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Localization and Distribution of Primary Cilia in the Adult Mouse Heart

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Abstract

Although primary cilia have been shown to play crucial roles in the development of embryonic mouse heart, their presence and function in adult mouse heart remains controversial. In this study, the presence of primary cilia in adult mouse heart was investigated. The presence of primary cilia was initially demonstrated in the surface of cardiac cells of mouse hearts from both young and adult mice by immunostaining with acetylated α-tubulin, a ciliary structural marker. The presence of cardiac primary cilia in 1-, 3-, 6- and 12-month old mice was further confirmed by staining heart tissues with an antibody against pericentrin, a marker for the basal body or the centriole. Ciliary polycystin-2 is a calcium channel and mechanosensory molecule, was demonstrated for the first time to localize to primary cilia of cardiac cells in both early and late stages of mouse adulthood, thus proposing a role of primary cilia in adult mouse heart calcium signaling. Primary cilia presence in different heart chambers was further confirmed by immunostaining. Furthermore, the abundance and length of cardiac primary cilia during mouse adulthood in three different age groups (< 3 month, 3-6 month, and >6 month old mice)was studied and found out that cardiac cells with primary cilia in the first age group (< 3 month) accounted for 37% of the total number of cells; however, the number of primary cilia in the 3-6 month and > 6 month age groups accounted for 29% and 25%, respectively, suggesting that primary cilia abundance is age dependent.

Keywords: Primary cilia; Cardiac myocytes; Mechanosensation.

Introduction

Primary cilium is a sensory organelle that senses mechanical signals on the apical membrane of cells [1-4]. Mechanical signals that produce enough force on the top of cells will bend and activate sensory cilia. These biomechanical properties play a very important role in visceral organs that support bodily fluid perfusion, including the heart. Of note is that traditional mouse models of homozygous cilia mutants do not survive at birth, pointing towards a possibility of heart dysfunction among others[5-10]. Aside from the kidney phenotype, the frequencies of phenotypic manifestations of patients with ciliopathy include congestive heart failure/hypertension (78%), hepatic cysts (75%), diverticulosis (70%), ovarian cysts (40%), cardiac valve disorders (25%), inguinal hernias (15%) and intracranial aneurysms (10%) [11].

Polycystic kidney disease (PKD), affecting 1 in 1,000 individuals, is an example of a ciliopathy resulting from the abnormal function and/or structure of primary cilia due to mutation in *Pkd2* gene encoding for polycystin-2 (PC2). Even with a successful renal transplant or replacement therapy, patients with PKD will eventually die due to cardiovascular complications, including heart failure and congestive heart disease[12-18]. Cardiovascular complications, including heart failure, contribute significantly to morbidity and mortality of PKD patients[19]. Although PKD has been categorized as a ciliopathy[20], the role of cilia in the heart has never been studied.

Polycystin-2 is involved in mechanosensation in the primary cilium of renal epithelia[21, 22], vascular endothelia[23, 24], cholangiocyte epithelia[25], and embryonic neural cells[26]. Most recently, polycystin-2 has been proposed to function as pressure sensor within the vascular system[27].Cilia have direct roles in heart development. *Pkd2* is required for the development of left-right asymmetry of the heart[28]. Cilia are also important for the development and function of the heart. Different mutations of cilia have led to abnormal cardiac development. For example, ventricular dilation and abnormal outflow tract development are seen at E11.5 in *IFT-88*-null mouse where cilia are absent. Cobblestone mutant mice, a hypomorphic allele of the gene *IFT-88*, have also shown numerous heart defects including persistent truncus arteriosus and hyperplasia of the myocardium at E14.5 and E16.5. Myocyte contraction is initiated by calcium influx through voltage gated L-type calcium channels (CaV1.2) triggering calcium-induced calcium release from the sarcoplasmic reticulum. Zebrafish with mutated *CaV1.2* do not have a functional beating heart[29, 30]. Furthermore, a recent study demonstrated that CaV1.2 and PC2 are co-localized to primary cilia of cardiac myocytes and that the loss of function of CaV1.2 and PC2 complex is manifested by cardiac edema and hypertrophy in zebrafish[2].

Primary cilia with 9+0 axoneme in myocytes of different species such as chicken, lizard, rabbit and mice were first described by Rash and colleagues decades ago [31]. Primary cilia in embryonic human heart were seen in epicardial, myocardial, and endocardial cells while adult human heart showed few cilia in non-muscular cells of the myocardial layer. Moreover, embryonic mouse heart at embryonic day E9.5 and E12.5 displays primary cilia in different areas. At E9.5, cilia are seen in left and right atria primordia, endocardial layer around ventricular trabeculations, endothelial cells of the atrial side of the endocardial cushion and mesenchymal cells within endocardial cushion (ECC). They are also found in early compact myocardial layer but with less abundance. At E12.5, cilia are found in locations similar to those in E9.5 in addition to the epicardial layer; however, they are less in number in the atrial endocardial layer [28]. Cilia length in embryonic mouse hearts ranges between 2-5μm in wild type animals according to Slough and colleagues [28]; however, others have also reported cilia at the E12.5 stage as 1-2μm length.

Although the presence of primary cilia in myocytes has been known for over 40 years[31], the presence and function of primary cilia in the adult cardiac system is still controversial. In fact, there is currently very little information about the roles of cilia in the adult heart function. Thus, cilia are mistakenly thought to be vestigial organelles with passive, nonfunctional remnants in the cardiac system. In this study, we demonstrate for the first time the presence of primary cilia in the adult mature mouse heart. We further propose primary cilia as a new organelle required for myocyte signaling and contraction. Furthermore, primary cilia could potentially be a novel and attractive therapeutic target for various cardiac diseases. Thus, our studies have a broad implication in basic science and in future therapeutic discoveries.

Materials and Methods

All experiments involving research animals are approved by The University of Toledo's Institutional Animal Care and Use Committee (IACUC). Wild type (WT) C57BL/6 mouse strain is used in all experiments. Mice are euthanized by asphyxiation using carbon dioxide for 5 minutes. Cervical dislocation was used as a second method of euthanasia to confirm death.

Immunofluorescence Microscopy

After dissecting the animals, mice hearts are excised and fixed in about 15 ml of 10% formalin overnight. Fixed tissues are embedded in paraffin and sectioned longitudinally at 4 μm thickness. Paraffin-embedded heart sections are baked in an oven at 60°C for 2 hours to remove the paraffin wax. Slides are then restored and rehydrated using xylene substitute (Sigma, Inc.) and a series of ethanol (100% 3x3 min, 90% 1x3 min, and 70% 1x3 min).

Antigen retrieval is performed by incubating the tissue sections in Proteinase K (A.G. Scientific, Inc.**)** in 20mM Tris-HCL (1:50) for 20 minutes followed by incubation in 30% H_2O_2 for 15 minutes. Slides are then washed with 1XPBS (HyClone Laboratories, Inc.) for 5 minutes and tissues are permeabilized and blocked using 1% triton X-100 (Fisher Scientific) in 10% FBS in PBS for 1 hour. Primary mouse anti-acetylated-α-tubulin antibody 1:500 (Sigma,Inc.) and primary rabbit anti-polycystin-2 antibody (PC2) 1:100 (Santa Cruz Biotechnology, Inc.) are used as primary cilia markers. Primary rabbit anti-pericentrin antibody 1:500 (Covance, Inc.) is used to stain the basal body of cilia or centriole. Slides are then washed three times with 1XPBS for 5 minutes. Secondary fluorescein anti-mouse IgG 1:500 (Vector Labs, Inc.), secondary texas-red anti-rabbit IgG 1:500 (Vector Labs, Inc.), and fluorescein Wheat Germ Agglutinin (WGA) 1:500 (Vector Labs, Inc.) are added for 1 hour after washing with 1XPBS three times for 5 minutes. Before observation under a fluorescent microscope (Nikon TiU), the section was counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for 5 minutes to stain the nucleus/DNA. To minimize photo bleaching, the sections were imaged immediately under minimum exposure time.

Cilia Number

The presence of primary cilia is confirmed with anti-acetylated-α-tubulin antibody and cilia numbers are recorded as a percentage, by dividing the number of cells with primary cilia by the total number of cells (represented by DAPI or WGA staining) in each field of vision under a fluorescent microscope (Nikon TiU). At least three wild-type mice from each age group (<3months, 3-6months, and >6months old) are used in every experiment. Over 400 cells are counted in each group. Percentages are then averaged and compared between the three groups using SPSS software.

Cilia Length Measurement

Primary cilia structure is mainly composed of acetylated microtubules. Primary cilia length is measured by direct immunofluorescence staining using anti-acetylated α-tubulin antibody (1:1,000; *Sigma, Inc.*). Cilia length (μm) is recorded using confocal microscope (Leica Microsystems) and measured using calibrated image acquisition and analysis MetaMorph software. For statistical purposes, at least three wild-type mice are used in each experiment from three different age groups (<3months, 3-6months, and >6months old). More than 30 cilia are measured in each group. Lengths are averaged and compared between the three groups using SPSS software.

Dissection of Heart Chambers

In addition to staining the whole heart tissue, we also stained the individual heart chambers in some experiments. In order to dissect the heart chambers, the whole heart was excised first and positionedin a way similar to its position in the body. The right atrium is easily identified and cut at the top right of the heart and the left atrium is located to the top left of the heart but in a slightly lower plane than the right atrium. In order to dissect the ventricles, the heart is cut below the roots of aorta and pulmonary artery to expose the left ventricle and left ventricle chambers, respectively. Right ventricle is easily identified and cut from its attachment with left ventricle since it has the thinner layer wall. After dissecting right ventricle, left ventricle with atrioventricular septum attached to it is exposed. Left ventricle is isolated after cutting the borderline of septum along its attachment to the left ventricle.

Statistics

All images are analyzed using MetaMorph software. All quantifiable data are reported as mean ± SEM. Comparisons between means are performed using one way ANOVA test followed by Tukey post-test analysis and statistical significance implies $p< 0.05$. All data analysis is done using SPSS software.

Results

PrimaryCilia are Present in Adult Mouse Heart

In order to verify the presence of cilia in adult mouse heart, whole-heart tissue-sections from adult mice from threedifferent age groups (<3 months, 3-6 months, and >6 months old) are stained with anti-acetylated-α-tubulin antibody (red), a well-known ciliary marker, and Wheat Germ Agglutinin (WGA, green), a carbohydrate-binding protein used to mark the plasma membrane of cells in the cardiac tissues. DAPI (blue) is used to counterstain DNA/nucleus. The presence of primary cilia is confirmed in all age groups (Figure1).

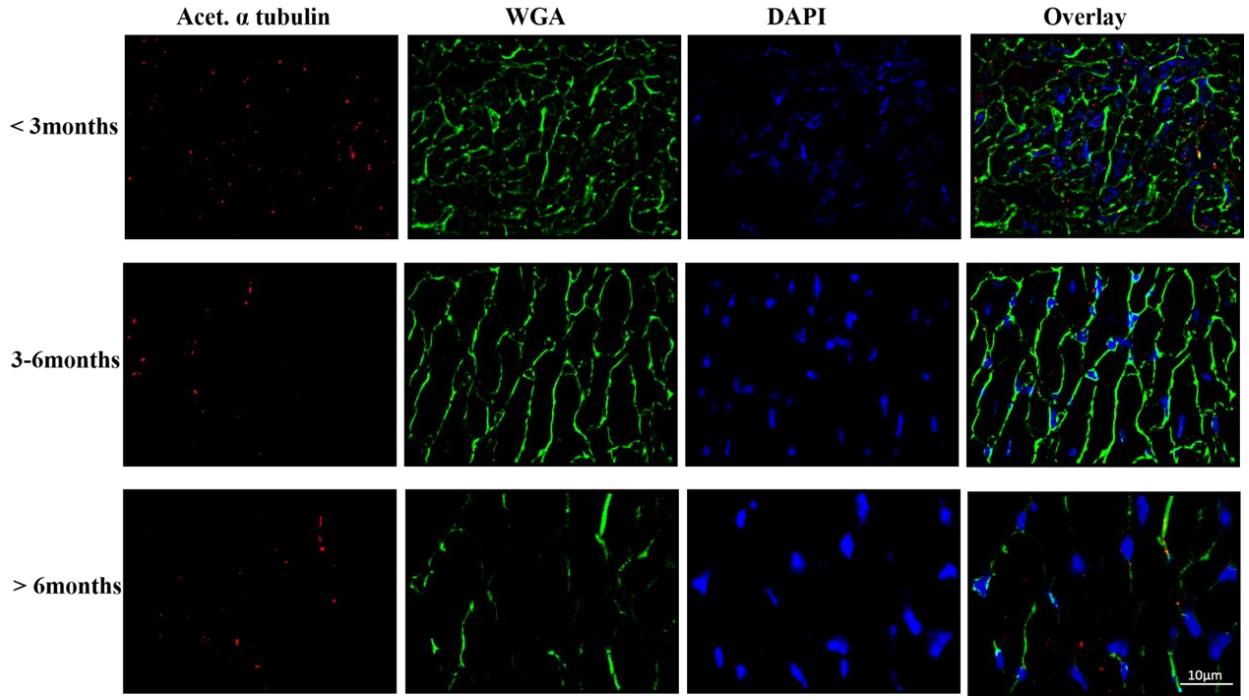

Figure 1: Primary cilia are present in adult mouse heart. Representative images from the whole heart showing primary cilia from different age groups of adult mice. Heart sections stained with anti-acetylated-α-tubulin antibody (red), a ciliary marker, and WGA (green), a plasma membrane marker and DAPI (blue) is used to counterstain DNA/nucleus. Images were captured at 60X 1.5- magnifications.

Primary cilia emanate from and are anchored to the cell body by the basal body or the centriole.

To further confirm the presence of cilia in adult mouse heart, anti-acetylated-α-tubulin antibody staining is accompanied with anti-pericentrin antibody (a marker for the centriole at the base of cilia). Representative images of the co-localization of anti-acetylated-α-tubulin (green)and anti-pericentrin (red)antibodies at the cardiac primary ciliaare obtained from 1-, 3-, 6-, and 12-month old mice (Figure 2).

For clarification purposes, the anti-pericentrin and WGA staining performed in the previous experimentswere merged and the co-localization with anti-acetylated-α-tubulin (red) within cardiac cells is clearly seen (Figure 3).

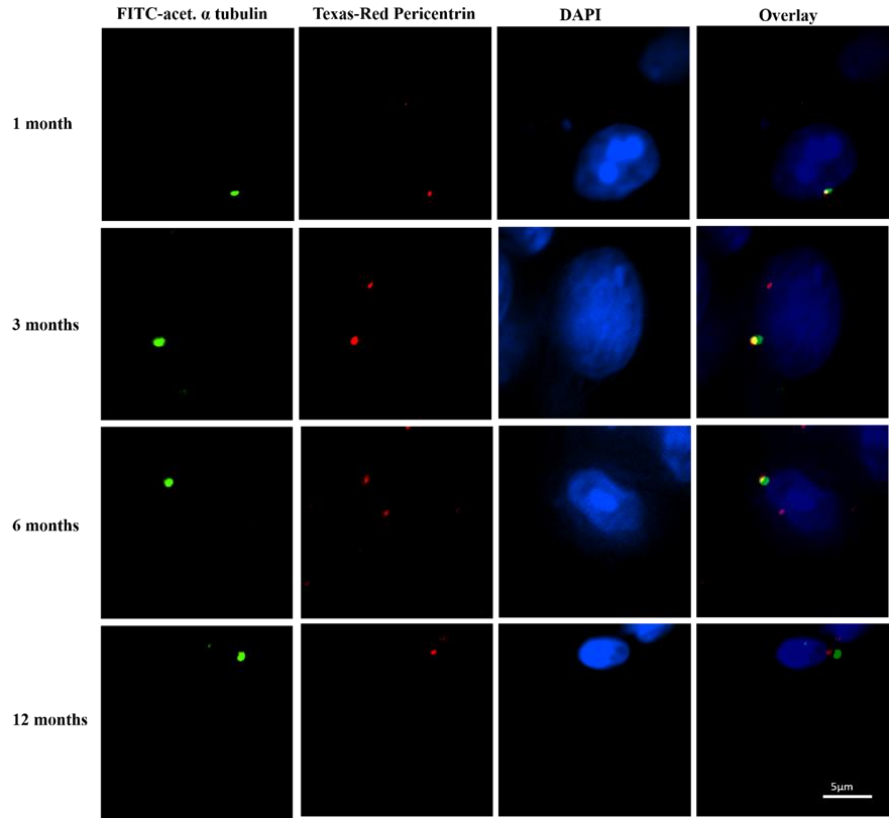

Figure 2: Confirmation of primary cilia presence in adult mouse heart with anti-pericentrin antibody. Co-localization of anti-acetylated-α-tubulin (green) and anti-pericentrin (red) antibodies at the cardiac primary cilia at different stages in mouse adulthood is shown through representative images from the whole heart. Images are captured at 100X magnification.

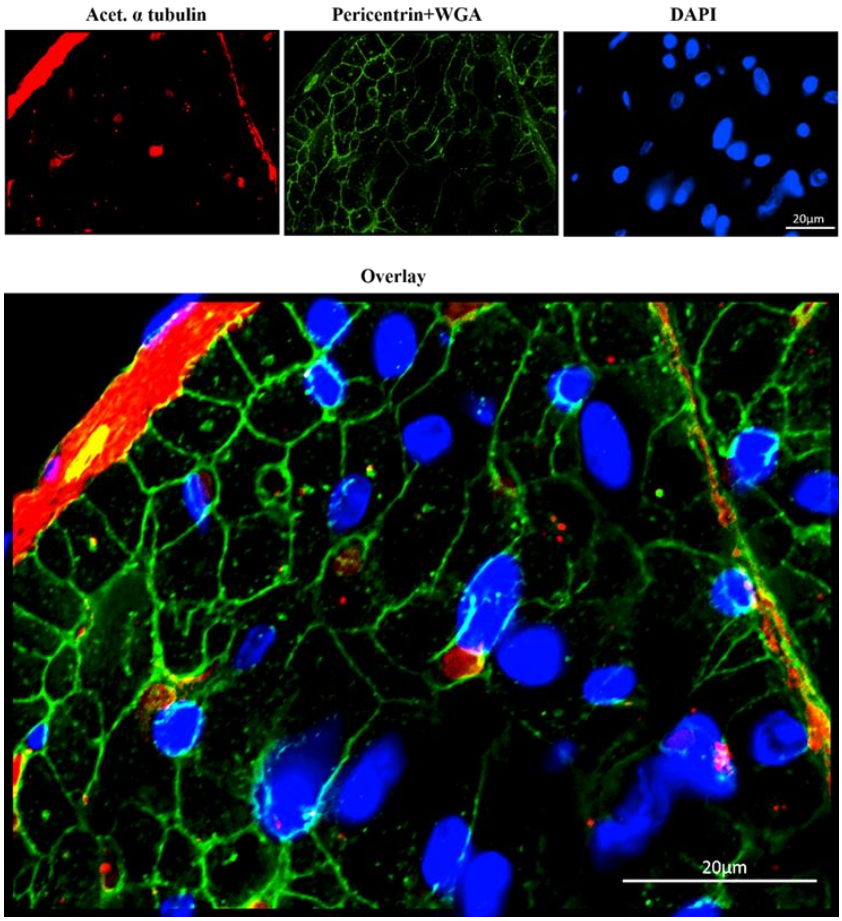

Figure 3: Confirmation of primary cilia presence in adult mouse heart with combined staining of WGA and anti-pericentrin antibody. Representative images from the whole heart showing the co-localization of anti-acetylated-α-tubulin and anti-pericentrin antibodies in the presence of WGA, a plasma membrane marker. DAPI (blue) is used to counterstain DNA/nucleus. Image is captured at 100X magnification.

Polycystin-1 and polycystin-2 are mechanosensory proteins that are known for their ciliary localization in different tissue- and cell-types throughout the body [21]. Polycystin-2 functions as a calcium channel and has been shown to play a role in calcium signaling in various cell types such as kidney and endothelia [23]. In order to test the hypothesis that polycystin-2 is a calcium channel involved in adult cardiac myocytes contraction, polycystin-2 localization to primary cilia of cardiac myocytes in the adult heart tissue sections was analyzed. This also provides anadditional confirmation of cardiac primary cilia presence. Immunohistochemistry is performed by staining heart tissues with anti-polycystin-2 antibody. Representative images of the co-localization of anti-acetylated-α-tubulin(green)and anti-polycystin-2 (red) antibodiesare attained from 1-, 3-, 6-, and 12-month old mice (Figure 4).

After confirming the cilia presence with different markers in the adult mouse heart sections, the presence of cilia in the isolated heart chambers i.e. left auricle (LA), left ventricle (LV), right auricle (RA) and right ventricle (RV)was then examined. The different heart chambers were isolated and stained with anti-acetylated-α-tubulin antibody (red) as a cilia marker, WGA(green) to mark the plasma membrane of the cells and DAPI (blue) to counterstain DNA/nucleus. Figure 5 shows representative images of the primary cilia in all heart chambers.

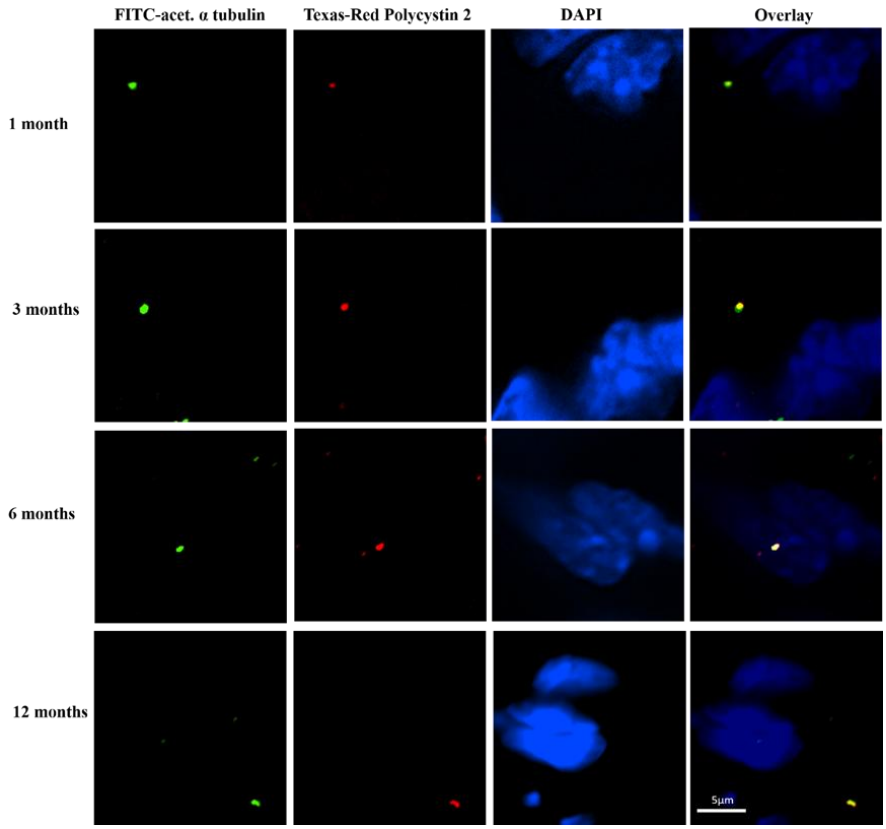

Figure 4: Polycystin-2 is localized to primary cilia of adult mouse heart. Representative images obtained from the whole heart of 1-, 3-, 6-, and 12-month old mice showing the co-localization of polycystin-2 (red) and acetylated-α-tubulin (green) at cardiac primary cilia. DAPI (blue) is used to counterstain DNA/nucleus. Images are captured at 100X magnification.

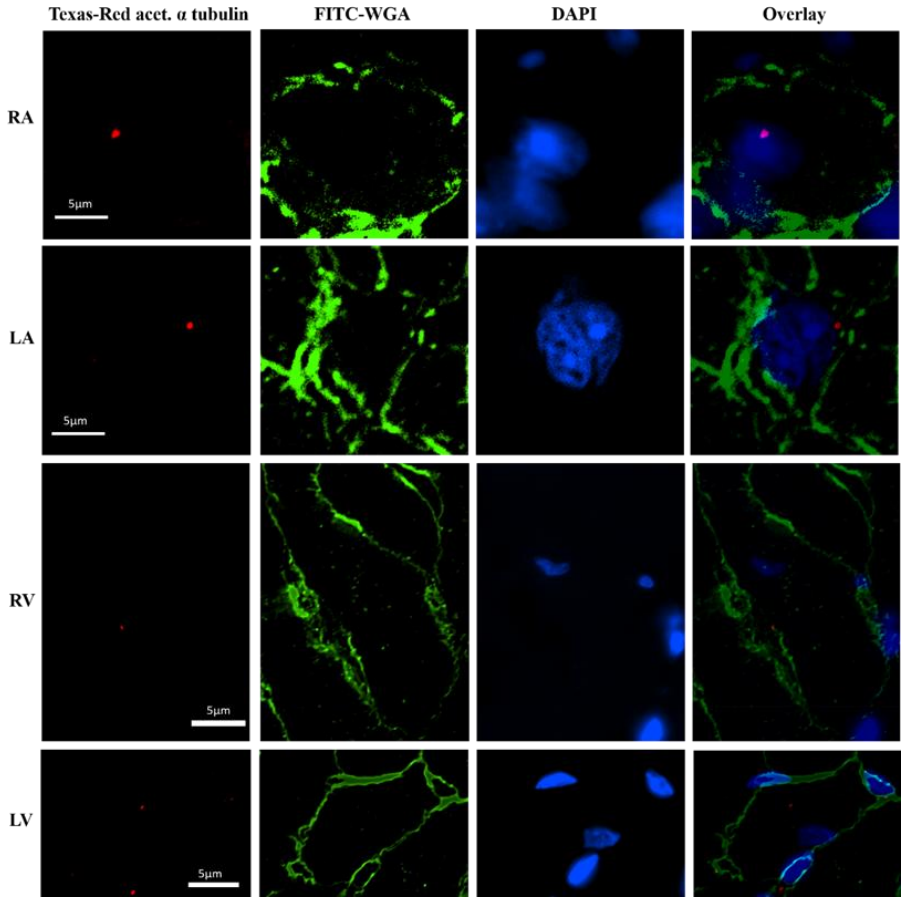

Figure 5: Primary cilia are present in adult mouse heart chambers. Representative images from individual heart chambers showing cardiac primary cilia in the different heart chambers of adult mouse tissues stained with anti-acetylated-α-tubulin antibody (red) and WGA (green). DAPI (blue) is used to counterstain DNA/nucleus. Images are captured at 100X magnification. RA= Right atrium, LA=Left atrium, RV=Right ventricle, LV=Left ventricle.

Similar to previous experiments and after confirming the cilia presence in heart chambers with anti-acetylated-α-tubulin antibody, the presence of cilia with anti-pericentrin antibody was then further confirmed. So, heart chambers are stained with anti-acetylated-α-tubulin (green) and anti-pericentrin (red) antibodies. Figure 6 shows representative images of the co-localization of the two markers to the cilia in all the heart chambers.

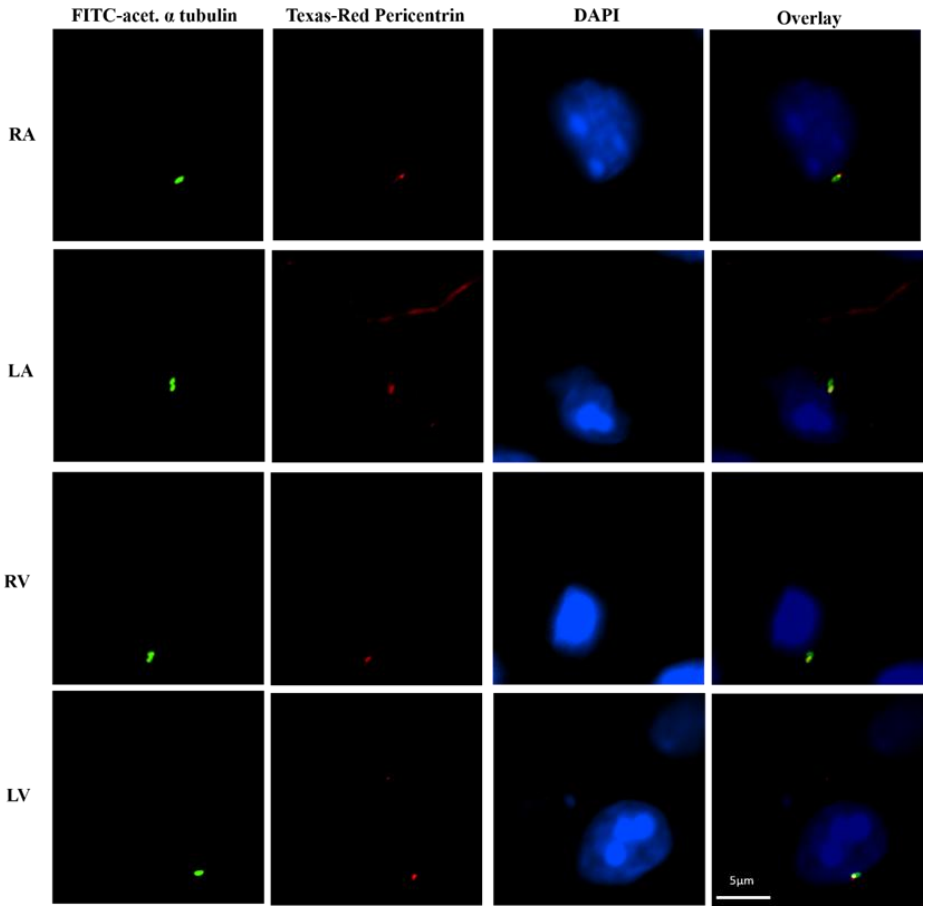

Figure 6: Confirmation of primary cilia presence in adult mouse heart chambers with anti-pericentrin antibody. Representative images from individual heart chambers showing the co-localization of anti-acetylated-α-tubulin (green)and anti-pericentrin (red) antibodies at cardiac primary cilia in the atria and ventricles. DAPI (blue) is used to counterstain DNA/nucleus. Images are captured at 100X magnification. RA= Right atrium, LA=Left atrium, RV=Right ventricle, LV=Left ventricle.

Primary cilia number in adult mouse heart

After confirming the existence of primary cilia in adult mouse heart with several markers, the abundance of primary cilia among different age groups (<3 months old, 3-6 months old, and >6 months old)was studied. The number of cilia as a percentage of the total number of cells within the same field of vision was recorded. In the first age group (<3 months old), cardiac myocytes with primary cilia accounted for about 37% of the total number of cells. Interestingly, the abundance of primary cilia declined to 29% and 25% in the second (3-6 months old) and third (>6 months old) age groups, respectively. Although our study shows that there is a decline in primary cilia abundance with increased age in adult mice heart, this decline in cilia number is only statistically significant between the first group (< 3 months) and the third group (> 6 months) (Figure 7).

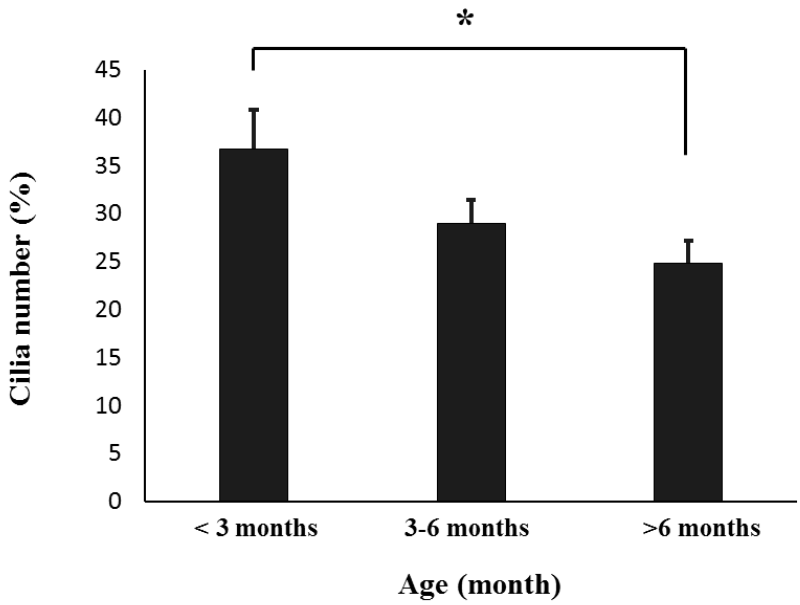

Figure 7: Primary cilia abundance in adult mouse heart is age dependent. Cilia number in the whole heart is represented in three age groups (<3 months, 3-6 months, and >6 months old mice) and our study shows a significant decrease in their abundance with age specifically between the first group (< 3 months) and the third group (> 6 months), *p<0.021*.

Primary cilia length in adult mouse heart

Studies on primary cilia in the renal epithelial system show that cilia length is highly regulated, with the cilia being longer in larger lumen and shorter in smaller lumen[32]. Many studies have also found that cilia length can be regulated under different physiological conditions[33]. Moreover, the length of primary cilia has been shown to play a significance role in their sensory function. So, cilia length was the next parameter that was studied. To accomplish this goal, cilia length from three different age groups (<3 months, 3-6 months, >6 months old mice) is measured and compared between the groups. Our data show that the length of primary cilia does not change significantly during different stages of mice adulthood with an average length of approximately 1µm (Figure 8).

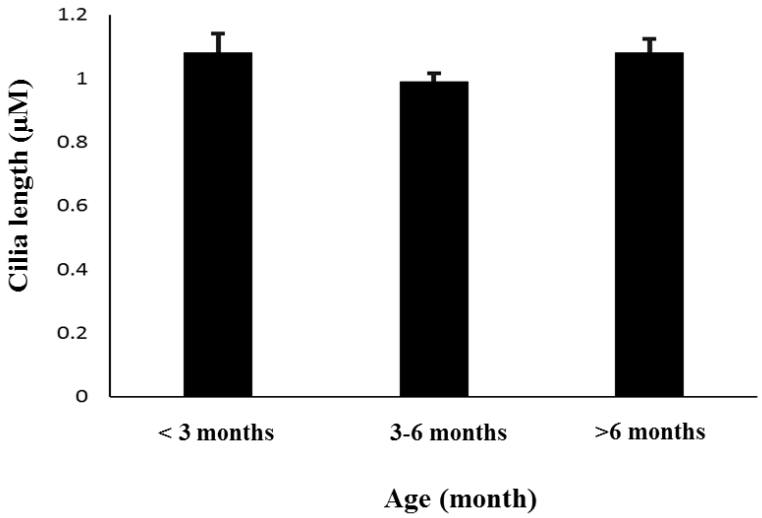

Figure 8: Primary cilia length in adult mouse heart is age independent. Primary cilia length does not change with age during mouse adulthood and the average length is around 1µm, *p<0.27*.

Discussion

Primary cilia in embryonic mouse heart have been reported several times to play crucial roles in cardiac development and function [28]. However, little is known and doubts are rising regarding the presence of primary cilia in adult mouse heart and their function. In order to confirm the presence of primary cilia in adult mouse heart, many approaches can be used such as electron microscopy, immunohistochemistry, or functional studies through knockdown or knockout approaches of genes that encode ciliary proteins. In our study, the presence of primary cilia in adult mouse heart and their potential role in the cardiovascular system through immunohistochemistry was investigated.

Our studies initially aimed at examining mouse heart sections from different adult age groups by staining with an antibody against acetylated-α-tubulin, a well-known ciliary marker, and WGA, a carbohydrate binding protein used to mark cells' plasma membrane. Our data confirms the presence of primary cilia within heart cells in adult mice aged between <3 months, 3-6 months, and >6 months old. This confirms the presence of primary cilia in adult mouse heart in early and late stages of adulthood indicating a possible function of cilia in the heart during mouse adulthood.

To rule out the possibility that our staining with anti-acetylated-α-tubulin antibody was not due to non-specific binding or artifacts and to further confirm cardiac primary cilia presence, adult mouse heart tissues from different stages in mouse adulthood were stained with anti-pericentrin antibody, a marker for the basal body of cilia from which cilia project, in addition to the staining with anti-acetylated-α-tubulin antibody (a ciliary marker). Figure 2 shows representative images of the co-localization of the two markers to cardiac primary cilia from 1-, 3-, 6-, and 12-month old mice. This additionally confirms that primary cilia continue to be present during mouse adulthood and suggests that they may still play a role in adult mouse heart in addition to their role during embryonic stage.

To further confirm the presence of primary cilia and get a better grasp of their possible function in adult heart, heart sections were stained with antibody against polycystin-2, a calcium channel found to be localized to cilia in different cell types mediating many calcium dependent pathways. Figure 4 provides representative images obtained from 1-, 3-, 6-, and 12-month old mice that show the co-localization of anti-polycystin-2 and anti-acetylated-α-tubulinantibodies to cardiac primary cilia, proposing a possible role of cilia in the adult heart through calcium signaling. Based on previous and ongoing studies from our laboratory, we propose that primary cilia might regulate myocardial contractility and blood flow within the heart chambers through maintaining sufficient calcium influx into cardiac myocytes. Also, their role could be similar to the one reported in endothelial cells where the activation of polycystin-2 increases the influx of calcium and triggers a series of calcium dependent signaling pathways.

Moreover, our data indicated the presence of primary cilia in all heart chambers. Figure 6 provide representative images of the co-localization of anti-acetylated-α-tubulinand anti-pericentrinantibodies to cardiac primary cilia in all heart chambers. The presence of primary cilia in all heart chambers might suggest a common function of primary cilia in each chamber. More importantly, the presence of primary cilia in an area with high shear stress such as the left ventricle contradicts some previous studies that report primary cilia reabsorption in response to shear stress. However, this might be explained partially by the very short and stubby nature of primary cilia in these chambers where the magnitude of shear stress is extremely high for the cilia to maintain their presence.

Furthermore, we studied the abundance of cardiac primary cilia during mouse adulthood. Cilia number was recorded as a percentage by dividing the number of cells with primary cilia by the total number of cells in each field of vision. Figure 7 in our results section compares the cilia abundance in adult mouse heart among three age groups (< 3 months, 3-6 months, and >6 months old). Our data shows that cells with primary cilia in early mouse adulthood comprise approximately one third of the total number of cells. Although this shows that not all cells in the heart are ciliated, this percentage of abundance of primary cilia must indicate a role of cilia in adult mouse heart. A decline in cilia number with increased age was seen in the older groups. This could be a sign that primary cilia abundance is age dependent.

Finally, Cilia length was the next parameter to be examined in our study due to its association with their sensory function. Cilia were confirmed with anti-acetylated-α-tubulinantibody and the length of primary cilia in adult mouse heart was measured and compared between three age groups (< 3 months, 3-6 months, and >6 months old). As shown in figure 8, we reported the average cilia length to be around 1μm and do not fluctuate much during adulthood. This shows slightly shorter cilia than the ones seen at the embryonic stage which could be simply a response of cilia towards the high shear stress inside the heart during adulthood and a mechanism by which cilia survive such a harsh condition.

Conclusions

In summary, our studies provide a novel insight into the localization and distribution of primary cilia in adult mouse heart. Without doubt, there are many more interesting future experiments that are revealed by our study. For example, studies of primary cilia abundance and cilia length in the different heart chambers are still needed in order to verify any difference in the distribution of cilia between heart chambers. Moreover, for better understanding of the role of primary cilia in adult mouse heart, other ciliary protein localizations and functional studies are warranted among which are CaV1.2, a voltage gated calcium channel, polycystin-1 and others. Finally, an *in vivo* model of cilia in the heart may reveal some translational importance of our studies.

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Declaration of Interest

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