



Published in final edited form as:

Nat Protoc. 2016 May ; 11(5): 872–881. doi:10.1038/nprot.2016.049.

Explant culture of adult zebrafish hearts for epicardial regeneration studies

Jingli Cao and Kenneth D. Poss*

Department of Cell Biology, Duke University Medical Center, Durham, NC 27710, USA

Abstract

Here we describe protocols to culture adult zebrafish hearts as explants, and to study regeneration of epicardial tissue *ex vivo*. Uninjured or injured adult hearts are excised, washed, and cultured in an incubator with gentle agitation. Preparation of heart explants is accomplished within 2 hours, and explants can be maintained in culture for 30 days or longer. Dynamic behaviors of epicardial cells are monitored through live imaging of fluorescent transgenes in the explant, using stereofluorescence microscopy. We also describe *ex vivo* procedures for genetic ablation of the epicardium, cell proliferation assays, tissue grafts, and bead grafts. Basic cell culture and surgical skills are required to carry out these protocols. As opposed to existing protocols for culturing isolated zebrafish epicardial cells on matrices, procedures described here maintain epicardial cells on an intact cardiac surface, better enabling *in vivo* cell behaviors. Our protocols complement and extend *in vivo* studies of heart regeneration.

Keywords

heart regeneration; epicardium; explant culture; genetic ablation; live imaging

INTRODUCTION

Zebrafish provide a valuable model system to study regeneration of cardiac tissue, as injured hearts can regenerate large portions of lost muscle through the proliferation of spared cardiomyocytes. Non-muscle supporting cells like epicardium, endocardium, vasculature, nerves, and inflammatory cells play critical roles during heart regeneration¹⁻⁶. The epicardium, a mesothelial layer covering the surface of all vertebrate hearts, is required for normal muscle regeneration after cardiac injury in zebrafish^{4, 5, 7-10}. Studies in zebrafish and mammals have led to many proposed roles for the epicardium: in vascularization of new

*Correspondence: kenneth.poss@duke.edu, Phone: 919-681-8457, Fax: 919-684-5481.

Key reference:

Wang, J., Cao, J., Dickson, A.L. & Poss, K.D. Epicardial regeneration is guided by cardiac outflow tract and Hedgehog signalling. *Nature* 522, 226-230 (2015).

AUTHOR CONTRIBUTIONS

J.C. and K.D.P. designed the study and wrote the manuscript. J.C. performed all experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

muscle, as a source of mitogens, as a contributor to muscle survival, and as a cellular target for direct reprogramming strategies¹¹⁻¹⁷.

Tissue regeneration is by definition an *in vivo* phenomenon. However, regeneration studies in adult zebrafish suffer from their long animal generation times (~3 months) and the dearth of molecular genetic tools for inducibly manipulating adult regenerative events. Moreover, the heart as an internal organ is technically challenging to access for live imaging of regenerative events. To address these challenges, we have developed an explant system that enables culturing of adult hearts for several weeks, while maintaining much of the morphology and contractility of the heart⁷. With this method and molecular genetic tools for genetic ablation and live monitoring of epicardial cells, we defined and dissected regenerative capacity of adult zebrafish epicardial tissue in a recent research report⁷. Additionally, this explant culture system can be adapted for tissue grafting and chemical screening approaches. Here, we describe in detail this protocol for explant culture, and important applications.

Development of the protocol

Our original methods for culturing heart explants incubated extracted hearts for 7 days with Liebovit's L-15 medium supplemented with 10% fetal calf serum and antibiotics¹⁰. With this method, heart explants tended to attach to the dish bottom, altering ventricular shape and challenging live visualization of epicardial cell behaviors on the cardiac surface. Spontaneous internal infarcts were formed within ~7 days inside the explants, suggesting poorly maintained cardiac muscles. An additional protocol exists to culture isolated epicardial cells from adult zebrafish on a fibrin matrix¹⁸. With this method, epicardial cells migrate into the fibrin gel from explanted chunks of ventricle apex. This method is advantageous for cellular and molecular analysis; however it is a more artificial environment than the cardiac surface. Several other methods exist for culturing mammalian cardiac slices for weeks while maintaining contractile activity¹⁹⁻²²; yet, these systems are not applicable for studies of the epicardium.

To visualize and manipulate epicardial cells on an intact cardiac surface for extended imaging experiments, we refined our explant culture protocol with the following specific modifications. a) Instead of non-carbonate buffered L-15 medium, we used carbonate buffered DMEM medium, which is ideal for a CO₂ incubator. We also tested DMEM/F12 and IMDM medium without noticing apparent differences. We ultimately chose to use DMEM medium, as it is inexpensive and sufficient for the experiments described here. b) We added 1% non-essential amino acids (NEAA) and 0.1% 2-mercaptoethanol, used previously for long-term heart slice cultures¹⁹. NEAA increases cell growth and viability, while 2-mercaptoethanol is a reducing agent that can limit oxygen radical levels. c) We used a cocktail of antibiotics - Primocin - for the first few days of primary culture to reduce the incidence of contamination¹⁸. We also tested a combination of penicillin-streptomycin and fungizone, which was inadequate in reducing contamination. d) Most importantly, we agitated the culture plate at 150 rpm in an orbital shaker while culturing, which allows the explant to remain suspended in the culture and prevents ventricular adherence and deformation. Agitating manually every 12 h without a shaker was typically not sufficient to

prevent attachment of explants to the dish bottom. These alterations in methodology allow the heart surface to remain largely intact during culture. With the refined protocol, we were able to longitudinally monitor epicardial cell migration and proliferation on the surface of a beating heart for at least one month. This protocol is suitable for studies that focus on the epicardium and epicardial regeneration. We have not tested pacing of the explanted hearts, different energy substrates and application of hyperoxygenation, which have the potential to further optimize the protocol.

Overview of the procedure

The overall experimental scheme is shown in Fig. 1. In this protocol, we will describe explant culture of intact hearts and *ex vivo* epicardial ablation. To ablate epicardial cells, we generated a BAC line by fusing a bacterial Nitroreductase (NTR) to an mCherry fluorescent reporter gene cassette, driven by *tcf21* regulatory sequences, which are epicardial and epicardial-derived-cell-specific in the heart (*tcf21:mCherry-NTR*)^{5,7}. After treatment with metronidazole (Mtz), which is processed by NTR into a cytotoxin, this line induced cell death specifically of epicardial and epicardial-derived cells^{23,24}. We typically use a *tcf21:nucEGFP*BAC reporter strain to mark epicardial cell nuclei⁵. In optional procedures (Box 1-3), we describe an assay for proliferation by epicardial cells in culture by incorporation of the nucleotide analog 5-ethynyl-2'-deoxyuridine (EdU) (Box 1); the engraftment of a freshly collected outflow tract to the base of an epicardially-ablated ventricle (Box 2); and embedding of beads with Hh proteins onto the ventricular base (Box 3).

Comparison with existing methods

Our original *ex vivo* explantation method was developed to study epicardial and endocardial cell gene expression after the formation of spontaneous internal infarcts¹⁰. This method is suitable only for short-term culture (7 days). Also, as heart explants attach to the culture dish and cardiac muscle undergoes rapid necrosis, there is considerable opportunity for artifacts. *In vitro* primary cell culture of isolated epicardial cells is effective to characterize epicardial cell-extracellular matrix interactions, and can be expanded to tests of cell-cell interactions by co-culture¹⁸. Although a more artificial environment than the cardiac surface, dissociated cell culture has certain advantages for cellular and molecular studies, including the capacities for gene transfection and live imaging. The refined protocol we describe here combines the advantages of these two systems. It is suitable for cellular and molecular analyses while largely maintaining the cardiac surface environment (described in the next section). Other methods for culturing cardiac slices are sufficient for testing cardiac muscle behaviors but are not applicable for studies of the epicardium¹⁹⁻²².

Applications of the method

Recently, by generating transgenic tools to ablate the epicardium in zebrafish, we found that the epicardium is required for normal regeneration of heart muscle after injury. Moreover, with the aid of this *ex vivo* explant culture system, we identified unexpected features by which the epicardium itself regenerates after its depletion. The procedures we describe have considerable advantages for investigating the cellular and molecular mechanisms of epicardial cell gene regulation, proliferation, migration, and overall regeneration in real time

while largely recapitulating *in vivo* environment. The heart explants are also suitable for assays of gene expression by *in situ* hybridization. We include procedures here that enable the study of tissue-tissue interactions, including tissue and bead grafting, which can potentially be expanded to include other tissues. A clear application for these methods is chemical screening; for instance, one can test hundreds or thousands of compounds for influences on epicardial cell behaviors while on the cardiac surface. Those factors that induce epicardial cell proliferation or the activation of markers of tissue regeneration have the potential to influence the efficacy of heart regeneration.

Limitations

The protocol described here has been successfully used in studying epicardial cell behaviors, but it is not suitable for detailed studies of cardiomyocytes. We detect very limited proliferation of cardiomyocytes during culture, possibly due to missing ingredients or suboptimal environmental conditions. Rates of contraction decrease during culture, and hearts occasionally show internal infarcts. Further development of the protocol is required to improve its applicability for *ex vivo* studies of cardiac muscle. Most applications can be completed in 2 weeks, during which the hearts appear grossly normal in morphology and beating. For long-term culture of more than 2 weeks, the heart shape becomes slightly altered. Shrinking is observable, and the pyramidal ventricle appears more rounded, likely secondary to the shaking of the culture. After grafting of cardiac chambers, the ventricular shape may change slightly due to pressure exerted from the agarose used in the procedure. However, no apparent difference in epicardial cell behavior (such as proliferation and migration) was observed in ventricles of different shapes.

Experimental design

Partial ventricular resection surgeries and inducible epicardial ablation

Depending on the experimental purpose, partial resection can be performed by removing 10-20% of the ventricular apex as described previously, before explant culture¹. Epicardial ablation can be performed before or during explant culture. Hearts can be collected at any time following *in vivo* manipulations. Examples can be found in our recent publication⁷. Hearts after resection or ablation injury will be adhesive and may be difficult to separate from pericardial material. In this report, we will describe explant culture of intact hearts and those undergoing *ex vivo* epicardial ablation. As heart sizes, surgery size and positions, and ablation effects are variable among different fish and clutches, at least 10 explants per treatment are required for each experiment. Five sham-treated (uninjured or unablated) explants should be included each time as a system control for culture procedures. At least two independent replicates are required for conclusions. If treatments will be applied during explant culture, such as chemical treatment and bead embedding, uninjured explants and/or explants with surgery or ablation should be randomized into different groups for each experiment. Finally, only clutchmates (or hearts collected from clutchmates) will be used for each experiment.

Choice of culture dish

While shaking, explants will gather in the middle of the culture dish or well. If epicardial cells are ablated or heart surgeries are performed prior to culturing, explants are adhesive and will become attached to each other when cultured as multiples. In this situation, one heart per well is recommended and multi-well plates are economical. On the other hand, small wells of 24- or 48- well plate are not conducive for live imaging. As the samples must remain covered while imaging, only the center of these small wells can provide an undistorted view. The 12-well plate is the best choice for most experiments. Uninjured hearts can be cultured together in larger wells or petri dishes.

Proliferation assay

There are various ways to assay indices of proliferation in cells on the cardiac cell surface. To mark cells undergoing DNA synthesis, we use the nucleotide analog EdU. A 1-hour period of EdU incorporation at 25 μ M is sufficient to label a large number of cells. Phosphorylated Histone H3 is a marker of cells in G2/M phase, recognized by several commercially available antibodies. We choose EdU as an example here. Epicardial cell nuclei are best identified by the *tcf21:nucEGFP* strain⁵. Two independent replicates and 10 explants per treatment are required for quantification of proliferation indices.

Chemical treatment

Small molecule compounds can be included in the incubation protocols to examine effects on epicardial proliferation and regeneration⁷. It is important to maintain sterile medium, accomplished either by filter sterilization of the medium with diluted drug, or by keeping the drug stocks sterile. For each chemical compound, two independent replicates with at least 10 explants of uninjured or epicardially ablated hearts per replicate are treated, and 10 more vehicle treated explants of hearts from the same clutch of zebrafish served as a control. Medium should be changed every other day with fresh chemicals. For uninjured hearts, effects on epicardial proliferation are assessed by quantification of cell numbers using the *tcf21:nucEGFP* reporter at 2-6 days of culture. For effects on epicardial regeneration after ablation, chemicals are added at 2 days post Mtz treatment, and explants from the *tcf21:nucEGFP* strain are imaged every day for up to 14 days to assess epicardial proliferation and migration.

Tissue grafts

We have successfully grafted the bulbous arteriosus (cardiac outflow tract) or ventricular apex to the base or apex of ventricles that have had their epicardium genetically depleted⁷. Here we describe the engraftment of freshly collected outflow tract to the base of an epicardially-ablated ventricle. In this experiment, about 50% of the explants will be tightly grafted together after 24 h contact in agarose. For 10 successful grafts, at least 20 attempts are required. As the grafted positions vary across grafts, we prefer to make conclusions based on 3 independent replicate experiments. The protocol should also be applicable to other tissue combinations.

Bead grafts

Agarose beads can be used to test factor function by controlled protein release. Here we will embed beads soaked with Hh proteins onto the ventricular base as an example. Beads soaked with BSA protein serve as a control. At least 50% of ventricles successfully engraft and maintain beads during culture. Three independent replicates with at least 20 attempts per replicate are required for each experiment. Heparin acrylic beads can also be used²⁵.

MATERIALS

Zebrafish

Adult zebrafish of the Ekkwill and Ekkwill/AB strains were used, maintained as described¹. Animals between 4 and 12 months of both sexes are generally used. Transgenic lines used in this study were *Tg(tcf21:mCherry-NTR)^{pd108}* (ref. 7), *Tg(tcf21:nucEGFP)^{pd41}* (ref. 26), *Tg(cmlc2:actin3-EGFP)^{sd10}* (ref. 27) and *Tg(fli1a:EGFP)^{y1}* (ref. 28). All transgenic strains were analyzed as hemizygotes. Animal procedures were approved by the Institutional Animal Care & Use Committee at Duke.

REAGENTS

Fish water (aquarium water from the zebrafish facility)

Tricaine (Sigma, cat. no. A5040) CAUTION Avoid inhalation and direct contact with eyes and skin.

DMEM cell culture medium (Invitrogen, cat. no. 11965)

FBS (Thermo Scientific, cat. no. SH30071.03)

Primocin (InvivoGen, cat. no. ant-pm-2)

PBS (Corning, cat. no. 46-013-CM)

Ethanol (VWR, cat. no. 89125-172)

Metronidazole (Mtz, Sigma, cat. no. M1547) CAUTION Mtz is a carcinogen. Avoid contact with skin and eyes. Avoid inhalation. Wear gloves and eye protection.

L-Glutamine (Invitrogen, cat. no. 25030-081)

MEM-NEAA (Invitrogen, cat. no. 11140-050)

Penicillin-streptomycin (Invitrogen, cat. no. 15140-122)

Low-melting agarose (Fisher Scientific, cat. no. BP1360-100)

Affi-Gel Blue beads (Bio-Rad, cat. no. 1537301)

5-ethynyl-2'-deoxyuridine (EdU, Life Technologies, cat. no. A10044) CAUTION Avoid contact with skin and eyes. Avoid inhalation. Wear gloves and eye protection.

Recombinant mouse Sonic Hedgehog (C25II), N-terminus protein (R & D Systems, cat. no. 464-SH-025)

Bovine serum albumin (BSA, VWR, cat. no. 97061-420)

Paraformaldehyde (Fisher Scientific, cat. no. O4042-500) CAUTION Avoid contact with skin and eyes. Avoid inhalation. Wear gloves and eye protection.

Fluoromount G mounting medium (Fisher Scientific, cat. no. 0100-01)

EQUIPMENT

Finger bowls (90 × 50 mm; VWR, cat. no. 89000-288)

1.7 ml Eppendorf tubes (EP tubes, Genesee Scientific, cat. no. 24-281)

12-well plates (Corning, cat. no. 3737)

60 mm Petri dishes (Corning, cat. no. 351007)

Millex-GP Filter units (0.22µm, Millipore, cat. no. SLGP033RB)

Syringes (BD, cat. no. 302831)

Sterile pipette tips (Corning, cat. no. 4135, 4139 and 4140)

Sterile disposable plastic transfer pipettes (VWR, cat. no. 414004-016)

Cover slips (Fisher Scientific, cat. no. 12-544-E)

Stainless steel microforceps (Dumont, cat. no. 5-inox-H)

Stainless steel microscissors (World Precision Instruments, cat no. 14124)

Sponges (1.5 × 5 × 3 cm) with a single center groove cut using scissors (0.5 × 2.5 cm)

Plastic spoons, heavy duty (Staples)

Falcon tubes (15 ml, 50 ml, Corning, cat. no. 430790, 430828)

Dissecting microscope (Leica)

Water bath (Fisher Scientific, ISOTEMP 150)

125 ml flasks (Corning)

Biological safety cabinet with laminar flow and UV light (Labconco, Class II, type A2)

Cell culture incubator (28°C, 5% CO₂, humidified; Fisher Scientific, Model 3530)

Orbital shaker (Troemner, cat. no. 12620-938)

GeneMate GyroMixer, Variable Speed (BioExpress, cat. no. R-3200-1XL)

Leica MZ05FA stereofluorescence microscope with camera

REAGENT SETUP

Tricaine stock solution

The stock solution contains 15 mM ethyl 3-aminobenzoate methanesulfonate (tricaine) and 20 mM Tris-HCl, pH 7.4. Store this stock solution at 4°C for as long as one month, and at -20°C for longer term storage. To anaesthetize adult zebrafish, dilute 4.2 ml of the stock solution into 100 ml of fish water.

Culture medium

Culture medium consists of DMEM plus 10% fetal bovine serum, 1% MEM-NEAA (non-essential amino acids), 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol. Primocin™ was added just before use to prohibit microbial contaminants during the first few days primary culture. Sterile medium can be stored at 4°C for up to one month without effects on culture results. CAUTION Medium and PBS must be sterile filtered.

Ablation medium

For epicardial ablation, freshly prepare 1 mM Mtz solution in culture medium for use. First make 10-20 ml of a 10 mM Mtz solution in a 50 ml Falcon tube by vortexing and pipetting. Then dilute 1:10 to the desired volume, and filter sterilize with a 0.22 µm filter unit.

CAUTION As Mtz is difficult to dissolve, ensure it is fully dissolved before use.

Low-melting agarose

Keep a separate bottle of agarose powder for culture use only, to reduce chance of contamination. To make 30 ml 1% low-melting agarose, weigh 0.3 g of powder and put into autoclaved 125 ml flask and add 30 ml sterile PBS. Microwave for several minutes until agarose is fully dissolved. Store the agarose in a 37°C water bath until use.

CRITICAL Use sterile PBS and flask.

PROCEDURE

Preparation TIMING 1 h

1. Prepare reagents as described above. Warm DMEM culture medium and PBS to 28°C (or room temperature, 25 °C), and prepare ablation medium if required. Place the orbital shaker in the incubator.
2. Sterilize forceps and scissors by immersing in 70% ethanol. Clean bench and dissecting scope stage with 70% ethanol. Clean sponge with ethanol and squeeze off residual ethanol prior to moistening with fish water (Fig. 2a).

CAUTION A sponge that is used repeatedly could accumulate contaminating microorganisms. Washing with EtOH will reduce the chance of contamination.

CRITICAL STEP Steps 2–7 must be performed on a clean lab bench using sterile tools and filter pipette tips.

3. Prepare a 60 mm petri dish with 5 ml culture medium, and EP tubes with 1 ml culture medium per tube. All samples will be placed in one tube (Fig. 2a).

Extracting hearts TIMING 2-5 min per heart

4. Euthanize the fish with Tricaine in a glass bowl until gill movements end, and there is no response to a tail pinch. Use a plastic spoon to transfer the fish, dry briefly on a paper towel, and then place fish ventral side up onto the groove of moist sponge under a dissecting microscope.

CRITICAL STEP Animal procedures must be performed in accordance with institutional guidelines.

5. Open the ventral wall of the fish with a longitudinal cut at the gill area. Locate the outflow tract. Grab the distal end of outflow tract with forceps and gently pull the heart out of the chest cavity. Sever the ventral aorta and sinus venosus with forceps. Place the heart into the 60 mm Petri dish with culture medium (Fig. 2b). A video demonstrating a similar heart extraction procedure can be found in a previous protocol¹⁸.

CRITICAL STEP If more than one heart is processed, store hearts in a Petri dish with medium at room temperature. Keep the lid of the culture dish closed.

Clean hearts TIMING 5 min

6. Carefully remove blood clots on the heart surface by using forceps while holding the end of the outflow tract with the other forceps. Remove the atrium or outflow tract if required by experiment.

CRITICAL STEP Steps 6-10: Be careful not to damage the epicardium, which may influence experiments.

7. After all hearts are dissected, cut off the tip of a 1 ml pipette tip, and use this (or a sterile plastic transfer pipette) to transfer hearts to the EP tube containing 1 ml culture medium (Fig. 2c).

CRITICAL STEP All explant transfer procedures should be performed in this way.

8. In a biological safety hood, wash hearts three times, using 1 ml PBS per wash.

CRITICAL STEP Steps 8–12 must be performed in a biological safety hood using sterile pipette tips and PBS.

Heart culture and epicardial ablation TIMING 5 min

9. Add medium to 12-well plate using 1 ml per well. If planning to ablate epicardium, use the ablation medium.

CRITICAL STEP Medium must be warmed before use. Ablation medium must be freshly prepared just before dissecting.

10. Transfer heart to culture dishes with 1 ml pipette tips.
11. Place culture plate onto the horizontal shaker in a 28°C incubator and shake at 150 rpm (Fig 2d). Culture medium should be changed every other day. If ablation medium is used, replace ablation medium with normal culture medium after 24 h.

CRITICAL STEP Ensure the shaker is working properly throughout the culture period.

TROUBLESHOOTING

Box 1. Proliferation assay

Release of Mtz treatment TIMING 30 min

12. Warm PBS and culture medium to 28°C in water bath. After 24 h culture in ablation medium with 1 mM Mtz, gently remove medium with a 1 ml tip. Ensure not to touch the hearts, which may injure the epicardium. Add 1 ml PBS to the well, gently shake a few times and then pipette out the PBS. Repeat washing with 1 ml PBS and then add 1 ml fresh medium without Mtz to each well. Return to the incubator and continue culturing.

CAUTION Follow institutional guidelines to dispose of medium with Mtz.

TROUBLESHOOTING

Box 2. Chamber grafts

Box 3. Bead grafts

Imaging TIMING up to 30 days

13. Images can be acquired every day using a Leica M205FA stereofluorescence or other similar dissecting scope (Fig. 2e, f). We typically image two sides of a heart, the atrial side and the opposite side.

CRITICAL While taking images, keep the lids of culture plates closed.

TROUBLESHOOTING

TROUBLESHOOTING

Step	Problem	Possible reason	Solution
11-13, b5, c6	Contamination with bacteria	Bench is not clean	Clean bench with 70% ethanol or perform procedures in a cell culture hood
		Tools are not sterile	Sterilize tools with 70% ethanol, use sterile pipette tips or filter tips
		Reagents are not sterile	Filter sterilize reagents

Step	Problem	Possible reason	Solution
		Plate lid is open during culture or imaging	Keep lid closed while culturing and imaging
		Primocin is not added	Add Primocin for the first few days of culture
11	Heart explant is attached to dish bottom	Culture plate is not shaking properly	Make sure the shaker is always working at 150 rpm during culture
13	Absent or weak epicardial ablation effect	Mtz is not fully dissolved	Ensure Mtz is fully dissolved when preparing 10 mM solution
b5	Chamber engraft fails	Chambers are not contacted closely	Use smaller hole to hold chambers tightly
c6	Beads floating	Too much medium	Add less medium

TIMING

Steps 1-3, Preparation: 1 h

Steps 4-5, Extracting hearts: 2-5 min per heart

Steps 6-8, Clean hearts: 5 min

Steps 9-11, Heart culture and epicardial ablation: 5 min

Step 12, Release Mtz treatment: 30 min

Step 13, Imaging: up to 30 days

Optional Steps a1-a3: Proliferation assay: 5-8 h

Optional Steps b1-b5: Chamber engraftment: 25-50 h

Optional Steps c1-c5: Beads embedding: about 26 h

ANTICIPATED RESULTS

During culture, cardiac contraction will gradually slow, although explants can continue beating for more than one month (ref. 7, Supplementary video 1)⁷. Sarcomeric structure, visualized using a transgenic *cmc2:actinin3-EGFP* line, is largely maintained during the first 2 weeks of culture, and muscle necrosis is rare (Fig. 4). The heart shape will also change gradually, shrink slightly and become rounded. Despite these minor changes, epicardial proliferation and migration are not affected. Epicardial cells will proliferate spontaneously without any treatment, with cell density increasing during the first 2 days, as assessed by staining with an antibody against Raldh2, and EdU incorporation and staining (Fig. 4; Fig. 5 and ref. 7)⁷. Proliferating endocardial cells were very rare, and were not detected in most hearts after 24h EdU incorporation (day 2-3) and assessment at day 3 by using a *fli1a:EGFP* reporter line (Fig. 5b). Some EGFP⁺/EdU⁺ endothelial cells were detected, but a nuclear marker of endothelial cells is required for further confirmation.

The cultured explants are suitable for *in situ* hybridization (Fig. 6), and we detected epicardial induction of *raldh2*, *tbx18* and *wt1* in uninjured hearts during explant culture. For most explants, *raldh2* was only detected in epicardial cells, while some explants displayed endocardial expression.

With Mtz treatment, epicardial cell death can be observed from day 2 (1 day post Mtz incubation, dpi) with debris protruding from the heart surface. Full ablation can be achieved at day 3 (2 dpi) with a significant portion of the ventral surface demonstrating absent *pcf21:nucEGFP* fluorescent. Epicardial regeneration is visualized by daily imaging; epicardial cells located at the ventricular base proliferate and migrate toward the ventricular apex (Fig. 7a). It takes approximately 2 weeks for epicardial cells to completely repopulate the ventricular surface (Fig. 7a and ref. 7, Fig. 2). In chamber engraftment experiments, about 50% of the explants will be tightly connected after 24 h contact in agarose (Fig. 7b, c). The ratio will be higher after 48 h contact in agarose. The ventricular epicardium loses regenerative capacity after removal of the outflow tract; however, a fresh donor outflow grafted to the ventricular base can rescue the defect (Fig. 7c and ref. 7, Fig. 3). In bead-grafting experiments, some ventricles lose the beads after release from agarose; these samples must be discarded. Ventricles also lose beads during 1-week culture and also must be discarded. At least 50% of ventricles successfully engraft and maintain beads during culture. Beads soaked with Hh protein can induce epicardial cell regeneration within the host ventricle after outflow tract removal (ref. 7, Fig. 4), implicating this factor in mechanisms of regeneration.

In summary, we describe in detail an explant culture system that maintains aspects of cardiac physiology and recapitulates *in vivo* observations of epicardial regeneration, while better representing such observations than cultured primary cells⁷. At the same time, cellular and molecular studies are convenient and efficient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank J. Wang and S. Singh for generating transgenic fish; J. Burris, N. Lee, A. Dunlap, S. Davies, T. Thoren, and N. Benkaci for fish care; A.L. Dickson for artwork; and A. Shoffner for comments on the manuscript. This work was funded by a postdoctoral fellowship from the American Heart Association to J.C. and grants from NIH (R01 HL081674 and R01 HL131319) to K.D.P.

REFERENCES

1. Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. *Science*. 2002; 298:2188–2190. [PubMed: 12481136]
2. Jopling C, et al. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature*. 2010; 464:606–609. [PubMed: 20336145]
3. Kikuchi K, et al. Primary contribution to zebrafish heart regeneration by *gata4(+)* cardiomyocytes. *Nature*. 2010; 464:601–605. [PubMed: 20336144]
4. Lepilina A, et al. A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell*. 2006; 127:607–619. [PubMed: 17081981]

5. Kikuchi K, et al. tcf21+ epicardial cells adopt non-myocardial fates during zebrafish heart development and regeneration. *Development*. 2011; 138:2895–2902. [PubMed: 21653610]
6. Mahmoud AI, et al. Nerves Regulate Cardiomyocyte Proliferation and Heart Regeneration. *Dev. Cell*. 2015; 34:387–399. [PubMed: 26256209]
7. Wang J, Cao J, Dickson AL, Poss KD. Epicardial regeneration is guided by cardiac outflow tract and Hedgehog signalling. *Nature*. 2015; 522:226–230. [PubMed: 25938716]
8. Gemberling M, Karra R, Dickson AL, Poss KD. Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish. *Elife*. 2015; 4
9. Wang J, Karra R, Dickson AL, Poss KD. Fibronectin is deposited by injury-activated epicardial cells and is necessary for zebrafish heart regeneration. *Dev. Biol*. 2013; 382:427–435. [PubMed: 23988577]
10. Kikuchi K, et al. Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev. Cell*. 2011; 20:397–404. [PubMed: 21397850]
11. Zhou B, et al. Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J. Clin. Invest*. 2011; 121:1894–1904. [PubMed: 21505261]
12. Huang GN, et al. C/EBP transcription factors mediate epicardial activation during heart development and injury. *Science*. 2012; 338:1599–1603. [PubMed: 23160954]
13. Smart N, et al. De novo cardiomyocytes from within the activated adult heart after injury. *Nature*. 2011; 474:640–644. [PubMed: 21654746]
14. Smart N, et al. Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. *Nature*. 2007; 445:177–182. [PubMed: 17108969]
15. Ieda M, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell*. 2010; 142:375–386. [PubMed: 20691899]
16. Qian L, et al. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature*. 2012; 485:593–598. [PubMed: 22522929]
17. Song K, et al. Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature*. 2012; 485:599–604. [PubMed: 22660318]
18. Kim J, Rubin N, Huang Y, Tuan TL, Lien CL. In vitro culture of epicardial cells from adult zebrafish heart on a fibrin matrix. *Nat. Protoc*. 2012; 7:247–255. [PubMed: 22262006]
19. Habeler W, et al. An in vitro beating heart model for long-term assessment of experimental therapeutics. *Cardiovasc. Res*. 2009; 81:253–259. [PubMed: 18980954]
20. Brandenburger M, et al. Organotypic slice culture from human adult ventricular myocardium. *Cardiovasc. Res*. 2012; 93:50–59. [PubMed: 21972180]
21. Stuckmann I, Evans S, Lassar AB. Erythropoietin and retinoic acid, secreted from the epicardium, are required for cardiac myocyte proliferation. *Dev. Biol*. 2003; 255:334–349. [PubMed: 12648494]
22. Pillekamp F, et al. Force measurements of human embryonic stem cell-derived cardiomyocytes in an in vitro transplantation model. *Stem Cells*. 2007; 25:174–180. [PubMed: 16973834]
23. Singh SP, Holdway JE, Poss KD. Regeneration of amputated zebrafish fin rays from de novo osteoblasts. *Dev. Cell*. 2012; 22:879–886. [PubMed: 22516203]
24. Curado S, et al. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. *Dev. Dyn*. 2007; 236:1025–1035. [PubMed: 17326133]
25. Brito JM, Teillet MA, Le Douarin NM. An early role for sonic hedgehog from foregut endoderm in jaw development: ensuring neural crest cell survival. *Proc. Natl. Acad. Sci. U S A*. 2006; 103:11607–11612. [PubMed: 16868080]
26. Wang J, et al. The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion. *Development*. 2011; 138:3421–3430. [PubMed: 21752928]
27. Lin YF, Swinburne I, Yelon D. Multiple influences of blood flow on cardiomyocyte hypertrophy in the embryonic zebrafish heart. *Dev. Biol*. 2012; 362:242–253. [PubMed: 22192888]
28. Lawson ND, Weinstein BM. In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev. Biol*. 2002; 248:307–318. [PubMed: 12167406]
29. Gupta V, Poss KD. Clonally dominant cardiomyocytes direct heart morphogenesis. *Nature*. 2012; 484:479–484. [PubMed: 22538609]

Box 1. Proliferation assay TIMING 5-8 h

Following Steps 1-11. To detect proliferation, EdU should be added to the culture medium at a concentration of 25 μ M.

- a1.** Warm DMEM culture medium to 28°C and add EdU to 25 μ M. Change medium of the explants with EdU medium and return to the incubator. A one-hour incorporation will efficiently label cells undergoing DNA synthesis. If explants will not be fixed immediately after the 1 h treatment, the medium with EdU should be filter sterilized before use. If planning to wash out EdU and keep culturing, pipette out EdU medium and wash twice with PBS as described in Step 12, and then change to fresh medium without EdU.
- a2.** Wash hearts with PBS twice and fix hearts in 4% paraformaldehyde. EdU staining can be done as described⁷.

CRITICAL STEP PBS washes are important; otherwise unincorporated EdU will produce high background.

- a3.** After staining, place the explants on a coverslip and add a drop of Fluoromount G. Compress the explant with another cover slip, which allows imaging of both ventricular surfaces²⁹. Dry samples at room temperature for a few hours before imaging with a confocal microscope (Fig. 4b).

Box 2. Chamber grafts TIMING 25-50 h

Following Steps 1-12. Here, we graft freshly collected outflow tracts to an epicardially ablated ventricle as an example. The ventricle was treated with 1 mM Mtz for 24 h and cultured for 2 more days before engraftment.

CRITICAL STEP Steps b1-b5 should be performed in a biological hood with sterile tools.

- b1.** Prepare culture dishes with low melting agarose. Under a dissecting scope, construct a hole in the middle of the agarose by using forceps, just large enough to hold the ventricle (Fig. 3a).

CRITICAL STEP Several holes with different sizes can be made in case heart size varies among your animals.

- b2.** Collect outflow tracts by following Steps 4-8.
- b3.** Transfer ventricular explants on top of the agarose by using a 1 ml pipette tip with a cut off end (or a plastic transfer pipette). Gently place the ventricle into the hole with the ventricular base positioned laterally, using forceps. Then, transfer an outflow tract to the same hole, modifying the size of the hole to ensure the tissues contact each other at their bases (Fig. 3c, e; Supplementary Video 1).

CRITICAL STEP Ensure the size of the hole is appropriate - not too small to squeeze chambers.

- b4.** Add sufficient medium carefully to the dish to cover the agarose with explants, without agitating the explants. Culture in the incubator without shaking.

CRITICAL STEP Gently place the dish in the incubator, and prevent agitation until explants are released from the agarose in subsequent steps.

- b5.** After 24-48 h of culture, release the grafted explants from the agarose by disrupting the agarose with forceps and flushing gently with culture medium. Transfer the explants to a new culture dish with fresh medium and culture while shaking. Images are taken every day as described in Step 13.

TROUBLESHOOTING

Box 3. Bead grafts TIMING about 26 h

Following Steps 1-12. Here we graft beads soaked with Hh proteins onto the ventricular base as an example.

CRITICAL STEP Steps c1-c6 can be performed in a biological hood using sterile tools.

- c1.** Prepare culture dishes with low melting agarose. Make a round hole in the middle of the agarose as described above (Fig. 3a; Supplementary Video 2).
- c2.** Affi-Gel Blue beads were prepared by thoroughly washing the beads in PBS for 5 times in an EP tube. During each wash, allow beads to settle by gravity, and then gently remove PBS without disturbing beads at the bottom (Fig. 3b). After the last washing, remove as much PBS as possible by using a 20 μ l pipette tip.

CRITICAL STEP Beads must be washed thoroughly to remove preservative.

- c3.** Recombinant mouse Sonic Hedgehog (C25II), N-terminus protein was reconstituted at 100 μ g/ml in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Then 20 μ l Shh solution is added to the beads and incubated for 2 hours at room temperature while rotating on a shaker (GeneMate GyroMixer). A solution with the same concentration of BSA protein is used as the control. The bead solutions can be stored at 4°C.
- c4.** Transfer ventricular explants to the agarose, and gently place the ventricle into the hole with the ventricular base on top, using forceps.
- c5.** Tilt the dish and pipette out extra medium before adding beads. Use a 20 μ l pipette tip to pipette beads and apply to the base of ventricular explants. Add medium carefully to cover only the explant and beads without allowing the beads to float (Fig. 3d, e). Transfer the dish to the incubator.

CRITICAL STEP Two to three beads can be applied to one site. Not all beads will be successfully grafted.

CRITICAL STEP Do not add too much medium, as this may cause the beads to float.

CRITICAL STEP Gently place the dish in the incubator, and keep still until the release of explants from the agarose.

- c6.** After 24 hours, release the ventricles from the agarose with the attached beads and transfer to a new dish with culture medium and culture while shaking. Images are taken every day as described in Step 13.

TROUBLESHOOTING

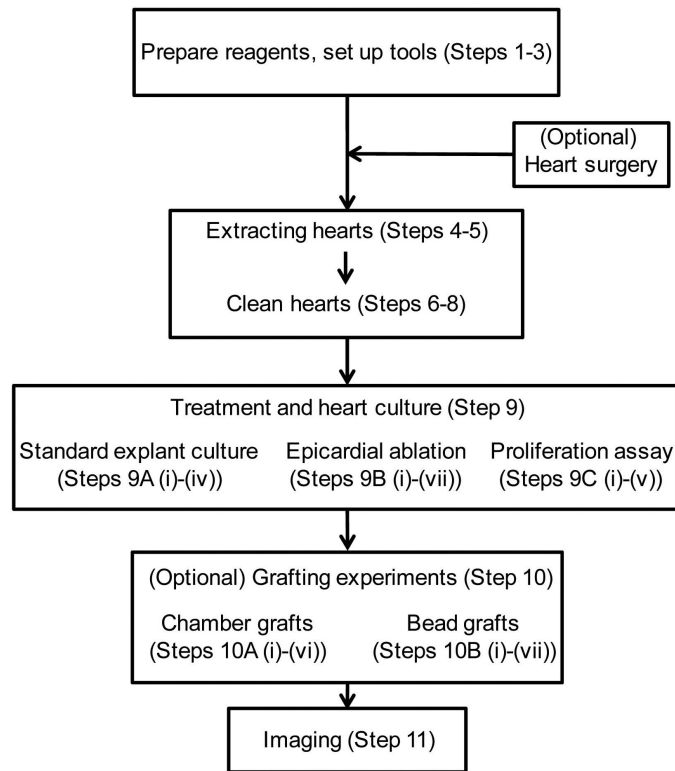


Figure 1.
Flowchart of experimental procedures.

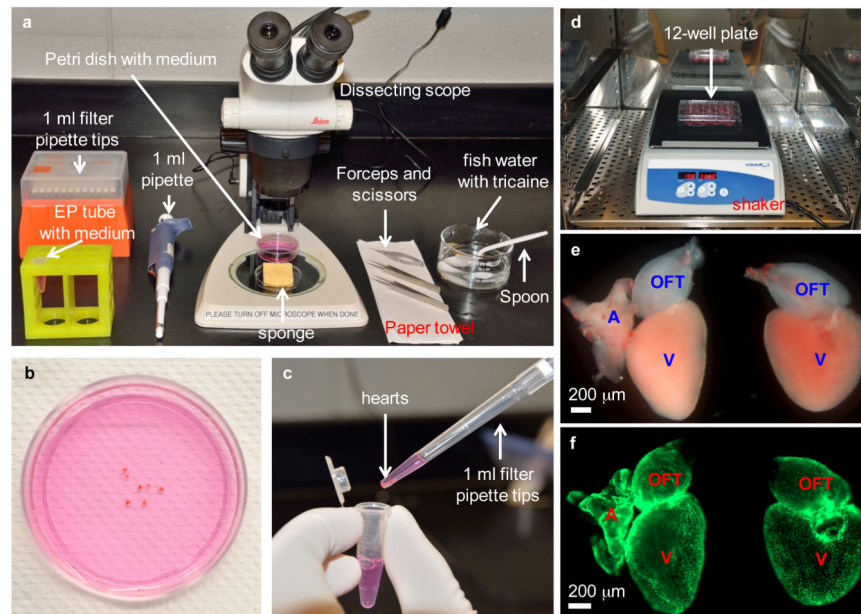


Figure 2. Heart dissection and explant culture. **(a)** Equipment setup for heart dissection, including dissecting scope, forceps, scissors, pipette and filter tips, grooved sponge, 60 mm Petri dish with culture medium, 1.5 ml EP tube with culture medium, finger bowl with diluted Tricaine in aquarium water, spoon and paper towel. **(b)** Collected hearts are placed in a 60 mm Petri dish to remove blood clots and extra tissues. **(c)** Cleaned hearts are transferred to an EP tube by using a 1 ml pipette tip with the end cut off. **(d)** Washed heart explants are transferred to a 12-well culture plate, and the plate is placed on a shaker in a CO₂ incubator, shaking at 150 rpm. **(e)** Close-up view of two heart explants with (left) or without (right) outflow tract in a culture plate. Hearts are from *tcf21:nucEGFP* zebrafish. A, atrium; V, ventricle; OFT, outflow tract. **(f)** Fluorescent view of explants in (e), showing nuclear EGFP signals. Work with zebrafish was approved by the Institutional Animal Care & Use Committee at Duke.

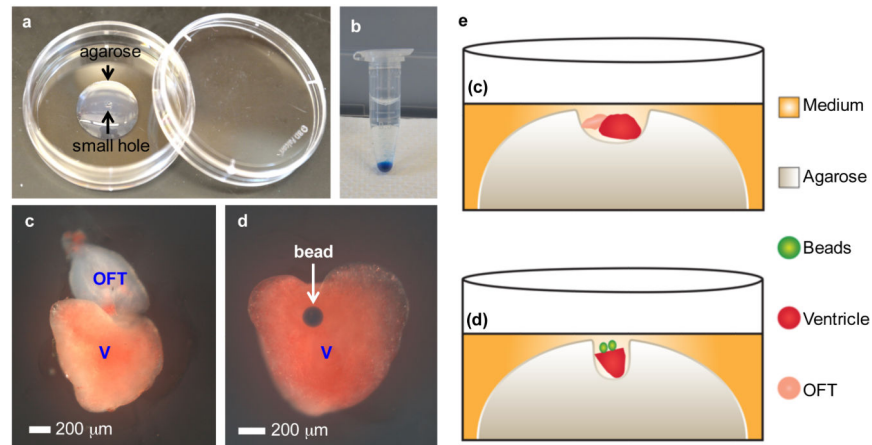


Figure 3.

Heart chamber and bead grafts. **(a)** A 35 mm Petri dish with a drop of 1% low-melting agarose in the center. A small hole is made at the top of the gel using forceps. **(b)** Washed Affi-Gel Blue beads settle to the bottom of EP tube. **(c)** A ventricle and an outflow tract from two hearts are placed together closely in the small hole at the top of the agarose gel. V, ventricle; OFT, outflow tract. **(d)** A ventricular explant inside the small hole is grafted with a bead at the base. **(e)** Cartoon of sectional view of the experimental samples in (c) and (d).

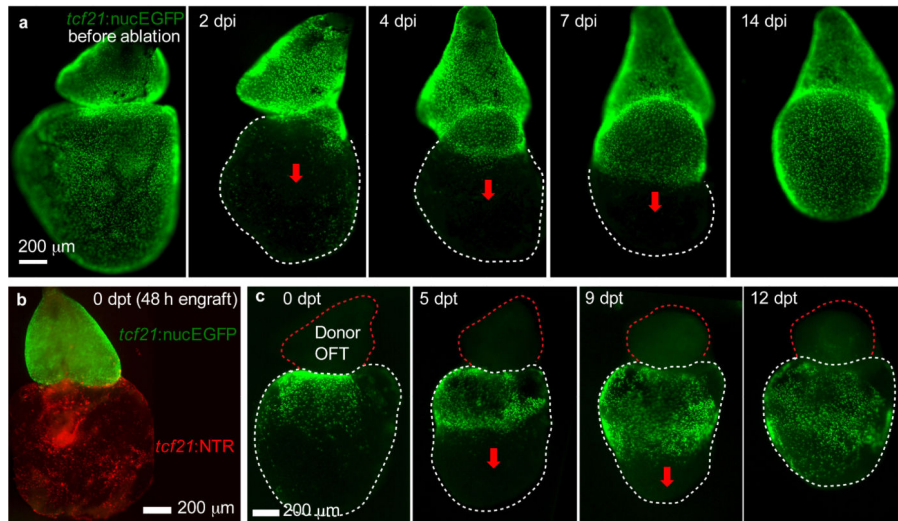


Figure 4. Sarcomere morphology and expansion of epicardial cells during explant culture. Cultured hearts from *cmhc2: actinin3-EGFP* fish were fixed at the indicated time point. (Top panels) Heart sections were stained with an anti-Raldh2 antibody to denote epicardial and endocardial cells (red) and DAPI for DNA (blue). Sarcomeric structure (green) was largely maintained in the first 2 weeks, and the epicardial cell population expanded. Cardiac muscle compresses somewhat at day 14, which likely causes tissue shrinkage. (Bottom panels) DAPI (white) staining showed largely normal myocardial nuclei. Necrosis was rare within 14 days of culture.

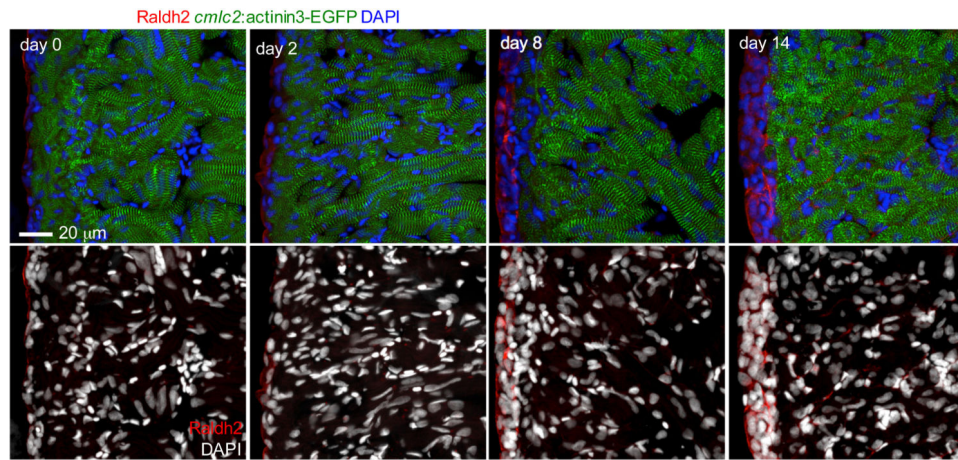


Figure 5.

Spontaneous proliferation of epicardial cells during explant culture. **(a)** Spontaneous proliferation of epicardial cells after 48 h in culture. This heart explant from a *tcf21:nucEGFP* fish was treated with 25 μ M EdU for 1 h at 47-48 h of culture. The framed region is enlarged to show details. Arrowheads indicate EGFP⁺/EdU⁺ nuclei. **(b)** Section of a 72 h heart explant from a *fli1a:EGFP* fish treated with 25 μ M EdU for 24 h at 48-72 h of culture. Most EdU⁺ cells were in the ventricular wall. EGFP⁺/EdU⁺ endocardial cells were very rare. There are some EGFP⁺/EdU⁺ endothelial cells (arrowhead).

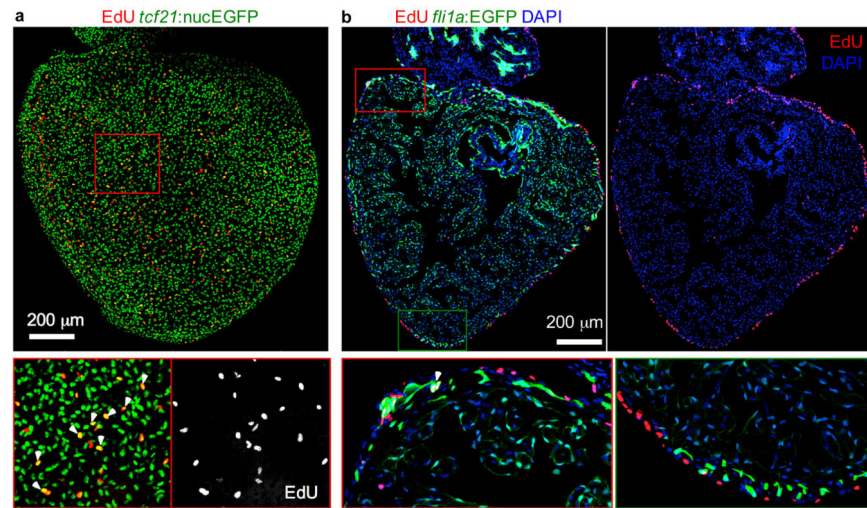


Figure 6.
In situ hybridization on sections of ventricular explants after 2 days of culture. *raldh2*, *tbx18* and *wt1* were induced during explant culture. For most explants, *raldh2* was only detected in the wall (top left), while some explants showed endocardial expression (top right), suggestive of endocardial activation by injury.

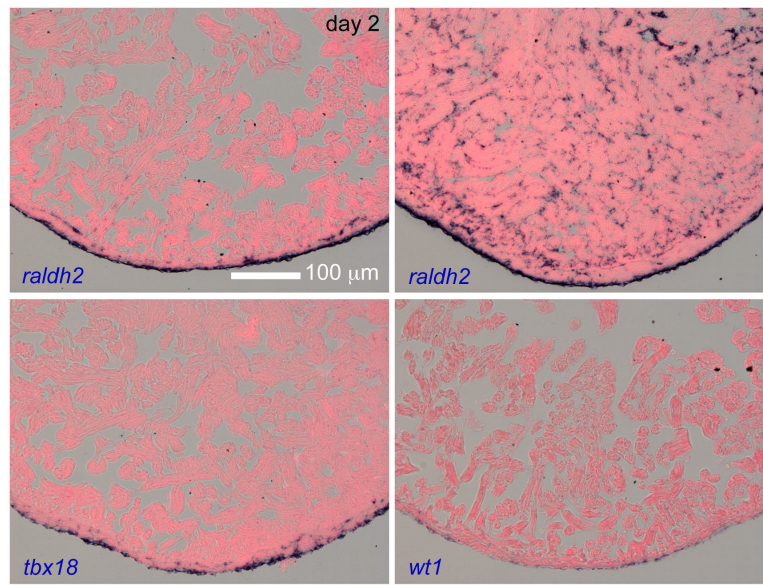


Figure 7.

Ex vivo epicardial regeneration and chamber engraftment. **(a)** Ventricular epicardium regenerates in a base-to-apex direction (arrows). Hearts from *tcf21:NTR; tcf21:nucEGFP* adults were incubated with 1 mM Mtz for 24 h. Whole mount images were taken daily. dpi, days post Mtz incubation. **(b)** A freshly collected outflow tract from a *tcf21:nucEGFP* fish (green) was grafted to the base of a ventricle from *tcf21:NTR* fish (red), by 48 h contact in agarose. The ventricle was treated with 1 mM Mtz for 24 h and cultured for 2 more days before engraftment. **(c)** A non-transgenic donor outflow tract (OFT) was transplanted to the base of an epicardially ablated ventricle. Base-to-apex epicardial regeneration (arrows) was observed from host tissue. dpt, days post-transplantation.