CHAPTER

5

Methods for studying primary cilia in heart tissue after ischemia-reperfusion injury

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Chapter outline

Abstract

Cardiovascular diseases are the leading cause of death and disability worldwide. After heart injury triggered by myocardial ischemia or myocardial infarction, extensive zones of tissue are damaged and some of the tissue dies by necrosis and/or apoptosis. The loss of contractile mass activates a series of biochemical mechanisms that allow, through cardiac remodeling, the replacement of the dysfunctional heart tissue by fibrotic material. Our previous studies have shown that primary cilia, non-motile antenna-like structures at the cell surface required for the activation of specific signaling pathways, are present in cardiac fibroblasts and required for cardiac fibrosis induced by ischemia/reperfusion (I/R) in mice. I/R-induced myocardial fibrosis promotes the enrichment of ciliated cardiac fibroblasts where the myocardial injury occurs. Given discussions about the existence of cilia in specific cardiac cell types, as well as the functional relevance of studying cilia-dependent signaling in cardiac fibrosis after I/R, here we describe our methods to evaluate the presence and roles of primary cilia in cardiac fibrosis after I/R in mice.

1 Introduction

Primary cilia are non-motile organelles present in most vertebrate cells (Malicki & Johnson, 2017). They are 2–10 μm in length and ~200 nm in diameter (Nachury & Mick, 2019) and play a vital role in the coordination of multiple signaling pathways critical for tissue homeostasis (Anvarian, Mykytyn, Mukhopadhyay, Pedersen, & Christensen, 2019; Avalos et al., 2022; Senatore et al., 2022). Defects in primary cilia structure or function lead to several syndromic disorders, known as "ciliopathies," that are marked by a wide range of phenotypes, such as neurodevelopmental disorders, obesity, retinal degeneration, and cystic kidney disease (Braun & Hildebrandt, 2017; Reiter & Leroux, 2017).

The core structure of the primary cilium comprises nine microtubule doublets radially arranged, known as a 9+0 "axoneme," that protrudes in a single copy from the plasma membrane (Satir, 2017). The axoneme elongates from the apical surface of the basal body, which is a modified mother centriole of the centrosome (Lattao, Kovacs, & Glover, 2017). The ciliary basal body consists of nine triplets of microtubules radially arranged, and its docking to the plasma membrane depends on the transition fibers that derive from distal appendages localized in the mother centriole (Garcia-Gonzalo & Reiter, 2017; Sorokin, 1962; Sorokin, 1968). The axoneme is physically separated from the basal body by an ultrastructure known as a transition zone, which is a typically 0.5 μm region characterized by Y-shaped links that connect the axoneme to the cilia membrane (Fisch & Dupuis-Williams, 2011; Garcia-Gonzalo & Reiter, 2017; Goncalves & Pelletier, 2017). Fibers along with the transition zone form a ciliary gate, where the entrance and exit of ciliary lipids and proteins are controlled, preventing protein-free diffusion, thereby contributing to the compartmentalization of the primary cilium (Garcia-Gonzalo & Reiter, 2017) (Fig. 1). Ciliary proteins are imported and exported across the transition zone and transported along the axoneme by the intraflagellar transport (IFT) machinery. IFT transport is mediated by two protein complexes, IFT-A and IFT-B, in which IFT-B mediates anterograde trafficking from the base to the tip of the cilia under the control of the kinesin-2 motor (Kif3 motor complex) (Funabashi, Katoh, Okazaki, Sugawa, & Nakayama, 2018) (Fig. 1). Conversely, IFT-A mediates retrograde trafficking from the tip to the base of the cilia using dynein-2 motors (Jordan, Diener, Stepanek, & Pigino, 2018). Additionally, Bardet-Biedl syndrome proteins, BBsome, a multimeric protein complex, mediates protein transport across the transition zone and acts as a cargo adaptor for IFT-dependent protein removal (Funabashi et al., 2018; Jordan et al., 2018) (Fig. 1). Multiple signaling pathways have been related to cilia, including Hedgehog, Wnt, Notch, Hippo, G-protein coupled receptors, GPCRs, receptor tyrosine kinase (PDGFRα, IGF-1), mTOR, and the TGFβ receptor (Anvarian et al., 2019; Wheway, Nazlamova, & Hancock, 2018). Whereas significant advances have emerged in the elucidation of primary cilium function in recent years, the role and mechanisms of cilia-dependent signaling in health and disease remain to be fully elucidated.

The presence of primary cilia in the heart and their physiological function have only recently coming to light. Some studies have revealed the presence of cilia in adult and embryonic hearts, however it remained unclear which cardiac cell types harbor the organelle (Bystrevskaya, Lichkun, Krushinsky, & Smirnov, 1992; Diguet, Le Garrec, Lucchesi, & Meilhac, 2015; Myklebust, Engedal, Saetersdal, & Ulstein, 1977; Rash, Shay, & Biesele, 1969). We reported that primary cilia in cardiac tissue are critical to promote fibrosis in mice after ischemia/reperfusion (I/R) (Villalobos et al., 2019). Our work uncovered that not only cilia but also polycystin-1, PC1, a ciliary protein involved in the activation of particular signaling pathways and whose mutation underlies ciliopathies (Lee & Somlo, 2014; Ta, Vien, Ng, & DeCaen, 2020) are required to induce heart fibrosis (Villalobos et al., 2019). Indeed, we observed increased presence of ciliated cells, identified as cardiac fibroblasts, in injured areas after I/R (Villalobos et al., 2019). Additionally, we showed that

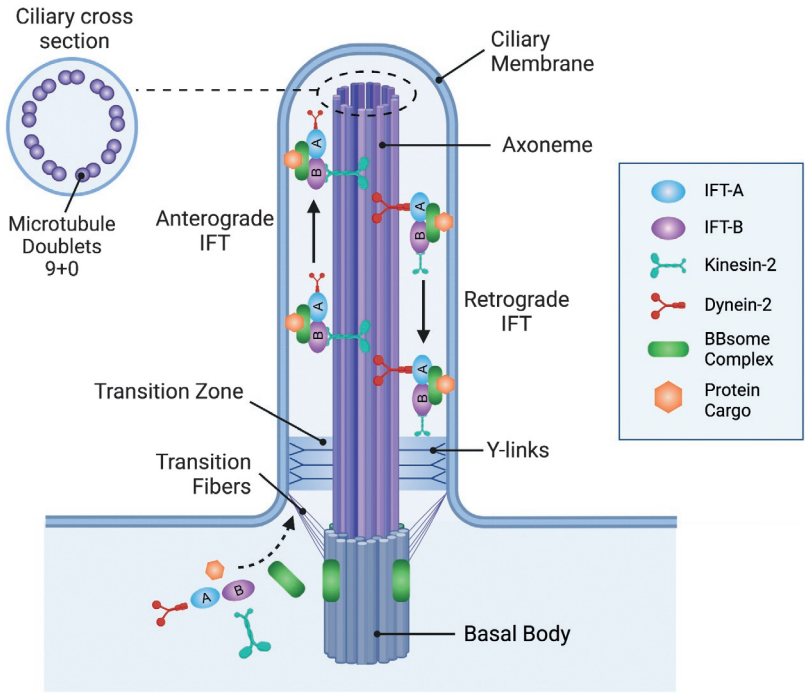

FIG. 1

Primary cilia structure. The primary cilium is composed of a structure bone called "axoneme" which is disposed of nine microtubule doublets radially arranged (9+0). At the base of cilia is located the ciliary "basal body," which consists of 9 triplets of microtubules radially arranged, and it is physically separated from the axoneme by a structure called "transition zone." Thus, proteins can traffic through the transition zone and transport along the axoneme by an intraflagellar transport, IFT, protein complex system. IFT transport system is mediated by IFT-A and IFT-B complexes, in which IFT-B mediates anterograde trafficking from the base to the tip of the cilia. Contrary, IFT-A mediates retrograde trafficking from the tip to the base of the cilia. In addition, BBsome, a multimeric protein complex, controls protein transport across the transition zone allowing selectivity on the ciliary protein composition. Thus, multiple signaling pathways have been specifically related to the control of cilia-dependent intracellular responses.

ablation of PC1 in cardiac fibroblasts blunts transforming growth factor-β1 (TGFβ)-triggered fibrogenesis (Villalobos et al., 2019). The presence of cilia in the heart had been a matter of debate, and in our work, we identified cilia in mouse cardiac fibroblasts and not in cardiomyocytes, both in neonatal and adult primary cultures (Villalobos et al., 2019). Thus, the primary cilium in cardiac fibroblasts

is key to driving extracellular matrix fibrogenesis, activating adaptative responses in the heart during cardiac remodeling in conditions of stress. In this article, we will describe the methods and protocols to study primary cilia in myocardial fibrosis after I/R in mice.

2 Myocardial fibrosis induced by ischemia/reperfusion (I/R) injury in mice

1. Anesthetize 10- to 12-week-old male C57BL/6 mice with 2.4% isoflurane and place the animals in a supine position on a heating pad at 37 °C (see Notes 1–2). Then, intubate animals with a 19G stump needle and ventilate them with room air using a MiniVent mouse ventilator (see Note 3).
2. Once animals are completely anesthetized, apply a sterile lubricant ointment on the mouse eyes to prevent dryness. Then, immobilize the animals on a warming pad.
3. Clean the chest using cotton swabs moistened with povidone-iodine or 70% Ethanol.
4. Perform a left thoracotomy through a small incision in the left chest skin in an orientation parallel to the sternum and cut the pectoralis muscles using small scissors (see Note 4). Then, identify the intercostal muscle and make a small incision to access the ribs, which are cut in a cross orientation. Using a small speculum, open gently the chest cavity by retraction of the ribs and identify the heart with its left anterior descending (LAD) coronary artery (see Note 5) (Fig. 2).
5. To perform the LAD ligation, use a tapered point surgical needle with a polyethylene suture, which is placed underneath the LAD. Then, place a small (~0.75 cm) portion of medical grade polyethylene tube, PE-90, over and along the LAD and tie the suture looped under the LAD together with the PE-90 tube for 45 min (ischemia, 45 min) (see Notes 6–7). To generate reperfusion, release the suture from the PE-90 tube, and immediately, suture the intercostal chest dissection (Fig. 2).
6. Use sham-operated mice as controls and submit them to the same procedure without occlusion of the LAD.
7. To evaluate myocardial fibrosis, harvest the hearts at 1-, 4-, 7- or 14-days post-reperfusion. To this end, submit the animals to terminal anesthesia by injecting 60 mg/Kg of sodium pentobarbitone. After confirming the absence of a pedal reflex, perform a thoracotomy and quickly excise the hearts and place them in 20–30 mL of ice-cold *relaxing buffer* for 5 min (see Note 8).
8. Then, fix the hearts in 4% PFA-PBS for 48 h at 4 °C and wash them in cold PBS at 4 °C. Finally, embed the hearts in paraffin.

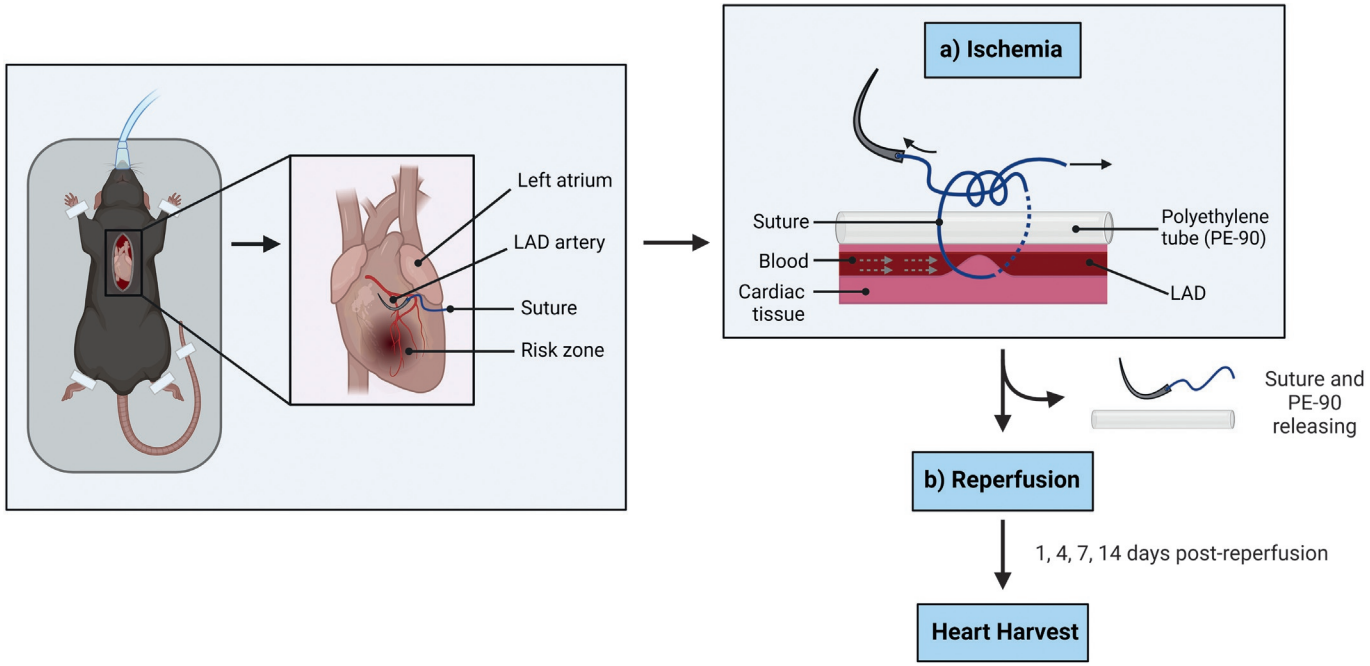

FIG. 2

Heart I/R procedure in mice. 10–12 weeks old male C57BL/6 mice are anesthetized, immobilized onto a warming pad, and intubated. Then, a thoracotomy is performed to expose the heart where the left anterior descending artery, LAD, is identified. To generate cardiac ischemia, LAD is ligated using a tapered point surgical needle and a polyethylene suture placed underneath the LAD. A small portion of polyethylene tube, PE-90, is placed over the LAD and the suture is tied looped under the LAD together with the PE-90 tube for 45 min. Then, to produce reperfusion, the suture and PE-90 tube is released, and the chest dissection is sutured. Finally, animals are sacrificed, and hearts are harvested at 1-, 4-, 7- or 14-days post-reperfusion. Sham surgery was considerated as a control.

3 Analysis of primary cilia in heart tissue after I/R

1. Slice the paraffin-embedded blocks of the heart using a tissue-microtome and prepare serial heart sections of 3- to 5-μm thickness in a two-chamber orientation (see Note 9) (Fig. 3).
2. To mount paraffin tissue sections, place the paraffin ribbon floating on a water bath at 40 °C and then mount the sections on microscope slides avoiding the presence of bubbles (Fig. 3). Then, place the slides on a metallic rack and heat them in an oven at 37 °C overnight (see Note 10).
3. Remove the paraffin from the tissue samples. We employ a system of automated deparaffinization using a Sakura DRS.601 Automatic Slide Stainer (Fig. 3). However, other methods or deparaffinization protocols can be used.

3.1 Masson trichrome staining to evaluate fibrosis

1. After deparaffinization, incubate samples in Bouin's solution (Merck HT10132-1L) for 1 h at 56 °C and then wash slides in H_2O until the yellowish color is eliminated.
2. Incubate slides in Weigert hematoxylin solution (Merck HT1079) for 10 min at room temperature and wash with H_2O. Then, stain samples with Biebrich Scarlet solution for 3 min and wash with H_2O (see Note 11).
3. Incubated the slides in a mixed solution of phosphotungstic/phosphomolybdic acid for 15 min at room temperature. Then, discard the solutions and wash slides in H_2O (see Note 12). Subsequently, stain slides in a blue aniline solution for 3–5 min and incubate them in acetic acid for 3 min at room temperature (see Note 13). Finally, dehydrate samples using alcohol solutions of ascending concentration, such as 95% ethyl alcohol, absolute ethyl alcohol, and xylene. To finish, place a resinous mounting medium over the samples and place a microscope cover glass over the samples. Store the samples at room temperature.
4. Collagen fiber deposition is observed as an intense blue color, whereas normal tissue is visualized as a reddish color (see Note 14) (Fig. 4).

3.2 Cardiac tissue immunofluorescence to study primary cilia

1. After deparaffinization, heat the samples in water at 50 °C for 30 min using a tissue microwave oven system, as heating helps to attach the tissue to the glass (see Note 15).
2. Of note, the protocol can be paused at this point. Samples can be maintained in PBS 1X overnight at 4 °C (see Note 16).
3. To perform antigen retrieval, we use the Biolegend® Retrieve-All Antigen Unmasking System1 Cat:927901. However, another with similar features can be used. To accomplish this, place the samples in a plastic container filled with antigen retrieval buffer and then heat tissues twice at 92 °C for 5 min using a tissue microwave oven. Then, cool down the samples at room temperature for 20–30 min and wash three times in 1X PBS at room temperature.

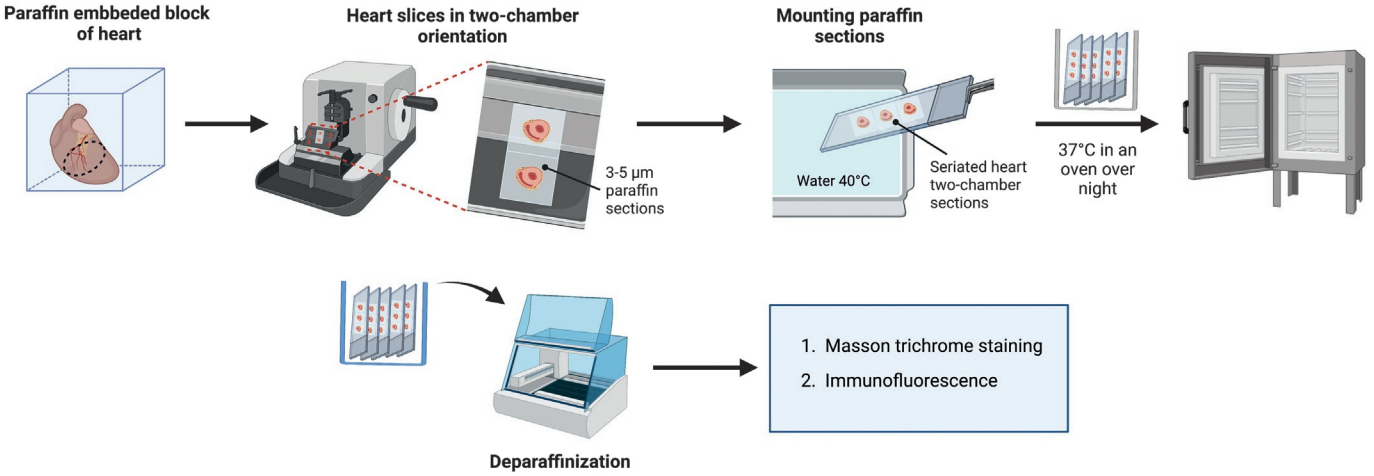

FIG. 3

Cardiac tissue processing to evaluate fibrosis and cilia. Hearts are embedded in paraffin, and blocks are sliced with a tissue-microtome. Serial heart sections are obtained in a two-chamber heart orientation. Then paraffin ribbon sections are placed floating on a water bath at 40 °C and then mounted on the slides carefully to avoid forming bubbles. Then, slides are heated at 37 °C overnight and paraffin is removed using an automated deparaffinization system. Subsequently, deparaffinized slices can be used both for Masson trichrome staining to evaluate fibrosis and cardiac tissue immunofluorescence in the study of primary cilia.

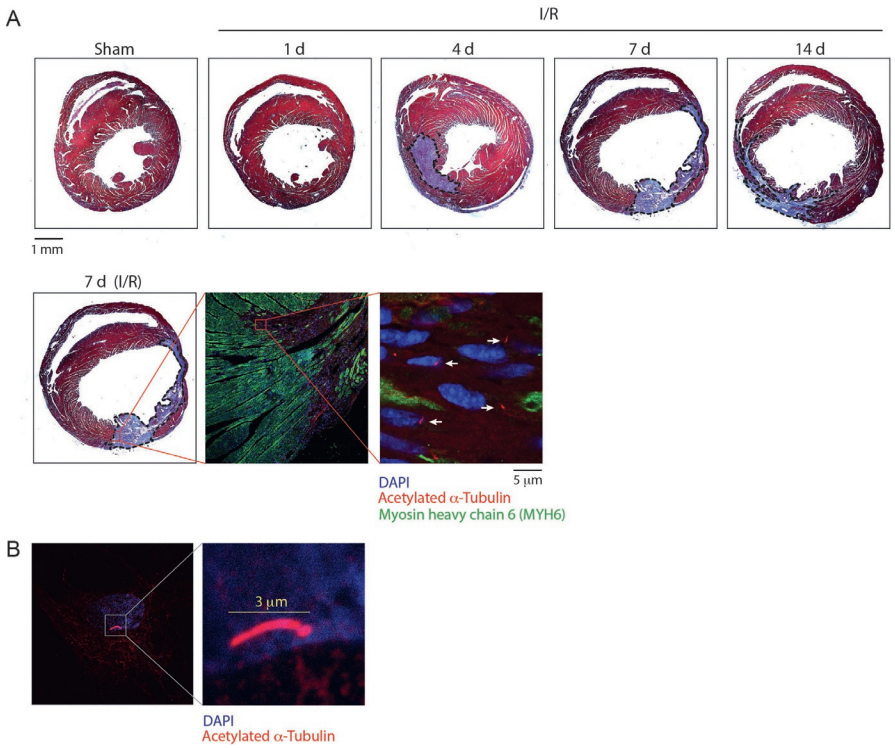

FIG. 4

Cardiac fibrosis and cilia after I/R. (A) Cardiac fibrosis, induced by I/R, was evaluated by Masson trichrome staining, in which blue staining indicates collagen deposition in the fibrotic areas. As it is observed, fibrosis started to be evident at 4-, 7-, and 14-days post-ischemia. Primary cilia were studied by immunofluorescence using the primary antibody acetylated α-tubulin (1:100), and ciliated cells, indicated by white arrows, are found in heart areas with fibrosis, and in which cells are negative for specific markers against cardiomyocytes, such as anti-Myosin heavy chain 6 (MYH6). Nuclei were stained with DAPI, and representative images of three independent experiments were obtained by confocal microscopy. (B) Cardiac fibroblasts were isolated from mice heart submitted to I/R and cilia stained with antibodies against acetylated-α-tubulin (T6793, Sigma-Aldrich, St. Louis, MO, EE.UU.). Nuclei were stained with 10 mg/mL Hoechst 33342 and images evaluated by confocal microscopy.

4. To permeabilize the tissue, place the slides in 0.1% Triton-X100, 1X PBS at room temperature for 45 min. Then, wash slides three times in 1X PBS (see Note 17).
5. Next, place the slides in a solution of 0.1% Sudan Black in 70% ethanol for 20 min at room temperature to quench autofluorescence, which is typically high in heart tissue. Then, wash samples three times in 1X PBS. Repeat the washing if excessive Sudan Black precipitates or if aggregates remain on the samples.

6. Block the samples in 5% BSA-PBS for 45 min at room temperature to avoid nonspecific binding of antibodies (see Note 18). After blocking, wash samples three times in 1X PBS.
7. We identify primary cilia using the primary antibody acetylated α-tubulin (1:100) (T6793, Sigma-Aldrich, St. Louis, MO, EE.UU.) in filtered 5% BSA-PBS at 4 °C. As noted above, we previously identified cilia in cardiac fibroblasts but not in cardiomyocytes (Fig. 4). In this regard, cilia can be identified in cardiac fibroblasts by co-incubating the samples with an antibody against fibroblast-specific markers, such as vimentin. To identify cardiomyocytes, we co-incubate samples with antibodies against cardiomyocyte-specific markers, such as anti-myosin heavy chain 6 (MYH6) (Sigma-Aldrich #HPA001349), anti-α-Actinin (Sarcomeric) (Sigma-Aldrich #7811), or anti-Troponin I (Fig. 4A). Incubate samples with primary antibodies at 4 °C, overnight. Then, wash the samples three times in 1X-PBS.
8. Next, incubate the samples with fluorescent conjugated secondary antibodies in a filtered solution of 5% BSA-PBS for 2 h at room temperature protecting the samples from light (see Note 19). Then, wash samples three times in 1X-PBS.
9. Place a drop of antifade mounting medium supplemented with DAPI on the samples and place a microscope coverslip over them.
10. Dry samples at room temperature overnight and protect them from light. Then, maintain samples at 4 °C. Samples can be stored at 4 °C for up to 3 months.
11. Cilia length is different depending on the cell type. In cardiac fibroblasts, we have observed that cilium length is between 3–6 μm (Fig. 4B). Cilia images can be taken by *Z*-stacking using a confocal microscope and a total of 15–20 Z-stack (0.14 μm/slice) images can be take in each sample. Quantification of cilia length, volume and surface can be performed using ImageJ's plugin CiliaQ (CiliaQ-0.1.4, CiliaQ, Editor_JNH-0.0.3, and CiliaQ Preparator_JNH-0.1.1) (Dummer, Poelma, DeRuiter, Goumans, & Hierck, 2016).

4 Isolation of adult mouse fibroblasts and cardiomyocytes for the study of cilia

Primary cardiac fibroblasts, as well as cardiomyocytes, can be isolated from hearts after I/R. In this regard, molecular and biochemical mechanisms driven by cilia can be studied in-vitro. We have not identified cilia in cardiomyocytes, however, cilia in fibroblasts could modulate intracellular responses in cardiomyocytes. For this reason, we describe methods to prepare not only primary adult cardiac fibroblasts but also cardiomyocytes.

1. To prepare cardiac fibroblasts and cardiomyocytes, submit animals to I/R, as we previously described (see Note 20). 7-days post-reperfusion, subject mice to terminal anesthesia by injecting 60 mg/Kg of sodium pentobarbitone together with 100 IU of heparin to prevent clot formation. Then, perform a thoracotomy,

excise the hearts quickly and submerge the heart in cold *Perfusion Buffer*. Immediately cannulate the heart and perform a retrograde perfusion through the aorta with *Perfusion Buffer* at 37 °C for 5 min (see Notes 21–22). Finally, perfuse the heart with *Digestion Buffer* for 10 min at 37 °C (see Notes 23–25).

2. Remove the heart from the cannula and place it into a 35 mm plastic petri dish, where the atrium and the non-cardiac tissue are excised and discarded. Ventricles should be gently disentangled using tweezers, and a pipette may be used to further promote tissue breakup. Add 3 mL of *Stop Buffer* at room temperature and filter the disaggregated tissue through a sterile mesh and place the collected material into a 15 mL tube, allowing to sediment the cells for 10 min at room temperature (see Notes 26–27).
3. Collect the supernatant, enriched in fibroblasts, and replace it gently with the *Calcium Restoration Solution 1*, without disturbing the pellet, rich in cardiomyocytes, for 10 min at room temperature (see Note 28). Then, remove the supernatant again and replace it with the *Calcium Restoration Solution* 2 for another 10 min at room temperature. Repeat this procedure with *Calcium Restoration Solutions* 3 and 4, collecting in each step the supernatant with fibroblasts. Finally, after collecting the supernatant containing *Calcium Restoration Solution* 4, resuspend the pellet containing cardiomyocytes, in 5 mL of complete M199 medium supplemented with 5 µL of 25 mM blebbistatin at 37 °C to inhibit cardiomyocyte contraction (see Notes 29–30). Place the cardiomyocytes in culture by spreading cells on culture plates coated with laminin and maintain the plates in a 5% CO_2 incubator at 37 °C for 1 h, to allow the attachment of cells. Then, cells are ready to be treated or fixed.
4. Centrifuge each collected fibroblast supernatant obtained in *step 3* at 1500 rpm for 5 min at room temperature and resuspend the cells in 10% FBS, DMEM-F12 at 37 °C. Then, place the fibroblasts in culture by spreading them in a 75 cm^2 cell culture flask and maintain the flask in a 5% CO_2 incubator at 37 °C.

5 Concluding remarks

Studies performed in the last decades have uncovered that loss of cilia, shortening, ablation, or mutations of ciliary proteins lead to several disorders in humans (Braun & Hildebrandt, 2017; Li et al., 2022; Senatore et al., 2022) and the vast plethora of phenotypes observed in these diseases are tightly related with the specific function of cilia in each tissue. Protein and membrane lipid composition in cilia are selectively regulated by both external stimuli and tissue environment, which can explain how cilia in different cell types can sense and then activate specific signaling pathways, depending on the organ (Nechipurenko, 2020; Wingfield, Lechtreck, & Lorentzen, 2018). The role of the heart is to provide a continuous supply of blood to the entire organism, and the high energy requirement for cardiac contractility renders the heart extremely sensitive to a deficit of nutrients and oxygen

(Lopaschuk, Karwi, Tian, Wende, & Abel, 2021). Total or partial interruption of blood supply to the heart, known as ischemia, induces cardiomyocyte death, which can be amplified after reperfusion by the sudden supply of oxygen with the subsequent robust generation of free radicals (Black et al., 1998; Forde & Fitzgerald, 1997; Myers, Bolli, Lekich, Hartley, & Roberts, 1985; Scarabelli et al., 2001; Zhao et al., 2000). Whereas there had been scant evidence about the presence of cilia in the heart, and precisely the type of cardiac cells that harbor cilia, we previously identified cilia in cardiac fibroblasts, but not in cardiomyocytes (Villalobos et al., 2019). In addition, we previously reported that ciliated fibroblasts are mainly observed in injured areas of myocardium and that cilia participate in fibrogenesis after I/R injury. In this article, we lay out the procedures and protocols for the study of cilia in the heart. It is important to mention that several studies have performed both ischemia or reperfusion for different periods of time (Redel et al., 2008), which impact the severity of fibrosis, extent of cell death, and the type of cell injury triggered, such as apoptosis, necrosis, autosis or a combination of them (Horstick et al., 1997; Matsumura, Jeremy, Schaper, & Becker, 1998; Nah, Sung, Zhai, Zablocki, & Sadoshima, 2022). It is essential to consider that fibrosis in the injured cardiac areas is not observed a few days after I/R, and that in our procedures at least 4- to 7-days are required to observe a significant fibrosis response. As mentioned, cilia have a particular, tissue-specific protein and lipid composition; as such it is important to consider in these methods the antibodies used to identify cilia. In our hands, an antibody targeting acetylated-α-tubulin, even from different suppliers, allowed us to observe cilia not only in mice but also in rat cardiac fibroblasts (Villalobos et al., 2019). Although the cilium can be distinguishable by its shape and, in most of cases, by its position near the nucleus, it is desirable to use alternative markers to ensure that cilia are efficiently identified. For instance, co-staining of acetylated-α-tubulin with γ-tubulin could be performed to observe not only the ciliary structure but also to identify the base of cilia (Villalobos et al., 2019). The BBsome, a multiproteic complex structure that controls traffic of proteins throughout cilia (Nechipurenko, 2020; Wingfield et al., 2018), can also be evaluated together to acetylated-α-tubulin, to confirm that the signal observed corresponds to the primary cilia.

Even if tubulin acetylation is enhanced in the ciliary compartment, it is important to mention that acetylation of tubulin is not exclusive of cilia, but it can be observed in the whole cytoplasmic compartment (Li & Yang, 2015). Thus, the best markers to identify cilia can vary among different types of tissues. Indeed, cilia in neurons are easily identified using antibodies against adenylated cyclase III, ACIII, instead of antibodies against acetylated-α-tubulin (Avalos et al., 2022). Interestingly, in HeLa cells, in which cilia are undetected when antibodies against acetylated-α-tubulin are used, cilia are detected using antibodies against the ciliary GTPase ARL13B (Kowal & Falk, 2015).

We hope these methods will be helpful in studying cilia-dependent molecular mechanisms that occur after I/R in the heart and, therefore, identify new modulators of cardiac fibrosis. Understanding how cilia are implicated in the crosstalk between fibroblasts and cardiomyocytes in disease-related myocardial remodeling may lead to identification of new strategies with therapeutic relevance.

6 Notes

1. Fur from the chest is removed 24–48 h before the surgery using a trimmer for small animals or Nair™ hair remover.
2. Depending on vaporizing system, sevoflurane can be used as an alternative.
3. We have performed experiments with C57BL/6 mice. However, parameters such as temperature, breath rate, and heart rate should be similar in other mouse strains.
4. Surgical instruments must be sterile. We use a glass bead sterilizer to sterilize instruments between each surgery procedure.
5. A stereoscopic microscope may be used to observe the left anterior descending (LAD) coronary artery.
6. Depending on the level of ischemia-induced injury, ligation can be performed for different time periods (Redel et al., 2008).
7. Ischemia is confirmed by a visual inspection with a stereo microscope, in which blanching of the myocardium, distal to the occlusion, is observed.
8. Relaxing buffer is required to avoid harvesting the heart contracted in systole. It contains 1 mM free Mg^{2+}, 100 mM KCl, 2 mM EGTA, 10 mM imidazole, and pH 7.0.
9. Sections in a two-chamber view, compared to four-chamber heart orientation, are preferred for observing damage and fibrosis provoked by I/R. Four-chamber heart orientation is recommended to evaluate hypertrophy.
10. After maintaining slides at 37 °C overnight, slides can be kept at 4 °C for 1 year.
11. Biebrich Scarlet solution is prepared with 1% fuchsin acid (10 mL), glacial acetic acid (1 mL) and 1% Biebrich Scarlet solution (90 mL).
12. To prepare a solution of phosphotungstic/phosphomolybdic acid, mix 5 g phosphotungstic acid, 5 g phosphomolybdic acid and add 200 mL H_2O.
13. Prepare a blue aniline solution mixing 2.5 g aniline blue, 2 mL acetic acid and 100 mL H_2O.
14. Given that two-chamber view images have an approximative area of 28.6 mm^2, we use a Zeiss trinocular stereoscopic microscope Stemi 305 Trino, zoom 0.8X-to-4X. However, any other microscope instrument can be used.
15. If the paraffin sections have been stored for more than 3 months, heating is not required.
16. Sterile PBS is not required. However, we recommend using sterile solutions to avoid the growth of bacteria.
17. To permeabilize, do not use a cold solution.
18. The solution of 5% BSA-PBS we used is filtered to avoid precipitation or insoluble products in the solution.
19. We used Alexa-Fluo secondary antibodies at a dilution of 1:300.
20. Before preparing primary fibroblasts and cardiomyocyte cultures, 12 well plates must be coated with 500 µL of laminin.
21. *Perfusion Buffer* is composed as follows: 113 mM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$-$7H_2O$, 12 mM $NaHCO_3$, 10 mM

$KHCO_3$, 0.922 mM HEPES sodium salt, 30 mM Taurine, 10 mM 2,3-butanedione-monoxime, 5.5 mM glucose. Adjust pH to 7.4 and prepare between 500 mL to 1 L of solution.

22. The rubber tubes of the perfusion system must be filled with the perfusion buffer before starting with heart perfusion to avoid the presence of bubbles, and the perfusion flow rate should be set at 2–3 mL per min.
23. *Digestion Buffer* is prepared as follows: use 30 mL of Perfusion Buffer and supplement it with 5 mg of Liberase™ and $CaCl_2$ at a final concentration of 12.5 μM. Then, to obtain a sterile buffer, filter the solution using a 0.45 μm filter.
24. While the digestion process is underway, a change in the color and an increase in the size of the heart due to swelling is observed.
25. The time required for tissue digestion could vary depending on the enzymatic activity of the enzyme lot.
26. *Stop Buffer* is prepared as follows: add 10% Fetal Bovine Serum to 30 mL of *Perfusion Buffer*.
27. All the described procedures should be performed in a laminar flow hood to avoid contaminations.
28. *Calcium Restoration Solutions* are prepared as follows: Prepare 4 solutions containing 112 μM (*solution 1*), 300 μM (*solution 2*), 710 μM (*solution 3*) and 1 mM (*solution 4*) of $CaCl_2$, each in 5 mL of *Stop Buffer*.
29. *Complete M199 medium* is prepared as follows: To supplement M199 medium with 100 IU/mL penicillin, 100 IU/mL streptomycin, 2 mM L-carnitine, 5 mM creatine, and 5 mM taurine.
30. The volume of complete M199 medium can be increased depending on the yield of cells.

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