Preparing parenchymal and cardiac decellularized extracellular matrix for transplantation and in vitro purposes

Running title: Generation of decellularized heart and liver scaffolds

Matteo Ghiringhelli[1], Yousef Abboud[1], Snizhanna V. Chorna[1], Irit Huber[1], Gil Arbel[1], Amira Gepstein[1], Georgia Pennarossa[3], Tiziana A. L. Brevini[3], Lior Gepstein[1,2].

[1]Sohnis Research laboratory for Cardiac Electrophysiology and Regenerative Medicine, the Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa, Israel, POB 9649, Haifa 3109601, Israel.

[2]Cardiology Department, Rambam Health Care Campus, 8 Haifa Haliya Hasniya St, Haifa 3109601, Israel.

[3]Laboratory of Biomedical Embryology, Department of Health, Animal Science and Food Safety and Center for Stem Cell Research, Università degli Studi di Milano, via Celoria 10, 20133 Milan, Italy.

Abstract

Tissue engineering provides unique opportunities for disease modeling, drug testing, and regenerative medicine applications. The use of cell-seeded scaffolds to promote tissue development is the hallmark of the tissue engineering. Among the different types of scaffolds (derived from either natural or synthetic polymers) used in the field, the use of decellularized tissues/organs is specifically attractive. The decellularization process involves the removal of native cells from the original tissue, allowing the preservation of the three-dimensional (3D) macroscopic and microscopic structures of the tissue and extracellular matrix (ECM) composition. Following recellularization, the resulting scaffold provides the seeded cells with the appropriate biological signals and mechanical properties of the original tissue. Here, we describe different methods to create viable scaffolds from decellularized heart and liver as useful tools to study and exploit ECM biological key factors for the generation of engineered tissues with enhanced regenerative properties.

Key words Decellularization, Recellularization, Cardiomyocytes, Hepatocytes, Engineer slice, Patch, Transplantation

1. Introduction

A remarkable clinical need exists for the development of various methods to facilitate the regeneration of injured or diseased tissues and organs. The recent developments in stem cell biology, molecular interventions, biopolymers, and other related biological and engineering disciplines have paved the way to the emerging research and clinical discipline of "Regenerative Medicine". Regenerative medicine seeks to harness methods for the replacement or repair of dysfunctional cells, tissues, or organs, in an attempt to restore normal function [1].

As part of the emerging regenerative medicine discipline [2]., tissue engineering is a multidisciplinary field that combines functional cells with three-dimensional scaffolds (made from synthetic or biological polymers) to create tissue substitutes. The use of cell-seeded scaffolds to promote tissue development is the hallmark of a tissue engineering strategy. The scaffold serves many purposes, including the delivery of biological signals to control and enhance tissue formation, to provide adequate biomechanical support for the cell graft, to control graft shape and size, to promote angiogenesis, and to protect the cells from physical damage.

Among the different tissue engineering strategies, the technique of whole-organ decellularization and recellularization has attracted increasing attention in the last decade [3]. When a decellularized extracellular matrix (ECM) is derived from an organ, it provides a unique non-cytotoxic three-dimensional scaffold that allows cell adhesion and proliferation [4, 5]. Cell removal techniques include utilization of mechanical, thermal, chemical and enzymatic methods that simultaneously with cell detachment are preserving the micro- and macro- anatomy of the ECM [6]. The patient-like organ shape is exploited and the goal is to construct a personalized neo-organ with a low potential for immunological rejection after transplantation [7].

Historically, one of the limitations of tissue engineering was the paucity of human cell sources for creation of the tissue-constructs, and specifically the ability to derive patient-specific cells. The advent of human embryonic stem cells and more recently of the human induced pluripotent stem cells (hiPSC) technology may provide a solution to this cell-sourcing problem.

The latter technology allows to reprogram patient-specific somatic cells into hiPSC, which can then be coaxed to differentiate into any cell type. The desired cells can then be used for generation of functional tissue/organ like structures for *in vitro* and *in vivo* applications. Consequentially, hiPSC-derived cardiomyocytes and hepatocytes were combined with a variety of scaffolding polymer to generate functional liver and cardiac tissues using a variety of tissue engineering strategies [8-10]. Here, we describe two efficient methods for decellularization/recellularization to generate and utilize liver and heart tissue-constructs that resemble the 3D structural and functional characteristic of the native organ (Fig.1-2). In addition to the detailed description of the methods used to generate such tissues, we provide two examples for each organ type in which the engineered tissues can be used as 'models in a dish' or for regenerative medicine related in vivo transplantation projects.

In general, our approach can be used to generate relatively thin tissues termed engineered heart slices (EHSs) and engineered livers slices (ELSs) that are more suitable for *in vitro* modeling studies as well as very thick tissue patches that may be more suitable for *in vivo* transplantation applications (Fig.3). We next describe two approaches that can be used to enhance the regenerative capacity of the tissue patches. These include supplementing the liver and heart tissue-constructs with chemical agents or extracellular vesicles (EVs) respectively. The first strategy includes the use of new culturing protocols to maintain hepatocytes viability and vitality for extensive time periods. To this end, different compound solutions are added to the culture medium, which demonstrate stimulatory effects and increase hepatic progenitor cells (HPC) replication capabilities [11]. For the liver tissue-constructs we report on the use of cocktail medium, which is based on branched chain amino acids, that seems to support the hepatocytes/liver organoid functionality *in vitro* (Fig 2c, d).

For the cardiac patch model, in order to improve graft survival following tissue transplantation, we enrich the scafold's ECM with EVs to utilize their potential anti-apoptotic, anti-fibrotic and pro-angiogenic properties (Fig. 4). Extra-cellular vesicles (EVs) is a collective term for vesicles secreted or shed by cells, which are formed from the outer phospholipid bilayer and contain the specific cells' cytoplasmatic contents [12]. All examined prokaryotic and eukaryotic cells

release EVs [13,14] and they can be found in surrounding media in cell culture, or in body fluids such as blood [13]. EVs can carry different molecules in their payload: proteins, lipids, DNA, RNAs – most notably micro RNAs – and more [13,15]. The process of vesicle formation and its cargo content is rather selective than passive [14]. Hence, It has been suggested that EVs can mediate inter-cellular communication and serve as potential theraupetic agents [13]. Recent Studies demonstrated the therapeutic effect of EVs from different cell sources in an animal model of myocardial infarction [16]. Furthermore, the feasibility of vesicular entrapment in and slow release from engineered tissues was also demonstrated [17]. This may serve as a method to enrich engineered tissues, creating a fertile ground for recellularization. EVs are poorly immunogenic, a feature that may facilitate their use in regenerative therapies [18].

2. MATERIALS

2.1 Liver Decellularization

2.1.1 Tissue retrieval

1. Ice container
2. Chlorhexidine skin disinfectant
3. Surgical gaze
4. Surgical blade handle # 3
5. Surgical blades #15
6. Metzenbaum scissor
7. Surgical tweezers/ Watchmaker’s forceps
8. 100 mm Petri dish

2.1.2 ELS

1. Optimal cutting temperature compound (OCT)

2. Cryomolds
3. Cryostat
4. 6 wells dish for cells culture
5. Distilled water (DW)
6. 1% sodium dodecyl sulfate (SDS) solution in DW
7. 1% Triton X-100 solution in DW
8. Dulbecco's Phosphate Buffer Saline solution (PBS)
9. Orbital shaker
10. Sterilized glass coverslip 12 mm
11. 70% ethanol
12. 1% penicillin/streptomycin solution (P/S)

2.1.3 Liver patch

1. Watchmaker's forceps
2. - 80°C freezer
3. 0.05% ethylenediaminetetraacetic acid (EDTA) solution in DW
4. 0.1 % ammonium hydroxide in DW
5. 3% Triton X-100 in DW
6. PBS
7. 1% P/S

2.2 Liver Recellularization

2.2.1 ELS

1. Surgical blade # 15
2. Trypsin-EDTA solution
3. 40 μm cell strainer

4. Centrifuge
5. Pipette single channel manual adjustable 1000 μL
6. Sterile tips for Pipette single channel manual adjustable 1000 μL
7. Culture medium for the liver organoid (CMLO): Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/12) supplemented with 20% fetal bovine serum (FBS), 2% Antibiotic Antymicotic Solution, 2mM L-Glutamine solution, 87.4 μg/ml Hepatic Growth Factor.
8. Albumin (ALB) ELISA kit.
9. Factor 7 (FVII) ELISA kit.

2.2.2 Liver patch

1. 70% ethanol
2. PBS
3. Pipette single channel manual adjustable 200 μL
4. Sterile tips for Pipette single channel manual adjustable 200 μL

2.2.3 Liver enriched medium

1. Branched Chain Amino Acid (BCAA +) medium: CMLO supplemented with 25 mM Leucine, 12.5 mM Isoleucine, 12.5 mM Valine.

2.3 Heart Decellularization

2.3.1 Tissue retrieval

1. Chlorhexidine skin disinfectant
2. Surgical gaze
3. Surgical blade handle # 3
4. Surgical blades #15

5. Metzenbaum scissor
6. Surgical tweezers/ Watchmaker's forceps
7. 100 mm Petri dish

2.3.2 EHS

1. Optimal cutting temperature compound (OCT)
2. Cryomolds
3. Cryostat
4. 6 wells dish for cells culture
5. Distilled water (DW)
6. 1% sodium dodecyl sulfate (SDS) solution in DW
7. 1% Triton X-100 solution in DW
8. Dulbecco's Phosphate Buffer Saline solution (PBS)
9. Orbital shaker
10. Sterilized glass coverslip 5 mm
11. 70% ethanol
12. 1% penicillin/streptomycin solution (P/S)

2.3.3 Cardiac patch

1. Potts scissors
2. - 80°C freezer
3. 0.05% ethylenediaminetetraacetic acid (EDTA) solution in DW
4. 0.1 % ammonium hydroxide in DW
5. 3% Triton X-100 in DW
6. PBS
7. 1% P/S

8. Rotamix RM 1

2.4 Heart Recellularization

2.4.1 EHS

1. TrypLE Express solution
2. DNase I, Bovine pancreas 10 mg/ml in water.
3. Pipette single channel manual adjustable 1000 μL
4. Sterile tips for Pipette single channel manual adjustable 1000 μL
5. 15 ml conical tube
6. RPMI 1640 Medium
7. B27 supplement minus insulin
8. 2μM Thiazovivin

2.4.2 Cardiac patch

1. 70% ethanol
2. PBS
3. Sylgard® 184 Silicone Elastomer kit
4. Stainless minutien pins 10 mm base x tip 0
5. RPMI 1640 Medium
6. B27 supplement

2.4.3 EVs enrichment of the cardiac patch

1. Centrifuge that can reach 100,000 g at 4°C.
2. Filtered PBS (0.022 μm)
3. Nanoparticles tracking analysis system.
4. 30G neadle

5. 100 μL syringe

2.5 In vivo transplantation

2.5.1 Liver patch transplantation

1. Cyclosporine
2. Methylprednisolone
3. Buprenorphine
4. Carprofen
5. Isoflurane
6. Isoflurane vaporizer
7. Piperacillin/tazobactam
8. 0.9% Saline Solution
9. 21 G needle
10. 10 mL syringe
11. Chlorhexidine skin disinfectant
12. Surgical gaze
13. Surgical blade handle # 3
14. Surgical blades #15
15. Metzenbaum scissor
16. Surgical tweezers/ Watchmaker's forceps
17. PROLENE® 7-0 suture
18. Nylon 4-0 suture.

2.5.2 Cardiac patch engraftment

1. Cyclosporine
2. Methylprednisolone

3. Buprenorphine
4. Carprofen
5. Isoflurane
6. Isoflurane vaporizer
7. Piperacillin/tazobactam
8. 0.9% Saline Solution
9. 21 G needle
10. 10 mL syringe
11. Chlorhexidine skin disinfectant
12. Surgical gaze
13. Surgical blade handle # 3
14. Surgical blades #15
15. Metzenbaum scissor
16. Surgical tweezers/ Watchmaker's forceps
17. Electric shaver
18. 18 G intravenous cannula
19. Rodent ventilator
20. Finocchietto rib spreader
21. PROLENE® 8-0 suture
22. PROLENE® 3-0 suture
23. Nylon 5-0 suture

2. METHODS

All procedures were carried out according to the 3R rules of animal experimentation.

2.1 Liver Decellularization

3.1.1Tissue retrieval

1. Collect adult female New England white rabbit 3 kg not eviscerated carcasses from a local slaughterhouse, carrying it with a certified ice container.
2. Before the incision, treat the abdominal wall muscles with topical chlorhexidine skin disinfectant alternating with chlorhexidine-soaked gauze and dry gauze.
3. Perform a ventral midline laparotomy extending from the pubis to the xyphoid combined with a rooftop incision using the #3 handle with #15 blade.
4. Dissect the falciform ligament with a Metzenbaum scissor. Use a wet gauze bandage to hold the medial and left liver lobes cranially under the dome of the diaphragm.
5. Cut the bile duct 1.5 cm from the bile duct bifurcation.
6. Dissect the portal vein and hepatic artery from the surrounding tissue.
7. Transect the infrahepatic inferior vena cava.
8. Free the suprahepatic inferior vena cava.
9. Transfer the whole liver to a sterile 100 mm Petri dish.

3.1.2 ELS

1. Select and cut a proper area from the liver parenchyma (*see* Note 1).
2. Place few drops of OCT onto the center of the bottom of cryomold. Be careful to select the proper size embedding mold according to the size of the tissues to be embedded.
3. Place the unfrozen tissue sample in the drops and oriented. Make sure that the side touching the bottom of the cryomold is the side you want sectioned first. Gently push the tissue with a forceps to ensure that the bottom surface of the tissue is placed properly, level with the container, touching the face of the bottom and the tissue is located in the center of the mold.

4. Carefully drop more OCT onto the specimen until it is completely covered. None of the tissue should remain exposed (*see* Note 2).
5. Let it settle for 15-30 seconds to allow the OCT to completely wet the surface of the tissue.
6. Place cryomold with OCT covered sample in it in the vapor phase right next to the liquid nitrogen with the flat side down using a long forceps.
7. After hardening of the OCT compound (it will happen in 0.5-1 minute), store the samples at -80°C (months) or directly transter it to the cryostat stage for cutting.
8. Slice the speciment with the cryostat ensuring that all the tissue is present and intact (*see* Note 3).
9. Transfer the slices in a 6 well dish filled with 2 ml DW for each well and let the OCT dissolve completely after that wash two times with DW.
10. Then treated with 1 mL of each of the following solutions (diluted in DW) while being rotated at 150 rpm on an orbital shaker: 2 washes of 1% SDS for 2 h each, 1 wash of DW for 15 min, 1 wash of 1% Triton X-100 for 10 min, and 3 washes of PBS for 15 min each.
11. Leave the decellularized slices in PBS to rotate 80 rpm on an orbital shaker overnight (*see* Note 4).
12. Sterilized glass coverslips 12 mm in diameter sterilized by rinsing in 70% ethanol and exposing to UV for 10 min.
13. Spread the ELS on the coverslips with the outer perimeter of the slice hooked around the edges of the coverslip and place them in a standard 24-well.
14. If required, the decellularized tissue could be store at 4°C in PBS containing 1% P/S.

3.1.3 Liver patch

1. Section a liver lobe with a scalpel and obtain a small tile with a volume of 125 cm^3 (*see* Note 5).

2. Gently grab with a micro dissecting Watchmaker's forceps the Glissonian capsule and detach it from the parenchymatous portion of the liver.
3. Store the tile at -80°C for at least 24h.
4. Thaw the liver specimen at room temperature (RT) avoiding any drying of the tissue.
5. Then start the chemical decellularization phase in the following succession of solution where the tissue is always place in a 50 ml tube on a laboratory shake: 0.05% EDTA in DW (24 hours); 1% SDS/0.05% EDTA in DW (24 hours); 0.1% ammonium hydroxide in DW (24 hours); 3 % Triton-X/0.05% EDTA in DW (24 hours); DW (24 hours); DW two time for 30 minutes; PBS overnight (*see* Note 6).
6. If required, the decellularized tissue could be store at 4°C in PBS containing 1% penicillin/streptomicin.

2.2 Liver Recellularization

Authors describe the recellularization process for the liver with a primary cells line not purified as a rabbit cells allotransplantation.

2.2.1 ELS

1. Establish primary livers cells co-culture from fresh biopsies.
2. Cut liver fragments of approximately 200 mm^3 wiht a surgical blade.
3. Incubate the liver slivers in 30 mL of Trypsin-EDTA solution for 30 min at 37°C with gentle shaking.
4. Filtered the digested tissue with 40 µm cell strainer and collect the cells throught centrifugation at 150g for 5 minuntes.
5. Removed the supernatant and add the CMLO medium according to the size of the pellet (*see* Note 7).
6. Create the ELS slices plating at a density of 0.5-1 million cells/cm^2.

7. Add the specific culture medium.
8. Let the cells and medium solution dry/attach to the matrix for at least 6 hours.
9. ELS were maintained in culture for at least 7 days before evaluation by ALB and factor 7 FVII production as markers of liver organoid fuctionality.

3.2.2 Liver patch

1. Before any manipulation for cells seeding, sterilized the patch ECM in 70% EtOH for 30 min than wash it in PBS for 30 min and finally under UV for 10 min in a biosafety cabinet.
2. Put the liver ECM in a 35 mm Petri dish let it dry for 2-5 minutes (*see* Note 8).
3. Follow the step number 1-5 in the subchapter 3.2.1.
4. After collecting the cells pellet, plate $1x10^6$ cells directly onto the decellularized bioscaffold surface as a single drop (100-150 µL).
5. Let the cells and medium solution dry/attach to the matrix for at least 6 hours.
6. Add slowly the culture medium avoiding cells washing.
7. Change the medium every three days.

3.2.3 Liver enriched medium

1. Add to the ELS or liver patch after the 6 hours incubation with the isolated cells the BCCA+ medium.
2. Starve the ELS or liver patch for 12 hours in PBS and measure in it the ALB and FVII productions.

3.3 Heart Decellularization

3.3.3 Tissue retrieval

1. Lift the skin away from the abdominal cavity with forceps and then use scissors to incise the peritoneal cavity, following the curve of the diaphragm back to the posterior angle of the ribs.
2. Once the diaphragm is visible, using small scissors, cut along the anterior surface of the diaphragm following the direction of the prior cuts to allow for entry into the thorax. Extend each cut along the axillary line bilaterally to the axilla.
3. Retract the ribcage anteriorly from the xiphoid process using forceps. Incise the pericardium and pleura.
4. Identify the inferior vena cava (IVC) and aorta just above the diaphragm and retract them *en bloc* anteriorly using blunt forceps.
5. Using large, curved scissors rapidly make an incision across the IVC and the aorta, pulling the heart and lungs out of the chest *en bloc*. Cut the esophagus, trachea, brachiocephalic arteries and veins cephalad to remove the heart and lungs from the thorax. Excise the thymic tissue with this block of tissue.

3.3.4 EHS

1. Select and cut a proper area from the heart.
2. Follow the same steps reported in the subchapter 3.1.2, from point 2 to 13.

3.3.5 Cardiac patch

1. Isolate the ventricles from the atria trough a transversal section at the level of the heart base, then divide the left and right ventricles and keep the septum with the right portion.
2. Store the heart tissue for at least 24 hours in a - 80°C freezer.
3. Thaw the heart tissue at RT avoind an excessive drying (*see* Note 9).
4. Wash 3 times 15 min each the ventricles in a 50 ml tube in DW with a laboratory shaker (*see* Note 10).

5. Then start the chemical decellularization phase in the following succession of solution where the tissue is always place in a 50 ml tube on a laboratory shake: 0.05% ethylenediaminetetraacetic acid (EDTA) in DW (24 hours); 1% sodium dodecyl sulfate (SDS)/0.05% EDTA in DW (24 hours); 0.1% ammonium hydroxide in DW (24 hours); 3 % Triton-X/0.05% EDTA in DW (24 hours); DW (24 hours); DW two time for 30 minutes; PBS overnight (*see* Note 11).
6. If required, the decellularized tissue could be store at 4°C in PBS containing 1% penicillin/streptomicin.

3.4 Heart Recellularization

For the heart recellularization we used human induce pluripotent stem cells derived cardiomyocytes (hiPSCs-CM) differentiated using previously described protocols (*see* Note 12) [19].

3.4.3 EHS

1. On the day 10-12 of differentiation, dissociate the hiPSC-CM when the confluence and amount of hiPSC-CM positively beating reach 70% and 90% respectively.
2. Aspirate medium from the culture-well, and wash cell monolayer with 2 mL of PBS. Aspirate PBS and replace with 1 ml of TrypLE supplemented with 1µl/ml DNase (1:1000 dilution). Ensure complete coverage of cell monolayer with TrypLE.
3. Place plate at 37ºC incubator for 3-10 min (*see* Note 13).
4. Triturate the cells with a P1000 tip to dislodge them from the plate.
5. Add the cells with the TrypLE to a 15-mL conical tube with 5 mL pre-warmed complete medium for well (at least 1:2 dilution) supplemented with 1µl/ml DNase.
6. Centrifuge the cells at 1100 rpm (150 *x g*) for 5 min.

7. Discard supernatant and resuspend cell pellet in 60 µl volume of RPMI/B27 medium, supplemented with 2 µM Thiazovivin (1:1000 dilution).
8. Plated on EHS slices at a density of 2 million cells/cm2.
9. EHS were maintained in culture for 7-200 days before evaluation by optical mapping or contraction measurements.

2.4.2 Cardiac patch

1. Before any manipulation for cells seeding, sterilized the patch ECM in 70% EtOH for 30 min than wash it in PBS for 30 min and finally under UV for 10 min in a biosafety cabinet.
2. Localize the ventricle patch in a glass dish coated with silicone (*see* Note 14) and solidarized the ECM to the bottom with sterilizable thin pins (*see* Note 15).
3. Add the specific RPMI/B27 medium to the plate and let the patch absorb part of it in the incubator (T 37°C, 5% CO_2) overnight.
4. Add to the plate the iPS-CM previously collected as cells aggregates and keep it in the incubator (T 37°C, 5% CO_2) overnight (*see* Note 16).
5. Carefully and slowly, fulfill the plate with the RPMI/B27 plus insulin medium and keep the patch in the incubator (T 37°C, 5% CO_2).
6. Change the medium every other day, according to the phenol red color deviation.

2.4.3 Patch EVs enriched

1. Stimulate EVs production in cultured cells with a conditional medium.
2. Collect cells medium after two days of culture and centrifuge in 2,000 g for 10 minutes at room temperature to get rid of cell remnants and debris.
3. Collect supernatant, centrifuge in 100,000 g for 70 minutes at 4°C.
4. Get rid of supernatant, wash with filtered PBS and centrifuge in 100,000 g for 70 minutes at 4°C.

5. Get rid of supernatant, collect the pellet of EVs with the desired volume of filtered PBS.
6. Use freeze or screen sample with nanoparticles tracking analysis system to determine vesicle concentration.
7. Inject using 30 gauge needle into decellularized patch transplanted to the heart (*see* Note 17).

3.5 Transplantation

Animals received humane care in compliance with the Guide for the Principles of Laboratory Animals. The responsible local authority approved all animal protocols.

1. The days before the operation inject sub-cutaneous 15 mg/kg Ciclosporin and 4 mg/Kg Methylprednisolone. Then every 24 hours 15 mg/kg Ciclosporin and 2 mg/Kg Methylprednisolone.
2. Anesthetize the New Zealand White Rabbit weighing 4-4,5 Kg with isoflurane on cone mask (3% for the induction, 2% during the operation, 1 l/min air flow, FiO2 70%) and keep on a warm pad.
3. Inject 10 ml of 0.9% saline solution subcutaneously and 0.6 g of piperacillin/tazobactam intramuscular before laparotomy with a 21 G needle and a 10 mL syringe after induction of anesthesia.
4. Perform a midline xipho-pubic laparotomy.
5. Reverse the xiphoid process with an auto-static forceps in order to better expose the liver.
6. Dislocate the left liver lobe and surrounding it with a wet surgical gauze.
7. Remove a segment from the left lateral lobe of the normal liver using a surgical trocal with inner diameter 1 cm.
8. Transplant the recellularized liver patch in the site of the detached part using PROLENE® 7-0 suture.

9. Close the animal by layers with Nylon 4-0 sutures. Allow free water and food from wakening.
10. Administer effective analgesia according to local institutional protocols.

3.5.2 Cardiac patch transplantation

1. The day before the operation inject sub-cutaneous 15 mg/kg Ciclosporin and 4 mg/Kg Methylprednisolone. Then every 24 hours 15 mg/kg Ciclosporin and 2 mg/Kg Methylprednisolone.
2. Place the Wistar rat weighing 300-400 g in an induction chamber and anesthetize the animal with isoflurane check the depth of anesthesia by the lack of response to the toe-pinch.
3. Inject 0.05 mg/kg buprenorphine subcutaneously with a 27 G needle and a 10 mL syringe after induction of anesthesia.
4. Inject 10 ml of 0.9% saline solution subcutaneously and 0.03 g of piperacillin/tazobactam intramuscular before laparotomy with a 21 G needle and a 10 mL syringe after induction of anesthesia.
5. Place the rat on its back and keep anesthesia with a facemask covering mouth and nose.
6. Spread the rat's legs and fix the position using tape.
7. Shave the chest of the anesthetized animal with an electric shaver. Disinfect the area widely using iodine-based scrub, followed by 70% ethanol. Repeat this disinfection steps three times.
8. Perform an intubation with 18 G intravenous cannula and insert the flexible part of the cannula as a tracheal tube.
9. Connect the tracheal tube to an animal respirator to continuously ventilate the rat during the procedure.
10. Perform a 2 cm horizontal skin incision in the scar area of the left lateral side using scissors and tweezers.

11. Carefully open the pleural space with scissors. Insert a rib spreader to expose the heart.
12. Visually identify the region of interest where the heart patch will be suture over.
13. Secure it with four PROLENE® 8-0 sutures at all sides (ventral, dorsal, cranial and caudal). Inflate the lungs with pressure, to avoid atelectasis of the lung. Remove the retractor from the intercostal space.
14. Close the ribs with two 3-0 sutures. Close the muscles over the ribs with a 4-0 running suture. For closure of the skin use 5-0 suture single stitches.
15. Reduce the isoflurane to 1%. When the animal is breathing spontaneously, remove the tracheal tube, and continue to administer 100% O_2.
16. Use buprenorphine (0.05 mg/kg per 12 h) for pain medication for the following 5 days.
17. For Evs injection is preferable to use a Hamilton's needle 30G where a proper low volume

Notes:

1. For the ELS creation, the cryostat sectioning will control the thickness. The area and extension of the specimen is chose according by the type of the experiment and the size of the cryomold.
2. Try to avoid the formation of air bubbles. Remove any bubbles inside the OCT. This is important because the air bubbles will create problems when cutting sections.
3. A 100 µm thickess is suggested to obtain a durable and flexible ECM (*see* Note1).
4. If the ELS (or EHS) are store for up to two moth place the slides in a 1% penicillin/streptomycin and PBS solution.
5. To obtain the tile also a microtome blade or a hollow hole square punch 5mm cutter steel tool leather belt watch band could be utilized.
6. To prepare the decellularization solutions mix with a magnetic stirrer at RT all the detergent in DW and The laboratory shaker used by the authors is Rotamix RM 1, ELMI, and the set up suggested is 50 RPM function #5.

7. Consider the proper volume of seeding, for one ELS with 0.5-1 x 10^6 cells/cm^2 not exceed 150 µL of culture medium for the liver organoid.
8. This step could be critical, pay attention not to dry to much the tissue. Using a plastic culture dishes with any coating and let the bottom of the scaffold dry for few minutes will help the patch to adhere to the plate.
9. Check the tissue every 15 minute to be sure that the thawing phase is not too long.
10. The laboratory shaker used by the authors is Rotamix RM 1, ELMI, and the set up suggested is 50 RPM #five.
11. To prepare the decellularization solutions mix with a magnetic stirrer at RT all the detergent in DW.
12. In the present manuscript, the procedure to proliferate and differentiate the hiPS-CM lines are not describe because is not concerning the aim of the methodology paper.
13. Depending on the age of the cell culture.
14. Glass Petri dishes 60mm x 20mm.
15. Stainless steel minutien pins 10 mm base x tip 0.
16. Each cell aggregates could be formed by 0.5-3 x 10^6 cells. At this step the cells could be added directly in the empty medium plate or proceed in a double phase seeding. In the former case, the aggregates are lain in the plate medium, push them on the top of the matrix and then the medium is gently sucked from the plate.

References

1. Terzic A, Pfenning MA, Gores GJ, et al (2015) Regenerative Medicine Build-Out. Stem Cells Transl Med. 4(12):1373-9.
2. Ginsburg GS, Phillips KA (2018) Precision Medicine: From Science To Value. Health Aff (Millwood) 37(5):694-701.

3. Gilbert TW, Sellaro TL, Badylak SF (2006) Decellularization of tissues and organs. Biomaterials 27(19):3675-83.
4. Tong C, Li C, Xie B, et al (2019) Generation of bioartificial hearts using decellularized scaffolds and mixed cells. Biomed Eng Online 4;18(1):71.
5. Modulevsky DJ, Lefebvre C, Haase K, et al (2014) Apple derived cellulose scaffolds for 3D mammalian cell culture. PLoS One 9(5):e97835.
6. Hillebrandt KH, Everwien H, Haep N, et al (2019) Strategies based on organ decellularization and recellularization. Transpl Int. 32(6):571-585.
7. Moroni F, Mirabella T (2014) Decellularized matrices for cardiovascular tissue engineering. Am J Stem Cells. 3(1):1-20.
8. Lu TY, Lin B, Kim J, et at (2013) Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells. Nat Commun. 4:2307.
9. Palakkan AA, Nanda J, Ross JA (2017) Pluripotent stem cells to hepatocytes, the journey so far. Biomed Rep. 6(4):367-373.
10. Jaramillo M, Yeh H, Yarmush ML, et al (2018) Decellularized human liver extracellular matrix (hDLM)-mediated hepatic differentiation of human induced pluripotent stem cells (hIPSCs). J Tissue Eng Regen Med. 12(4):e1962-e1973.
11. Chen L, Zhang J, Yang L, et al (2018) The Effects of Conditioned Medium Derived from Mesenchymal Stem Cells Cocultured with Hepatocytes on Damaged Hepatocytes and Acute Liver Failure in Rats. Stem Cells Int. 2018:9156560.
12. van der Pol E, Coumans F, Varga Z, et al (2013) Innovation in detection of microparticles and exosomes. J. Thromb. Haemost. 11(1):36–45.
13. Ibrahim A, Marb E, Marbán E (2016) Exosomes : Fundamental Biology and Roles in Cardiovascular Physiology. Annu. Rev. Physiol. 78:1–17.
14. Fleury A, Martinez MC, Le Lay S (2014) Extracellular vesicles as therapeutic tools in cardiovascular diseases. Front. Immunol. 5:1–8.

15. Chistiakov DA, Orekhov AN, Bobryshevy YV (2016) Cardiac extracellular vesicles in normal and infarcted heart. Int. J. Mol. Sci. 17(1):1–18.
16. El Harane N, Kervadec A, Bellamy V, et al (2018) Acellular therapeutic approach for heart failure: in vitro production of extracellular vesicles from human cardiovascular progenitors. Eur Heart J. 39(20):1835-1847.
17. Liu B, Lee BW, Nakanishi K, et al. (2018) Cardiac recovery via extended cell-free delivery of extracellular vesicles secreted by cardiomyocytes derived from induced pluripotent stem cells. Nat Biomed Eng. 2(5):293-303.
18. Burridge PW, Matsa E, Shukla P et al (2014) Chemically defined generation of human cardiomyocytes. Nat Methods. 11(8):855-60.

Figure Captions

Fig. 1 Process of organ decellularized scaffolds creation. [a] Liver tile before and after decellularization. **[b]** Ventricle ECM patch obtained from the left wall of the rat heart. Note that the natural "V" shape of the ventricle is maintained. **[C]** EHS derived from the original heart slice. Note that the tissue before decellularization shows the typical myocardial morphological micro-architecture. **[d]** Engineered slices are hooked to the 12mm glass cover-slip to allow fixation of the tissue at the bottom of the well and also to gently stretch the collagen fibers. **e** The decellularization process allows to keep the vascular tree of the original organ intact.

Fig. 2 Scaffold recellularization and tissue characterization. [A] Hematoxylin and Eosin (H&E) staining of the liver patch. **[b]** Immunohistochemical staining of the hepatic tile for albumin (ALB) in order to characterize the seeded cell population. **[c-d]** Tissue culturing of the liver patch with the addition of BCAAs (BCAA+) significantly decreases the release of the ALB and factor VII (FVII) proteins compared to the standard medium (BCAA-) at 14 and 21 day of culture. **[e-f]** Representative confocal images depicting the cardiomyocytes cultured within the EHS, which were immunostained for human α-actinin (red) human troponin I (TnI. orange) and DAPI (blue nuclei). **[g]** Morphology of the hiPSC-CMs cultured as monolayer as controls. Notice the different morphology of these cardiomyocytes (round) compared to the cells cultured within the scaffold that are markedly more elongated.

Fig. 3 Transplantation of the cardiac patch. [a] Representative photograph of an implanted one week old cardiopatch (arrow) sutured on the anterior ventricle wall of the rat heart. **[b]** Masson's trichrome staining of the transplanted recellularized patch after 4 weeks. This image focuses on the border between the patch and the heart where neovascularization (arrows) and rat cardiomyocytes' invasion (arrow heads) can be appreciated. **[c]** Masson's trichrome staining focusing on the center

of the patch where relatively mature hiPSC-CMs (arrows) and blood vessels (arrow heads) are visible. **[d]** Immunohistochemical staining of the patch for human cardiac troponin I (TnI), positive cells are spotted (arrows).

Fig. 4 EVs isolation and supplementation to the cardiac patch. [a] Scheme describing the process used to produce and isolate EVs. **[b]** SEM images of the ECM-EVs enriched cardiac patch where the EVs (arrows) can be identified as the spherical heterogeneous structures on the ECM surface.