTTGACAAACAGTG	GGTCCTAACAAGTAAGCCCAGTGTT CTGCACCAGCCATTTCCTCTAAGTCATGTA	WT: 350 Cond: 370 Mut: 200
Kif3a	AGGGCAGACGGAAGGGTGG	TCTGTGAGTTTGTGACCAGCC TGGCAGGTCAATGGACGCAG	WT: 360 Cond: 490 Mut: 200
Mx1:Cre	AGCCATCTGAACTGGTGGTGTCC	AATCGCGAACATCTTCAGGT	300
mTmG	CTCTGCTGCCTCCTGGCTTCT	CGAGGCGGATCACAAGCAATA TCAATGGGCGGGGGTCGTT	WT: 330 mTmG: 250
Pecam1	ACACAAAGGCAAGGGTGTTC GGCGTTACTTAAGCTAGCTTGC	ACCTTCAGCCCCTGCTTAAT	WT: 203 Mut: 120
Pkd2	CCTTTCCTCTGTGTTCTGGGGAG	GTTTGATGCTTAGCAGATGGC CTGACAGGCACCTACAGAACAGTG	WT: 282 Cond: 318 Mut: 143
Smo	CCACTGCGAGCCTTTGCGCTAC CTTGGGTGGAGAGGCTATTC	CCCATCACCTCCGCGTCGCA AGGTGAGATGACAGGAGATC	WT: 160 Cond*: 280
Tek:Cre	GGGAAGTCGCAAAGTTGTGAGTT	AATCGCGAACATCTTCAGGT	450

Table 2. Genotyping primers for mouse strains used in this study. All primers are listed in a 5' – 3' orientation. WT = Wild Type. Cond = Conditional. Mut=Mutant and refers to the loss of function allele. * The *Smo* conditional genotyping reaction amplifies the Neomycin Resistance cassette present in the conditional allele and is not specific for the *Smo* locus.

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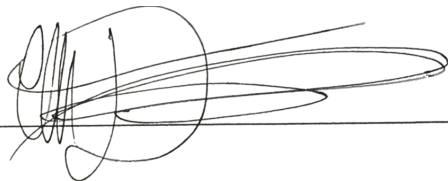46. Alam J, Cook JL. How Many Transcription Factors Does It Take to Turn On the Heme

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