2015

Localization and distribution of primary cilia in adult mouse heart

Ali Zarban
University of Toledo

A Thesis

Entitled

Localization and Distribution of Primary Cilia in Adult Mouse Heart

By

Ali Zarban

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in

Pharmaceutical Sciences

_____

Dr. Wissam AbouAlaiwi, Committee Chair

_____

Dr. Lijun Liu, Committee Member

_____

Dr. Andrea Kalinoski, Committee Member

_____

Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

August 2015

An Abstract of

Localization and Distribution of Primary Cilia in Adult Mouse Heart

By

Ali Zarban

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Master of Science Degree in
Pharmaceutical Sciences

The University of Toledo

August 2015

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 our study, we investigated the presence and the potential function of primary cilia in adult mouse heart through immunohistochemistry. Initially, heart tissues from different ages in mouse adulthood were stained with an antibody against acetylated-α-tubulin, a cilia marker, and WGA, a plasma membrane marker, and primary cilia were found to be on the surface of cardiac cells in early and late mouse adulthood. Cardiac primary cilia presence in adult mouse heart was further confirmed by staining heart tissues with an antibody against pericentrin, a marker for the basal body of cilia from which cilia arise, and the co-localization of anti-pericentrin and anti-acetylated-α-tubulin antibodies to primary cilia was seen in 1 month, 3 months, 6 months, and 12 months old mice. Moreover, the presence and possible function of primary cilia in adult mouse heart were also examined by staining heart tissues with an antibody against polycystin-2, a calcium channel that is found to be localized to cilia in different tissues, and the co-localization of

anti-polycystin-2 and anti-acetylated-α-tubulin antibodies to cardiac primary cilia was seen in early and late mouse adulthood, proposing a role of primary cilia in the adult mouse heart through calcium signaling. Moreover, primary cilia presence in the different heart chambers was next confirmed by staining the four heart chambers with antibodies against both acetylated-α-tubulin and pericentrin. Furthermore, we studied the abundance of cardiac primary cilia during mouse adulthood using three different age groups (< 3 months, 3-6 months, and >6 months old mice), and found out that cells with primary cilia in the first group accounted for about 37% of the total number of cells; however, the number of primary cilia in the late two groups was found to decrease to 29% and 25%, respectively, suggesting that primary cilia abundance is age dependent. Finally, cilia length was measured and compared between the same three age groups and our study reported an average cilia length of approximately one μm in all age groups, showing that cilia length in adult mouse heart does not change with increased age. In summary, our study provides for the first time a novel insight regarding the localization and distribution of primary cilia in adult mouse heart.

Acknowledgements

I cannot thank my advisor Dr. Aboualaiwi enough for making everything possible for me to finish my project and thesis on time. I deeply appreciate his patience, time, and continuous encouragement and guidance. I also thank Dr. Nauli, with whom this project started, for his continuous advice and support.

I sincerely thank my committee members, Dr. Liu and Dr. Kalinoski, for their continuous encouragement, advice and support. I thank Dr. Liu especially for providing some antibodies and animals and for helping me with animal dissection. I thank Dr. Kalinoski especially for giving me full access to all types of microscopes in her lab and for always making the time to teach me how to use them.

I thank Dr. Sari and his lab members for their assistance and patience. Also, I truly appreciate Dr. Steinmiller's help on reviewing my statistical analysis.

I thank my employer, Jazan University, for the financial support. I also thank the faculty members at the Department of Pharmacology and Experimental Therapeutics and the University of Toledo-College of Pharmacy and Pharmaceutical Sciences for their support and assistance during my Master's degree.

I thank all my lab mates Ashraf, Alzahra, Zubayer, Hannah and Laura for their help and for making the lab such a great environment to do research.

Finally, I am sincerely grateful to all my family members overseas for their unconditional love and support.

Table of Contents

List of Figures

List of Abbreviations

ADPKD …….Autosomal Dominant Polycystic Kidney Disease

ARPKD …….Autosomal Recessive Polycystic Kidney Disease

ANOVA ……Analysis of Variance

CaV1.2 ……. L-Type calcium channel

DAPI …….4', 6-diamidino-2-phenylindole

ECC ……..Endocardial Cushion

PC-1…….. Polycystin-1

PC-2 ……..Polycystin-2

PCD …….. Primary Ciliary Dyskinesia

PKD …….. Polycystic Kidney Disease

WGA …… Wheat Germ Agglutinin

WT ……Wild Type

Chapter 1

Introduction

1.1. Cilia

Cilia are sensory projections located at the cell surface of most cells in the body (Wheatley, 1996). Based on their axonemal microtubule arrangement, cilia are generally categorized into primary non-motile and motile cilia. They share nine outer pairs of microtubules but the primary cilia miss a central pair of microtubules; for this reason they are described as “9+0” cilia. On the other hand, motile cilia show “9+2” axonemal arrangement **(Figure 1)**. The clear deviation from this classification is seen in the nodal cilia which are now considered as a third category since they are motile yet have “9+0” axoneme. Nodal cilia are seen in the embryonic node where they are responsible for the left and right arrangement of internal organs (Tissir et al., 2010).

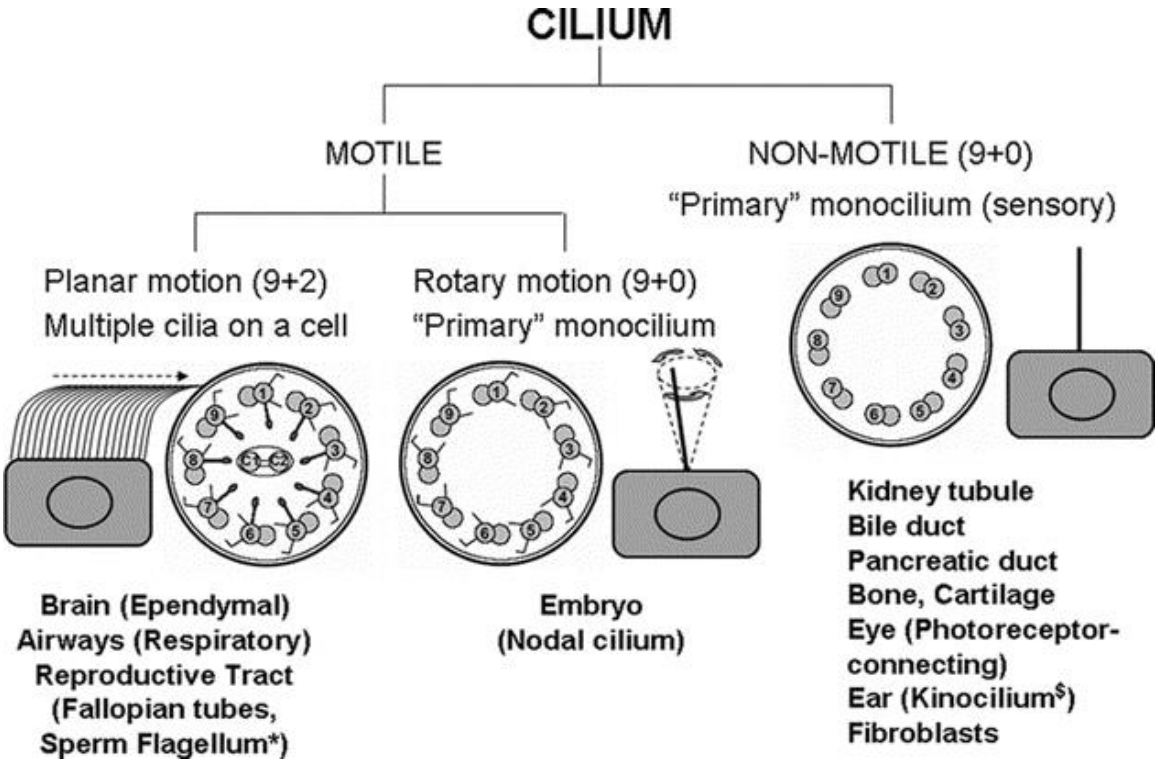

Figure 1. Types of cilia. Cross sections showing the different types of cilia classified depending on their axonemal arrangement of microtubules into primary non-motile (9+0) and motile with planar motion (9+2) or rotary motion (9+0). The figure also indicates the localization of each cilia type within body tissues. (Used with permission from Leigh et al., 2009)

1.2. Primary Cilia

Primary cilia, which are usually found as one cilium per cell, arise from a mother centriole or basal body and depend on an intraflagellar transportation (IFT) system for their formation, maintenance and signal transduction (Satir and Christensen, 2007). The ciliary doublet microtubules project from triplet microtubules in the basal body in an area referred to as the transition zone (Rohatgi and Snell, 2010). This transition zone contains many plasma membrane proteins known as the ciliary necklace which discriminate the

plasma cell membrane from the ciliary membrane (Gilula and Satir, 1972). Moreover, the IFT system is composed of many proteins and molecules that travel in two directions, upward and downward, along the cilium (Pedersen et al., 2012). The upward movement is mainly regulated by kinesin-2 while dynein-2 regulates the downward movement (Pedersen and Rosenbaum, 2008; Ishikawa and Marshall, 2011) **(Figure 2)**. Although primary cilia have been around for over a century, their role has not been appreciated until recently due to the discovery of primary cilia association with many diseases primarily polycystic kidney disease. Primary cilia are seen in tissues such as kidney tubules, retina, and aorta. They work as chemosensory (Handel et al., 1999) and mechano-sensory (Praetorius and Spring, 2005) organelles by which cells interpret external signals. They are also involved in many signaling pathways crucial to development such as Hedgehog (Clement et al., 2009) and Wnt signaling (Kestler and Kühl, 2008).

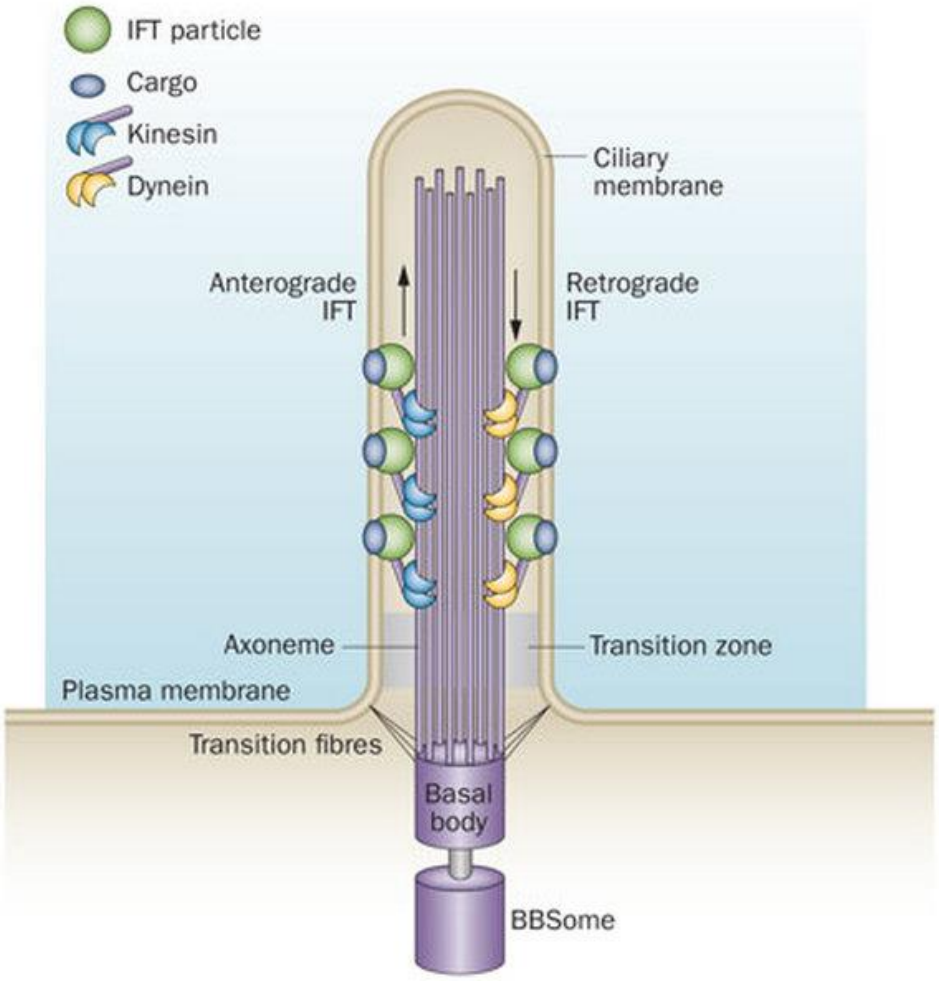

Figure 2. Ultrastructure of primary cilia. Primary cilia are protrusions engulfed in a ciliary membrane at the cell surface of most eukaryotic cells. They consist of nine pairs of microtubules axoneme and project from a basal body in the transition zone. Primary cilia rely on intraflagellar transportation system for transporting molecules in anterograde and retrograde pathways along the cilium where kinesin regulates the anterograde movement and dynein regulates the retrograde movement. (Used with permission from Valente et al., 2014).

1.3. Motile Cilia

Motile cilia are usually present as multi-ciliated organelles. The peripheral doublet microtubules of the motile cilia have outer and inner dynein arms attached to them. Furthermore, the motile cilia have rod like structures known as radial spokes that extend from the outer nine microtubules toward the central pair of microtubules. Motile cilia are seen in places such as the trachea, the sperms and the ependymal cells of the brain, serving as mucus and fluid moving machines through two dynein arms that bring about ciliary movement (Satir and Christensen, 2007). Moreover, the ependymal cells at the third ventricle of mouse brain are classified into three types depending on cilia movement patterns (Liu et al., 2014).

1.4. Cilia-Related Disorders

Due to cilia distribution within most tissues in the body, any dysfunction in cilia leads to a plethora of diseases. For example, mutations in the primary cilia in the epithelial cells of the kidney tubules leads to polycystic kidney disease (PKD) in addition to liver and pancreatic cysts. This occurs due to the inability of primary cilia to sense the shear stress exerted by the urine flow leading to randomization in the direction of cell division within the tubules. Normally, primary cilia bend in response to the direction of fluid flow sending signals to the cells. This bending in turn results in the influx of calcium to the intracellular compartment (Praetorius et al., 2003). Polycystin-1 and polycystin-2, proteins encoded by Pkd-1 and Pkd-2 genes, respectively, have been shown to be localized to primary cilia and function as a mechano-sensory complex (Nauli et al.,

2003). Another example of a cilia-related disease is primary ciliary dyskinesia (PCD), a genetic disease that affects motile cilia. It is manifested by defects in the ciliary movement mainly due to a deformity of the dynein arms, leading to many disorders such as bronchitis, infertility, and disruption of the internal organs arrangement (Afzelius, 2004). Other diseases that also involve cilia include retinitis pigmentosa and obesity.

1.5. Polycystic Kidney Disease

Polycystic kidney disease (PKD) is a disease that affects the nephron and is manifested by fluid filled cysts leading eventually to end stage renal failure. There are two forms of the disease, autosomal dominant and autosomal recessive PKD. The autosomal dominant polycystic kidney disease (ADPKD) is the most common form and in fact is considered the most common hereditary disease since it affects about 1:800 live births. Moreover, ADPKD is characterized by mutations in Pkd1 gene, which account for about 85% of cases, and mutations in Pkd2 gene, which are seen in 10-15% of the cases. Pkd1 and Pkd2 are the genes that encode for polycystin-1 and polycystin-2, respectively. On the other hand, autosomal recessive PKD (ARPKD) is characterized by mutations in Pkhd1 gene that encodes for fibrocystin. Cysts in PKD patients are also seen in extra-renal tissues such as liver and pancreas, along other early symptoms which include hypertension and back pain. Furthermore, ADPKD cysts are defined by abnormal epithelial cell division and ion and fluid secretion. Although some approaches have been proposed to treat PKD patients, the only available treatment is through dialysis and renal transplantation in addition to the aggressive control of hypertension (Wilson, 2004).

1.6. Cardiac Myocytes

The myocyte cells are the contraction force of the heart. According to Banerjee, they comprise approximately 56% of the total number of cells in the myocardium of mice (Banerjee et al., 2007). Myocytes produce their contractility through the sarcomere, a crucial unit that houses many proteins of the contractile apparatus like myosin, actin, and troponin complex. The contractility of the myocyte requires an enormous amount of energy which is provided by the mitochondria since they account for 40% of the myocyte cell volume. The mitochondria also preserve the myocyte from any Ca^{2+} overload effects by forming a complex with the excessive cytosolic calcium (Walker and Spinale, 1999). Calcium plays an important role in myocyte contractility. In the presence of cardiac action potential, L-type calcium channels are activated leading to an increase in the influx of calcium inside cardiac myocytes, releasing in return more calcium from the sarcoplasmic reticulum. The extra cytosolic calcium binds to Troponin C and triggers a synchronized movement of myofilaments which is then interpreted into myocyte contraction (Bers, 2000).

1.7. Cardiac Primary Cilia

Primary cilia with 9+0 axoneme in myocyte of different species such as chickens, lizards, rabbits and mice were first described by Rash and colleagues decades ago (Rash et al., 1969). Myklebust et al. have also shown primary cilia in normal embryonic and adult hypertrophied human heart tissues. 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 (Myklebust et al., 1977). 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 (Slough et al., 2008). Cilia length in embryonic mouse heart ranges between 2-5μm in wild type animals according to Slough and colleagues; however, others have also reported cilia at the E12.5 stage as 1-2μm length (Willaredt et al., 2012).

Cilia are also important to 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 (Clement et al., 2009). 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 (Willaredt et al., 2012). Furthermore, a recent study demonstrated that CaV1.2, a voltage gated calcium channel, and PC-2 are co-localized to primary cilia of cardiac myocytes and that the loss of function of CaV1.2 and PC-2 complex is manifested by cardiac edema and hypertrophy in zebrafish (Muntean et al., 2014).

1.8. Role of Primary Cilia in Adult Heart

The significance of studying cilia in the adult heart comes from the serious cardiovascular complications of polycystic kidney disease and the fact that they are the leading cause of death for PKD patients.

Hypertension is seen in about 70% of autosomal dominant polycystic kidney disease patients and it precedes the deterioration in the renal function (Chapman & Schrier, 1991; Kelleher et al., 2004). Primary cilia have been shown to play an important role in regulating pressure through sensing and bending in response to the flow inside endothelial blood vessels. This leads to the activation of polycystins complex and the release of the well-known vasodilator nitric oxide through triggering calcium dependent cascade (Aboualaiwi et al., 2009). Also, high left ventricle mass index is reported in patients with PKD indicating left ventricle hypertrophy and appears to be independent of systolic blood pressure (Lumiaho et al., 2003). Congestive heart failure is another example of cardiovascular complications of PKD. Patients with heart failure still die even after a successful renal transplant (Biagini et al., 1993; Torres & Harris, 2006). However, the role of primary cilia in heart failure remains unclear.

Chapter 2

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. After dissecting the animals, mice hearts are excised and fixed in about 15 ml of 10% formalin overnight. Fixed tissues are sent to the pathology lab to be embedded in paraffin and sectioned longitudinally at 4 µm thickness.

2.1. Immunofluorescence Microscopy

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 (PC-2) 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 Lab, Inc.), secondary texas-red anti-rabbit IgG 1:500 (Vector Lab, Inc.), and fluorescein Wheat Germ Agglutinin (WGA) 1:500 (Vector Lab, 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 with the minimum exposure time possible.

2.2. Counting 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 are used in each experiment from three different age groups (<3months, 3-6months, and >6months old). Over 400 cells are counted in each group. Percentages are then averaged and compared between the three groups using SPSS software.

2.3. Measurement of Cilia Length

Cilia are confirmed with anti-acetylated-α-tubulin antibody. Cilia length (μm) is measured using confocal microscope (Leica Microsystems). 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.

2.4. Dissection of Heart Chambers

In addition to staining the whole heart tissue, we also stained the different heart chambers in some experiments as indicated in the figures' legends in the results section. In order to dissect the heart chambers, we first excised the whole heart and put it in a position that is similar to its position in the body as you face the body. The right atrium is easily identified and cut at the top right of the heart and the left atrium will be located to the top left of the heart but in a slightly lower plane than the right atrium. In order to dissect the ventricles, 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.

2.5. 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.

Chapter 3

Results

3.1. Confirmation of Cilia Localization in Adult Mouse Heart by Immunostaining

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

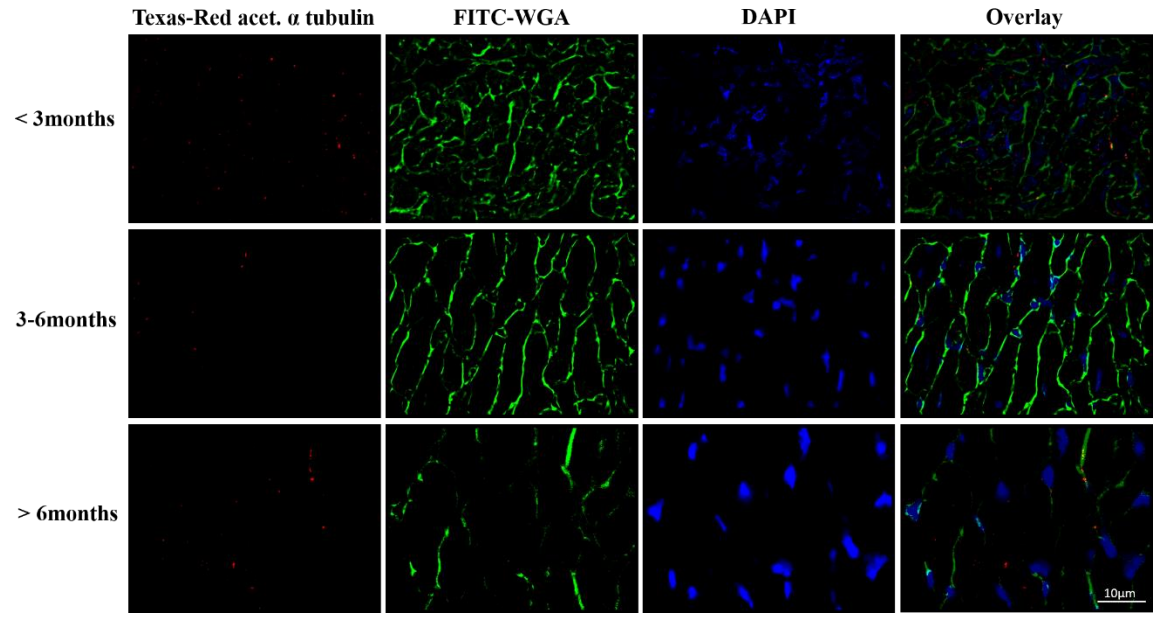

Figure 3. Confirmation of primary cilia presence in adult mouse heart with anti-acetylated-α-tubulin antibody. Representative images from the whole heart showing primary cilia from different age groups of adult mice. Heart sections stained with anti-acetylated-α-tubulin antibody (red), a ciliary marker, and WGA (green), a plasma membrane marker and DAPI (blue) is used to counterstain DNA/nucleus. Images were captured at 60X 1.5- magnification.

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 cilia are obtained from 1 month, 3 months, 6 months, and 12 months old mice. DAPI (blue) is used to counterstain DNA/nucleus (**Figure 4**).

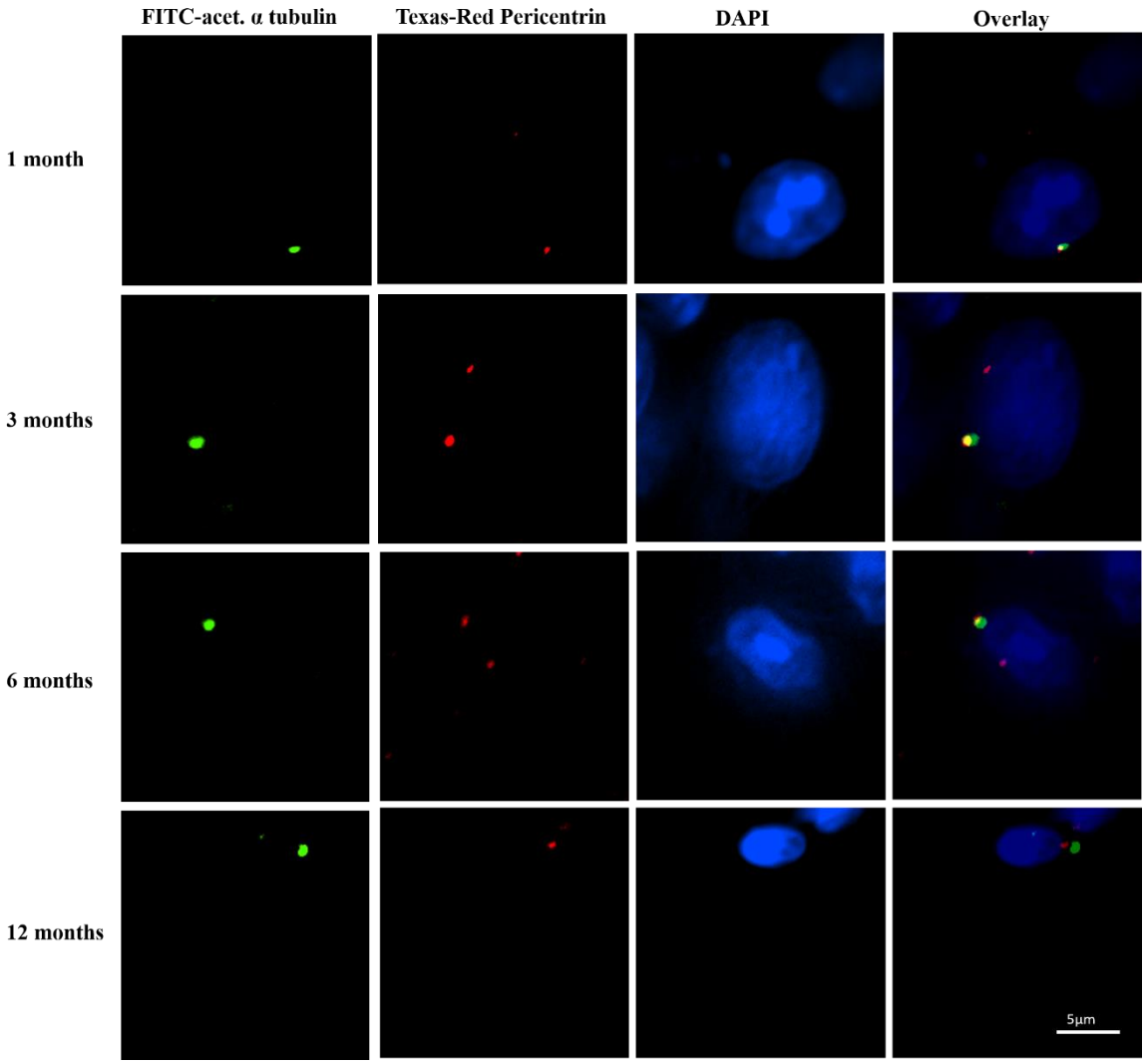

Figure 4. Confirmation of primary cilia presence in adult mouse heart with anti-pericentrin antibody. Co-localization of anti-acetylated-α-tubulin (green) and anti-pericentrin (red) antibodies at the cardiac primary cilia at different stages in mouse adulthood is shown through representative images from the whole heart. Images are captured at 100X magnification.

For more clarification of the staining, the anti-pericentrin and WGA were combined in one color (green) and the co-localization with anti-acetylated-α-tubulin (red) within cardiac cells is still seen. DAPI (blue) is used to counterstain DNA/nucleus (**Figure 5**).

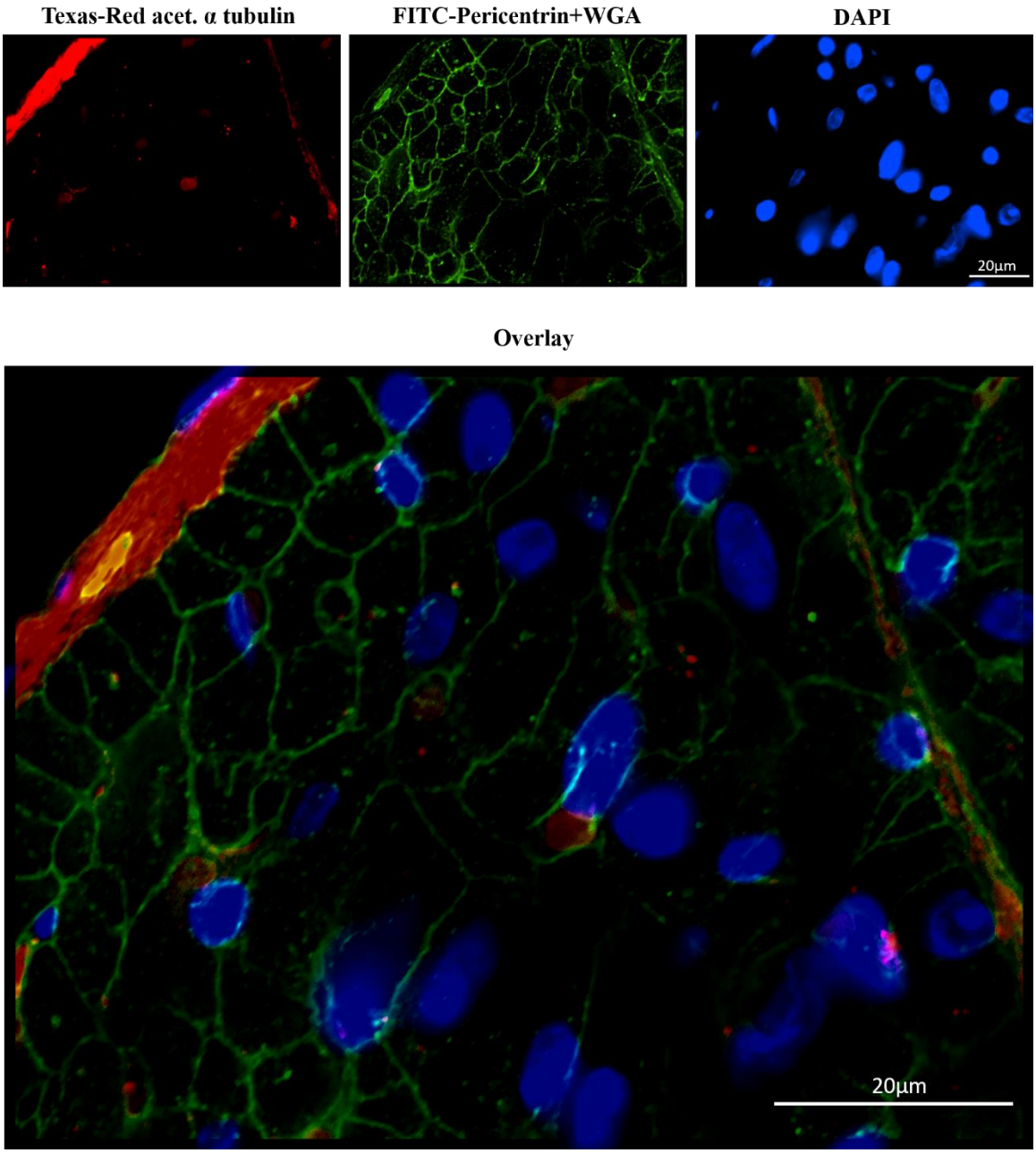

Figure 5. Confirmation of primary cilia presence in adult mouse heart with combined staining of WGA and anti-pericentrin antibody. Representative images from the whole heart showing the co-localization of anti-acetylated-α-tubulin and anti-pericentrin antibodies in the presence of WGA, a plasma membrane 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 tissues and cell types throughout the body (Nauli et al., 2003; Nauli and Zhou, 2004). Polycystin-2 functions also as a calcium channel and has been shown to play a role in calcium signaling in various cell types such as epithelial cells (Koulen et al., 2002) and vascular endothelia (Aboualiawi et al., 2009). In order to test the hypothesis about potential polycystin-2 function in adult cardiac myocytes, we decided to analyze polycystin-2 localization to primary cilia of cardiac myocytes in the adult heart tissue sections. This also provides an additional confirmation of cardiac primary cilia presence. Immunohistochemistry is performed by staining heart tissues with anti-polycystein-2 antibody. Representative images of the co-localization of anti-acetylated-α-tubulin (green) and anti-polycystein-2 (red) antibodies are attained from 1 month, 3 months, 6 months, and 12 months old mice. DAPI (blue) is used to counterstain DNA/ nucleus (**Figure 6**).

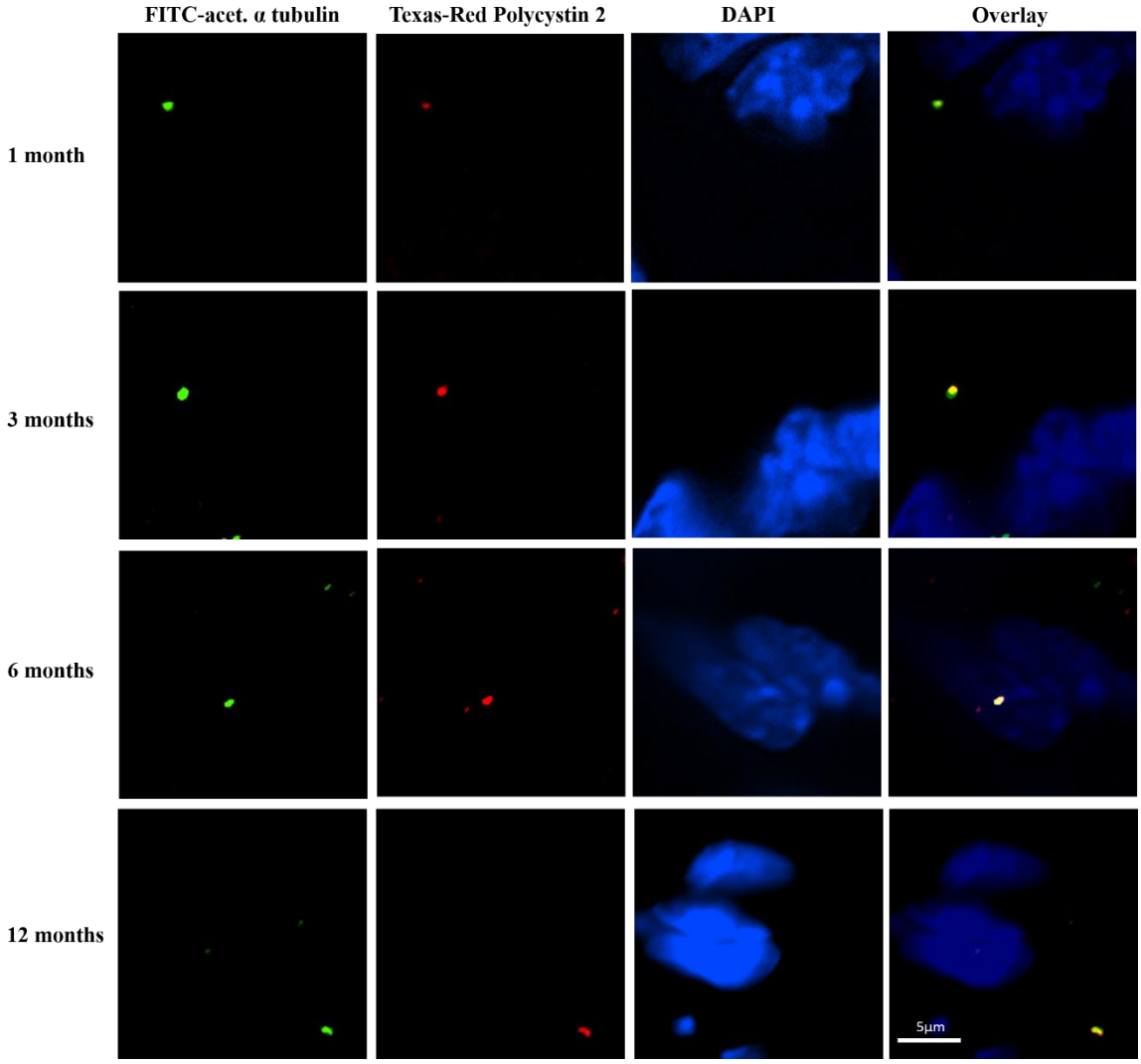

Figure 6. Confirmation of primary cilia presence in adult mouse heart with anti-polycystin-2 antibody. Representative images obtained from the whole heart of 1 month, 3 months, 6 months, and 12 months old mice showing the co-localization of anti-polycystin-2 (red) and anti-acetylated-α-tubulin (green)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

After confirming the cilia presence with different markers in the whole mouse adult heart sections, we then decided to examine the presence of cilia in the different heart chambers. So, all heart chambers are isolated and stained with anti-acetylated-α-tubulin antibody (red). WGA (green) is used to mark the plasma membrane of the cells and DNA/nucleus is counterstained with DAPI (blue). Figure 7 shows representative images of the primary cilia in all heart chambers **(Figure 7)**.

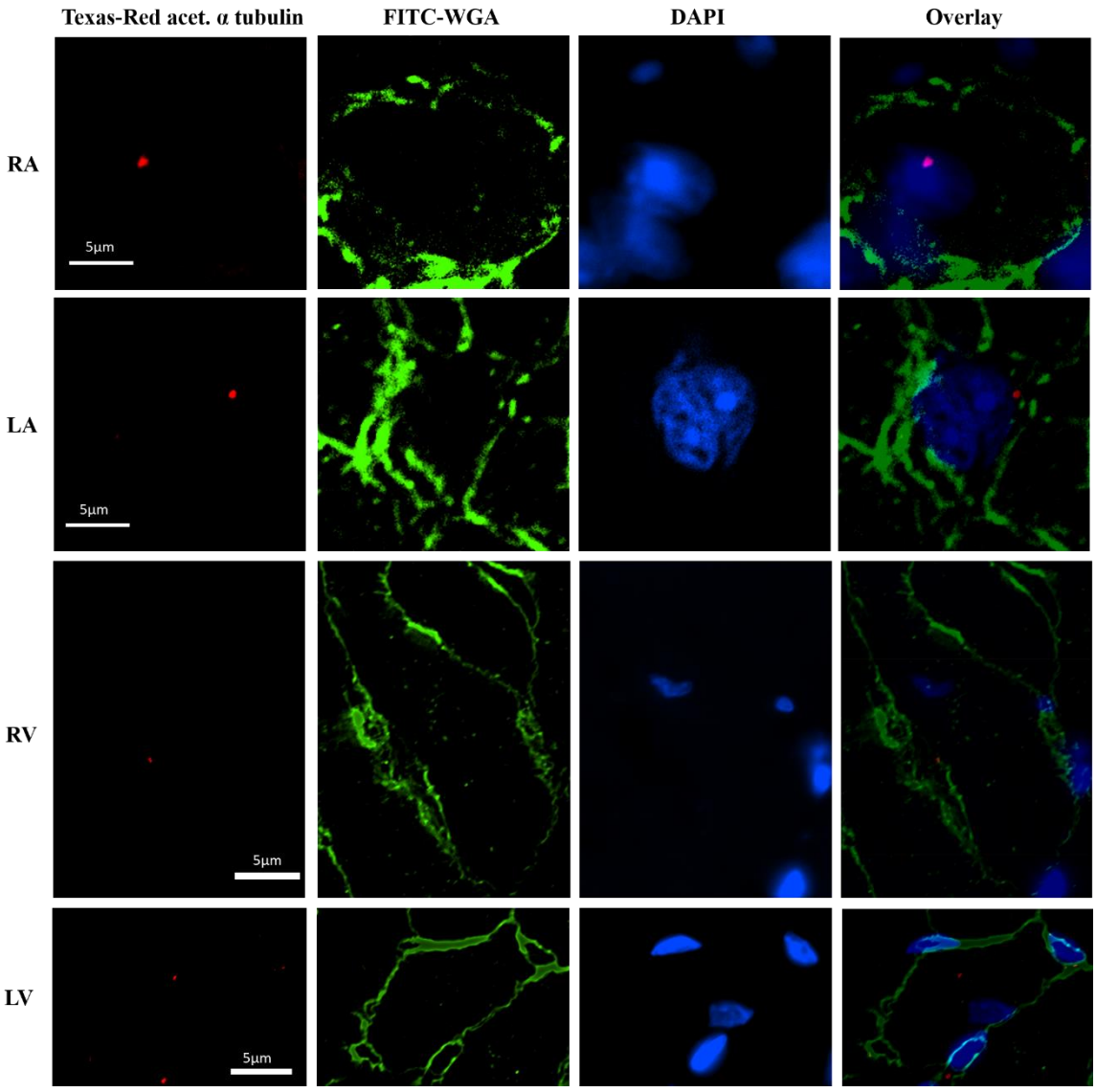

Figure 7. Confirmation of primary cilia presence in adult mouse heart chambers with anti-acetylated-α-tubulin antibody. Representative images from individual heart chambers showing cardiac primary cilia in the different heart chambers of adult mouse tissues stained with anti-acetylated-α-tubulin antibody (red) and WGA (green). DAPI (blue) is used 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, we then further confirmed the presence of cilia with anti-pericentrin antibody. 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 heart chambers. DAPI (blue) is used to counterstain DNA/nucleus (**Figure 8**).

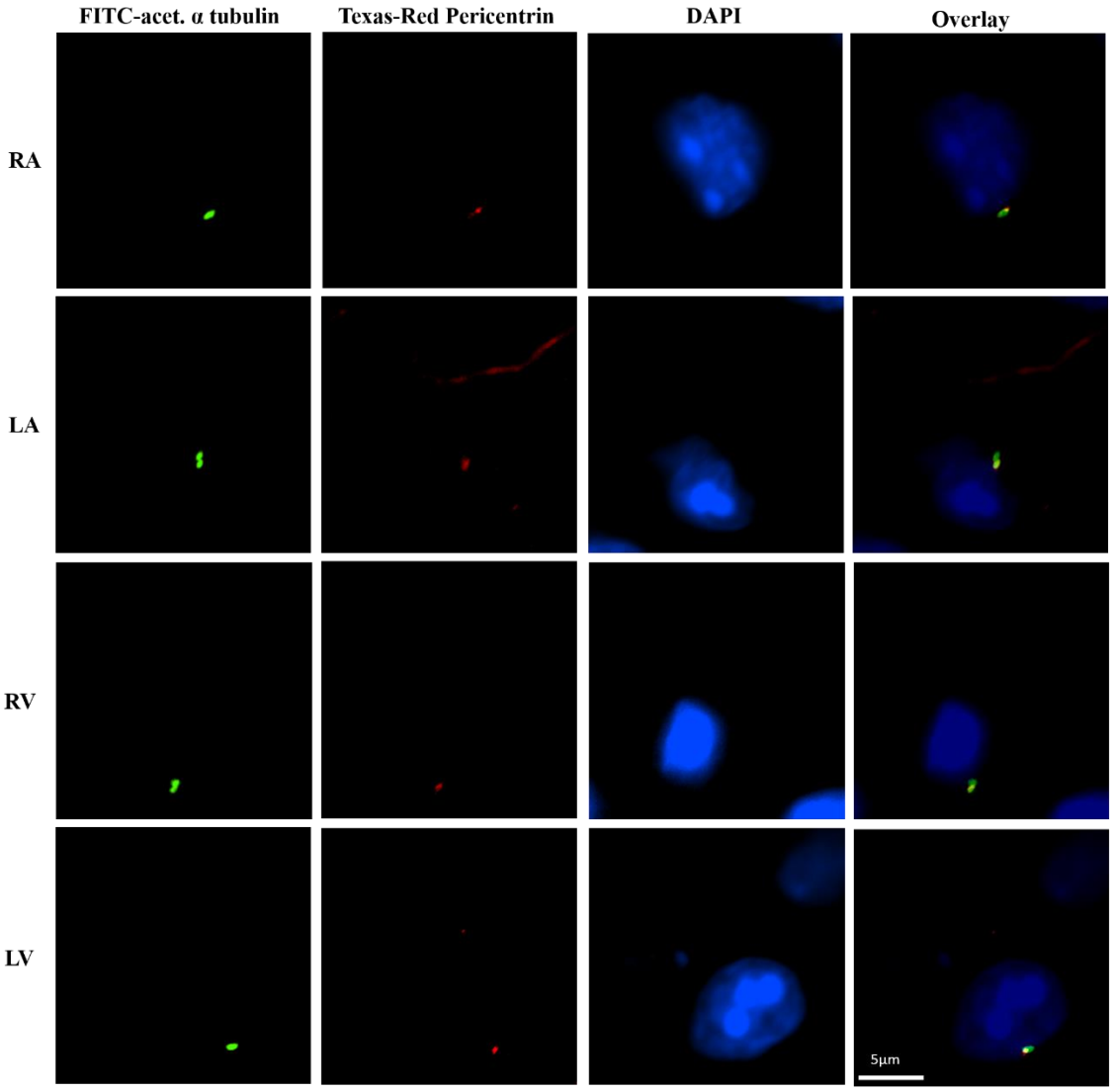

Figure 8. Confirmation of primary cilia presence in adult mouse heart chambers with anti-pericentrin antibody. Representative images from individual heart chambers showing the co-localization of anti-acetylated-α-tubulin (green) and anti-pericentrin (red) antibodies at cardiac primary cilia in the atria 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.

3.2. Primary Cilia Number in Adult Mouse Heart

After confirming the existence of primary cilia in adult mouse heart with several markers, we decided to study the abundance of primary cilia during different stages of mouse adulthood. We analyzed the abundance of primary cilia in the mouse heart from three different age groups (<3 months old, 3-6 months old, and >6 months old) with at least three wild type mice from each group. We counted the number of cilia as a percentage of the total number of cells within the same field of vision. 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 primary cilia percentage in the second group (3-6 months old) declined to 29%. Finally, the third group (>6 months old) displayed 25% primary cilia of the total number of cells. Although our study shows that there is a decline in primary cilia abundance with increased age in adult mice heart, this decline in number is only statistically significant between the first group (< 3 months) and the third group (> 6 months) (**Figure 9**).

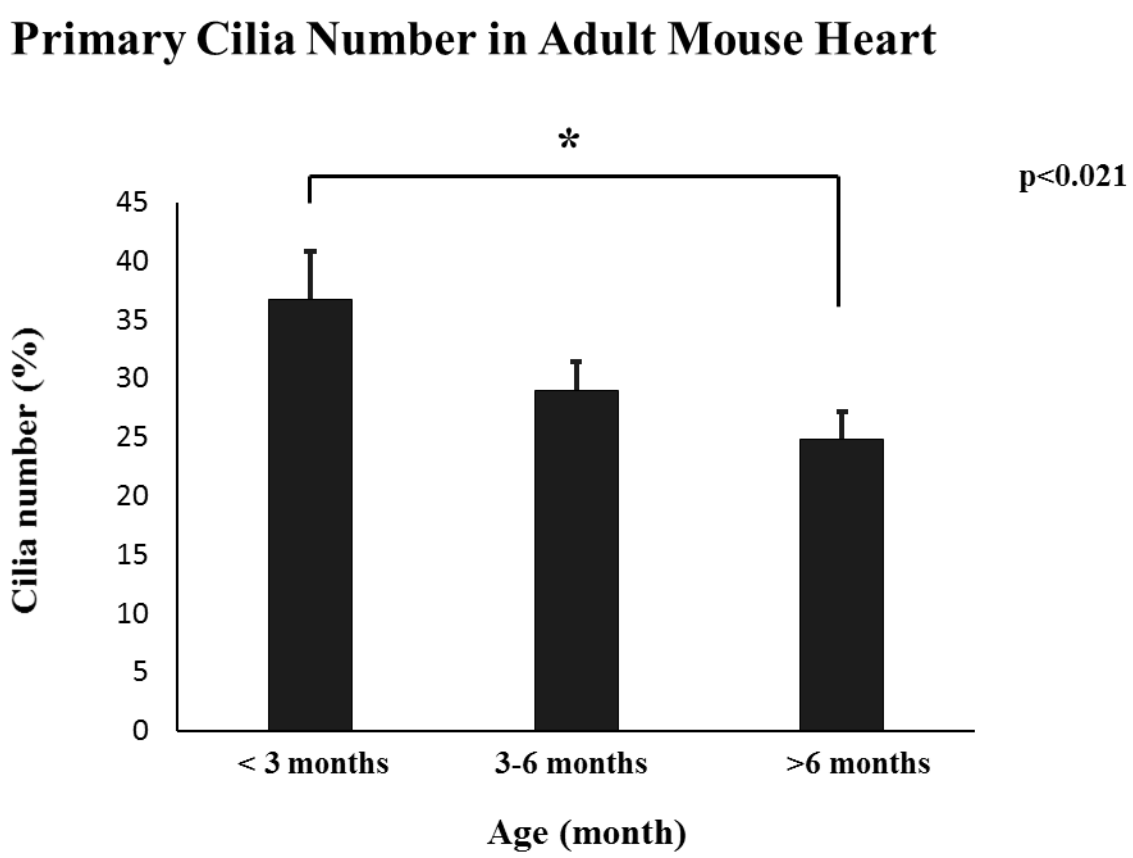

Figure 9. Primary cilia number in adult mouse heart. Cilia number in the whole heart is represented in three age groups (<3 months, 3-6 months, and >6 months old mice) and our study shows a significant decrease in their abundance with age specifically between the first group (< 3 months) and the third group (> 6 months).

3.3. Primary Cilia Length in Adult Mouse Heart

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 we decided to study. To accomplish this goal, cilia length from three age groups (<3 months, 3-6 months, >6 months old mice) is measured and compared between all groups. Our data show that the average

length of primary cilia does not change significantly during different stages of mice adulthood with an average length of approximately 1µm **(Figure 10)**.

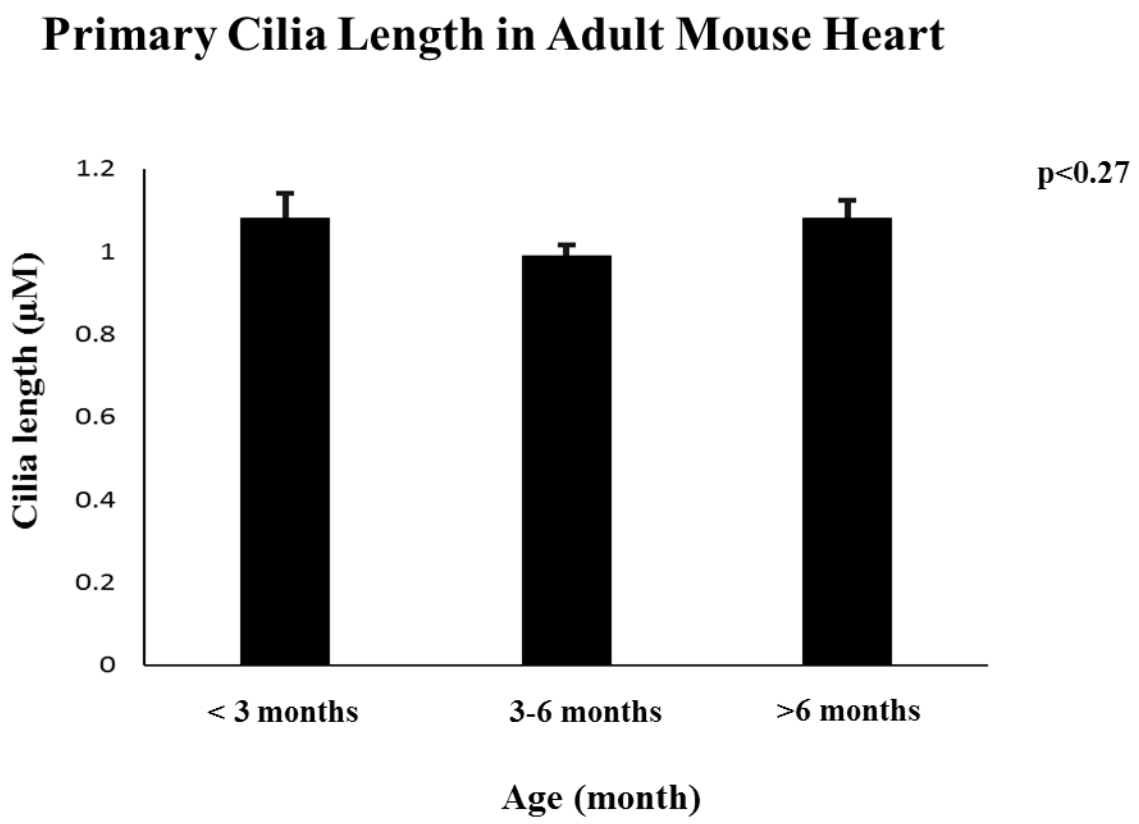

Figure 10. Primary Cilia length in adult mouse heart. Primary cilia length does not change with age during mouse adulthood and the average length is around 1µm.

Chapter 4

Discussion

Primary cilia in embryonic mouse heart have been reported several times to play crucial roles in cardiac development and function (Slough et al., 2008; Clement et al., 2009; Willaredt et al., 2012). 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 knock down or knockout approaches to some genes that encode ciliary proteins. In our study, we investigated the presence of primary cilia in adult mouse heart and their potential role in the cardiovascular system through immunohistochemistry.

So, we initially examined mouse heart sections from different ages in adulthood 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. Figure 3 in our results section shows representative images of the localization of anti-acetylated-α-tubulin antibody to 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 previous staining with anti-acetylated-α-tubulin antibody was only an artifact and not a real staining of primary cilia 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 4 in our results section shows representative images of the co-localization of the two markers to cardiac primary cilia from 1 month, 3 months, 6 months, and 12 months 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.

For clarification purposes, we then combined anti-pericentrin antibody and WGA staining, both labelled with FITC, and studied their co-localization with anti-acetylated-α-tubulin antibody to cardiac primary cilia. Figure 5 in our results section provides representative images attained from 6 months old mouse that show the co-localization of anti-pericentrin and anti-acetylated-α-tubulin antibodies to primary cilia within heart cells. This further proves that primary cilia are present in adult mouse heart and that they are actually found on the surface of cardiac cells.

To further confirm the presence of primary cilia and get a better grasp of their possible function in adult heart, we stained heart sections with polycystin-2, a calcium channel found to be localized to cilia in different cell types mediating many calcium dependent pathways. Figure 6 in our results section provides representative images obtained from 1 month, 3 months, 6 months, and 12 months old mice that show the co-localization of anti-

polycystin-2 and anti-acetylated-α-tubulin antibodies 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 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. Figures 7 and 8 in our results section provide representative images of the co-localization of anti-acetylated-α-tubulin and anti-pericentrin antibodies 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. Cilia were confirmed by anti-acetylated-α-tubulin antibody and cells were identified by DAPI or WGA. Figure 9 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 later 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 the sensory function of cilia. Cilia were confirmed with anti-acetylated-α-tubulin antibody 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 10 from our results section, we reported the average cilia length to be around one μm and does not change 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.

In summary, our study provides for the first time a novel insight regarding the localization and distribution of primary cilia in adult mouse heart. Further confirmation of cilia presence in adult mouse heart and their ultrastructure can be achieved through electron microscopy. Also, more studies of the cilia abundance and length in the different heart chambers are still needed 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 need to be studied among which are CaV1.2, a voltage gated calcium channel, polycystin-1 and others.

References

AbouAlaiwi, W. A., Takahashi, M., Mell, B. R., Jones, T. J., Ratnam, S., Kolb, R. J., & Nauli, S. M. (2009). Ciliary polycystin-2 is a mechanosensitive calcium channel involved in nitric oxide signaling cascades. *Circ Res, 104*(7), 860-869. doi: 10.1161/CIRCRESAHA.108.192765

Afzelius, B. A. (2004). Cilia-related diseases. *J Pathol, 204*(4), 470-477. doi: 10.1002/path.1652

Bers, D. M. (2000). Calcium fluxes involved in control of cardiac myocyte contraction. *Circ Res, 87*(4), 275-281.

Biagini, A., Maffei, S., Baroni, M., Piacenti, M., Terrazzi, M., Paoli, F., . . . Salvatore, L. (1993). Familiar clustering of aortic dissection in polycystic kidney disease. *Am J Cardiol, 72*(9), 741-742.

Chapman, A. B., & Schrier, R. W. (1991). Pathogenesis of hypertension in autosomal dominant polycystic kidney disease. *Semin Nephrol, 11*(6), 653-660.

Clement, C. A., Kristensen, S. G., Mollgard, K., Pazour, G. J., Yoder, B. K., Larsen, L. A., & Christensen, S. T. (2009). The primary cilium coordinates early cardiogenesis and hedgehog signaling in cardiomyocyte differentiation. *J Cell Sci, 122*(Pt 17), 3070-3082. doi: 10.1242/jcs.049676

Gilula, N. B., & Satir, P. (1972). The ciliary necklace. A ciliary membrane specialization. *J Cell Biol, 53*(2), 494-509.

Handel, M., Schulz, S., Stanarius, A., Schreff, M., Erdtmann-Vourliotis, M., Schmidt, H., . . . Hollt, V. (1999). Selective targeting of somatostatin receptor 3 to neuronal cilia. *Neuroscience, 89*(3), 909-926.

Ishikawa, H., & Marshall, W. F. (2011). Ciliogenesis: building the cell's antenna. *Nat Rev Mol Cell Biol, 12*(4), 222-234. doi: 10.1038/nrm3085

Kelleher, C. L., McFann, K. K., Johnson, A. M., & Schrier, R. W. (2004). Characteristics of hypertension in young adults with autosomal dominant polycystic kidney disease compared with the general U.S. population. *Am J Hypertens, 17*(11 Pt 1), 1029-1034. doi: 10.1016/j.amjhyper.2004.06.020

Kestler, H. A., & Kühl, M. (2008). From individual Wnt pathways towards a Wnt signalling network. *Philos Trans R Soc Lond B Biol Sci, 363*(1495), 1333-1347. doi: 10.1098/rstb.2007.2251

Koulen, P., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., . . . Somlo, S. (2002). Polycystin-2 is an intracellular calcium release channel. *Nat Cell Biol, 4*(3), 191-197. doi: 10.1038/ncb754

Liu, T., Jin, X., Prasad, R. M., Sari, Y., & Nauli, S. M. (2014). Three types of ependymal cells with intracellular calcium oscillation are characterized by distinct cilia beating properties. *J Neurosci Res, 92*(9), 1199-1204. doi: 10.1002/jnr.23405

Lumiaho, A., Pihlajamaki, J., Hartikainen, J., Ikaheimo, R., Miettinen, R., Niemitukia, L., . . . Laakso, M. (2003). Insulin resistance is related to left ventricular hypertrophy

in patients with polycystic kidney disease type 1. *Am J Kidney Dis, 41*(6), 1219-1224.

Muntean, B. S., Jin, X., Williams, F. E., & Nauli, S. M. (2014). Primary cilium regulates CaV1.2 expression through Wnt signaling. *J Cell Physiol, 229*(12), 1926-1934. doi: 10.1002/jcp.24642

Myklebust, R., Engedal, H., Saetersdal, T. S., & Ulstein, M. (1977). Primary 9 + 0 cilia in the embryonic and the adult human heart. *Anat Embryol (Berl), 151*(2), 127-139.

Nauli, S. M., Alenghat, F. J., Luo, Y., Williams, E., Vassilev, P., Li, X., . . . Zhou, J. (2003). Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat Genet, 33*(2), 129-137. doi: 10.1038/ng1076

Nauli, S. M., & Zhou, J. (2004). Polycystins and mechanosensation in renal and nodal cilia. *Bioessays, 26*(8), 844-856. doi: 10.1002/bies.20069

Pedersen, L. B., & Rosenbaum, J. L. (2008). Intraflagellar transport (IFT) role in ciliary assembly, resorption and signalling. *Curr Top Dev Biol, 85*, 23-61. doi: 10.1016/S0070-2153(08)00802-8

Pedersen LB, Schrøder JM, Satir P, Christensen ST. The ciliary cytoskeleton. *Compr Physiol*. 2012;2:779–803.

Praetorius, H. A., Frokiaer, J., Nielsen, S., & Spring, K. R. (2003). Bending the primary cilium opens Ca2+-sensitive intermediate-conductance K+ channels in MDCK cells. *J Membr Biol, 191*(3), 193-200. doi: 10.1007/s00232-002-1055-z

Praetorius, H. A., & Spring, K. R. (2005). A physiological view of the primary cilium. *Annu Rev Physiol, 67*, 515-529. doi: 10.1146/annurev.physiol.67.040403.101353

Rash, J. E., Shay, J. W., & Biesele, J. J. (1969). Cilia in cardiac differentiation. *J Ultrastruct Res, 29*(5), 470-484.

Ratnam, S., & Nauli, S. M. (2010). Hypertension in Autosomal Dominant Polycystic Kidney Disease: A Clinical and Basic Science Perspective. *Int J Nephrol Urol, 2*(2), 294-308.

Rohatgi, R., & Snell, W. J. (2010). The ciliary membrane. *Curr Opin Cell Biol, 22*(4), 541-546. doi: 10.1016/j.ceb.2010.03.010

Satir, P., & Christensen, S. T. (2007). Overview of structure and function of mammalian cilia. *Annu Rev Physiol, 69*, 377-400. doi: 10.1146/annurev.physiol.69.040705.141236

Slough, J., Cooney, L., & Brueckner, M. (2008). Monocilia in the embryonic mouse heart suggest a direct role for cilia in cardiac morphogenesis. *Dev Dyn, 237*(9), 2304-2314. doi: 10.1002/dvdy.21669

Tissir, F., Qu, Y., Montcouquiol, M., Zhou, L., Komatsu, K., Shi, D., . . . Goffinet, A. M. (2010). Lack of cadherins Celsr2 and Celsr3 impairs ependymal ciliogenesis, leading to fatal hydrocephalus. *Nat Neurosci, 13*(6), 700-707. doi: 10.1038/nn.2555

Torres, V. E., & Harris, P. C. (2006). Mechanisms of Disease: autosomal dominant and recessive polycystic kidney diseases. *Nat Clin Pract Nephrol, 2*(1), 40-55; quiz 55. doi: 10.1038/ncpneph0070

Walker, C. A., & Spinale, F. G. (1999). The structure and function of the cardiac myocyte: a review of fundamental concepts. *J Thorac Cardiovasc Surg, 118*(2), 375-382.

Wheatley, D. N., Wang, A. M., & Strugnell, G. E. (1996). Expression of primary cilia in mammalian cells. *Cell Biol Int, 20*(1), 73-81. doi: 10.1006/cbir.1996.0011

Willaredt, M. A., Gorgas, K., Gardner, H. A., & Tucker, K. L. (2012). Multiple essential roles for primary cilia in heart development. *Cilia, 1*(1), 23. doi: 10.1186/2046-2530-1-23

Wilson, P. D. (2004). Polycystic kidney disease: new understanding in the pathogenesis. *Int J Biochem Cell Biol, 36*(10), 1868-1873. doi: 10.1016/j.biocel.2004.03.012