

## Video Article

# Isolation of Rat Portal Fibroblasts by *In situ* Liver Perfusion

Jessica W. Wen<sup>1</sup>, Abby L. Olsen<sup>2</sup>, Maryna Perepelyuk<sup>2</sup>, Rebecca G. Wells<sup>2</sup><sup>1</sup>Division of Gastroenterology, Hepatology & Nutrition, Department of Pediatrics, The Children's Hospital of Philadelphia<sup>2</sup>Department of Medicine, University of PennsylvaniaCorrespondence to: Rebecca G. Wells at [rgwells@mail.med.upenn.edu](mailto:rgwells@mail.med.upenn.edu)URL: <http://www.jove.com/video/3669>DOI: [doi:10.3791/3669](https://doi.org/10.3791/3669)

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## Abstract

Liver fibrosis is defined by the excessive deposition of extracellular matrix by activated myofibroblasts. There are multiple precursors of hepatic myofibroblasts, including hepatic stellate cells, portal fibroblasts and bone marrow derived fibroblasts<sup>1</sup>. Hepatic stellate cells have been the best studied, but portal fibroblasts are increasingly recognized as important contributors to the myofibroblast pool, particularly in biliary fibrosis<sup>2</sup>. Portal fibroblasts undergo proliferation in response to biliary epithelial injury, potentially playing a key role in the early stages of biliary scarring<sup>3-5</sup>. A method of isolating portal fibroblasts would allow *in vitro* study of this cell population and lead to greater understanding of the role portal fibroblasts play in biliary fibrosis.

Portal fibroblasts have been isolated using various techniques including outgrowth<sup>6,7</sup> and liver perfusion with enzymatic digestion followed by size selection<sup>8</sup>. The advantage of the digestion and size selection technique compared to the outgrowth technique is that cells can be studied without the necessity of passage in culture. Here, we describe a modified version of the original technique described by Kruglov and Dranoff<sup>8</sup> for isolation of portal fibroblasts from rat liver that results in a relatively pure population of primary cells.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/3669/>

## Protocol

The entire procedure is carried out at room temperature unless otherwise specified.

### 1. Preparation of Enzyme Solutions

1. Prepare enzyme solutions according to **Table 1** and sterile filter using a 0.22 µm filter. Keep solution 1 and solution 2 at room temperature and solution 3 at 4 °C until time of use. All solutions and equipment coming into contact with isolated cells must be sterile.

### 2. Preparation of Perfusion Equipment

1. Prime the perfusion system with HBSS minus Ca<sup>2+</sup>/Mg<sup>2+</sup>, which has been warmed to 37 °C. Note: To ensure that the perfusate is 37 °C when it arrives at the liver, HBSS is warmed in a water bath set to 37 °C. After it passes through the perfusion pump, it goes through a jacketed glass condenser that is connected to a water circulator set to 40 °C (**Fig. 1**).
2. Sterilize surgical tools in a beaker with 70% ethanol.

### 3. Perfusion of the Liver

1. Anesthetize an adult male Sprague-Dawley rat by intraperitoneal injection of Nembutal (50-100 mg/kg body weight as necessary to obtain adequate anesthesia).
2. Place the animal in a supine position on a plastic tray and fix the limbs to the tray with tape.
3. Clean the abdomen with 70% EtOH.
4. Make a small incision in the skin on the abdomen in the midline. Dissect the skin away from the fascia by making a large U-shaped cut on the abdomen. Cut the abdominal muscle with scissors following the same shape to expose the peritoneal cavity.
5. Expose the portal vein by using 2 cotton swabs to gently move the abdominal viscera to the right side.
6. Pass two sterile 2.0 silk sutures underneath the portal vein and tie loosely.
7. Cannulate the portal vein with a 16-18 gauge IV catheter and secure the catheter in place by tightening the loosely tied sutures from the previous step (**Fig. 2**).

8. Inject diluted Heparin through the IV catheter (1 ml of Heparin 5000 USP units/ml diluted with 4 ml of HBSS minus  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ). The liver should blanch.
9. Connect the IV catheter to the perfusion system. Perfuse with HBSS minus  $\text{Ca}^{2+}/\text{Mg}^{2+}$  at a rate of 20 ml/min for 10 minutes. Transect the IVC inferior to the liver to allow drainage of blood and perfusate. Aspirate pooled blood from the abdominal cavity.
10. Change the perfusion fluid to a 0.3% collagenase solution (150 mg of type 2 collagenase in 500 ml HBSS plus  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ) and perfuse for 25 minutes. Keep the liver moist by covering it with the previously dissected abdominal flap. Put a Petri dish cover over the abdominal flap and position a heat lamp over the area to maintain the intra-abdominal temperature at approximately 37 °C.

#### 4. Preparation of the Biliary Tree

1. Remove the liver by lifting it out from the abdominal cavity with forceps and place it in a sterile tissue culture dish filled with cold Leibovitz's L-15 media.
2. Working in the tissue culture hood, peel off the liver capsule and gently tease the liver parenchyma apart with forceps. Move the liver through several dishes filled with Liebovitz's L-15 media while continuing to tease away the parenchyma, until finally left with the isolated biliary tree. The liver parenchyma is tan colored, whereas the remaining biliary tree is white.
3. Place the isolated biliary tree into a 50 ml falcon tube filled with 10 ml of penicillin/streptomycin (10,000 IU/ml penicillin and 10,000 µg/ml streptomycin); leave on ice for 15 minutes.

#### 5. Isolation of the Portal Fibroblast Fraction

1. Place the biliary tree into a sterile 50 ml falcon tube and mince with sterile scissors.
2. Add a small amount of enzyme solution #1 into the tube (about 5 ml) and continue to mince the biliary tree until it reaches the consistency of a slurry.
3. Pour the slurry into a sterile glass bottle. Wash out falcon tube with the remaining solution #1, then pour it into the glass bottle. Incubate at 37 °C with shaking at 100 rpm for 30 minutes.
4. Filter the slurry through a beaker covered with a single layer of nylon mesh (30-micron pore size).
5. Transfer any tissue remaining on top of the mesh to a sterile glass bottle by washing the mesh with enzyme solution #2. Start by pouring half (25 ml) of the solution into a sterile 15 cm Petri dish. Carefully remove the mesh from the beaker by holding the edges of the mesh without contaminating the center area, then inverting the mesh and dipping it into the solution in the Petri dish to remove any remaining tissue on the mesh. Discard the mesh. Transfer the solution in the Petri dish into a sterile glass bottle. Rinse the Petri dish with the remaining 25 ml solution #2 and transfer it to the same glass bottle. Incubate at 37 °C with shaking at 100 rpm for 30 minutes.
6. In the meantime, take the filtrate in the beaker and pour into a 50 ml falcon tube. Centrifuge at 1600 rpm (460 x g) for 5 minutes at room temperature.
7. Aspirate media and resuspend the pellet in 25 ml of solution #3.
8. Take the solution from step 5.5 and filter it into a second sterile beaker covered with 30-micron mesh.
9. Discard the mesh. Centrifuge the filtrate at 1600 rpm for 5 minutes at room temperature. Aspirate and resuspend the pellet with 25 ml of solution #3 as was done in step 5.7.
10. Combine the 2 fractions from steps 5.7 and 5.9 and centrifuge again at 1600 rpm for 5 minutes at room temperature.
11. Aspirate media and resuspend the pellet in 10 ml of portal fibroblast culture media (DMEM/F12 supplemented with 10% FBS, 1% penicillin/streptomycin, 0.3% gentamycin, and 0.2% fungizone).
12. Count cells for viability and plate in tissue culture dishes. Depending on the desired application, 0.5 to 5 x 10<sup>6</sup> cells per 10-cm tissue culture plate are appropriate. From an adult Sprague-Dawley rat, we typically obtain 2 to 10 million viable cells.
13. Replace the culture media the next day to remove non-adherent debris. Thereafter, replace culture media every 2 days.

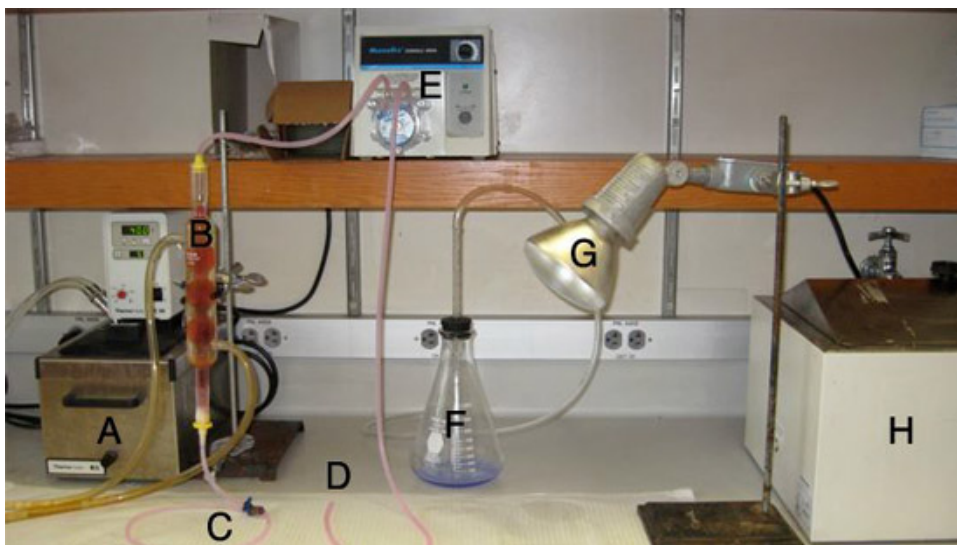
#### 6. Representative Results

Primary portal fibroblasts isolated from adult rat liver are shown at 1, 3 and 7 days after isolation (**Fig. 3**). Notice that the cells are elongated, with morphology typical of fibroblasts. Cells can be stained with anti-elastin antibody (CL55041AP, Cedarlane Labs, Burlington, NC) to confirm purity. Portal fibroblasts undergo myofibroblastic differentiation in culture. This can be demonstrated by immunostaining for α-smooth muscle actin (α-SMA; A2547, Sigma, St. Louis, MO).

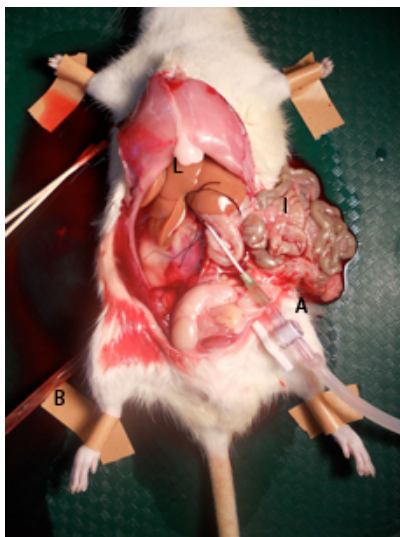
<b>0.3% collagenase solution</b>	Type 2 collagenase	150 mg
	HBSS with $\text{Ca}^{2+}/\text{Mg}^{2+}$	500 ml
<b>Solution #1 (Pronase)</b>	Bovine serum albumin	50 mg
	Collagenase type 2	25 mg
	Pronase	18 mg
	DNase	3 mg
	DMEM/F12	47.5 ml
	Pen/Strep	1 ml
	Fetal bovine serum	1.5 ml
<b>Solution #2</b>	Bovine serum albumin	50 mg
<b>(Hyaluronidase)</b>	Collagenase type 2	25 mg

	Hyaluronidase	22 mg
	DNase	3 mg
	DMEM/F12	47.5 ml
	Pen/Strep	1 ml
	Fetal bovine serum	1.5 ml
<b>Solution #3</b>	DNase	3 mg
	RPMI medium 1640	50 ml

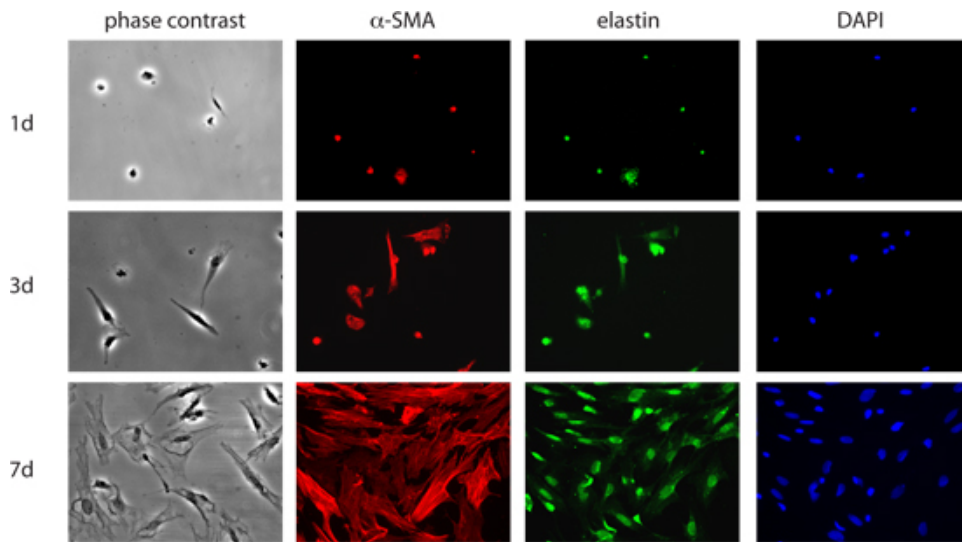
**Table 1.** Enzyme solutions.



**Figure 1.** Perfusion set up. A. Water circulator for warming column. B. Column containing perfusion media. C. Stopcock controlling pump outflow. D. Pump inflow. E. Variable-speed peristaltic pump. F. Vacuum flask with Bio-Clean detergent. G. Heat lamp. H. Water bath for warming perfusion media.



**Figure 2.** *In situ* perfusion. A. IV catheter inserted into the portal vein, secured with suture and connected to the perfusion tubing via stopcock. B. Glass pipette connected to vacuum flask and wall suction to remove perfusion fluid draining out of transected IVC. I. Intestines. L. Liver.



**Figure 3. Phase contrast and immunofluorescence stain of rat portal fibroblasts in culture.** Portal fibroblasts cultured for 1, 3, and 7 days after isolation were fixed and stained for elastin,  $\alpha$ -SMA and DAPI.

## Discussion

Portal fibroblasts play an important role in biliary fibrosis. Here we describe a modification of the original protocol published by Kruglov and Dranoff<sup>8</sup> for isolating portal fibroblasts from rat liver, providing a straightforward way to study this cell population *in vitro*.

This approach uses protease digestion and size-based filtration. The primary advantage of the method is that a relatively pure population of cells can be obtained without passage in culture, enabling the study of gene expression or functional cell behavior several days after isolation. This technique also offers the potential for isolating cells from both healthy and diseased livers.

One of the most critical steps in this protocol is the enzymatic digestion of the hepatic parenchyma. To ensure successful digestion, it is important to confirm that blood is completely drained out of the liver during the initial perfusion by making sure that there is even blanching of the liver. To facilitate this, it is possible to use a cotton swab to gently massage the liver while perfusing. Over-digestion of the liver parenchyma results in a low yield of viable cells, while under-digestion will make it difficult to separate the liver parenchyma from the biliary tree. Since preparations of pronase have variability in enzymatic activity between different lots, optimization of the amount used may be needed when there is low yield of viable cells.

This protocol may be modified for isolating portal fibroblasts from mouse liver or young rat liver by using a smaller gauge IV catheter to cannulate the portal vein, decreasing the volume of perfusion solutions in proportion to animal weight, and decreasing the liver perfusion rate to about 10 ml/min.

Portal fibroblasts in culture undergo myofibroblastic differentiation in 10-14 days, as evidenced by  $\alpha$ -SMA expression<sup>9</sup>. Various markers have been used to distinguish portal fibroblasts from hepatic stellate cells, including fibulin-2, IL-6, elastin, nucleoside triphosphate diphosphohydrolase-2 (NTPDase2), cofilin-1 and neuronal proteins such as synaptophysin<sup>2, 6, 10, 11</sup>. We have found that elastin is a good marker for portal fibroblasts, even after myofibroblastic differentiation, while NTPDase2 is lost after portal fibroblasts have undergone culture after several days<sup>9</sup>. Therefore, we typically confirm isolation of portal fibroblasts by immunofluorescence staining for elastin.

The limitation of this technique is that, immediately after portal fibroblast isolation, there is a small fraction of contaminating cells including Kupffer cells and biliary cells. Portal fibroblasts will outgrow these contaminating cells within 2-3 days, however, yielding a relatively pure population (>98%)<sup>9</sup>.

Since portal fibroblasts in culture undergo myofibroblastic differentiation on tissue culture plastic, we typically study these cells within 7 days of isolation. Cells are maintained in portal fibroblast culture media containing 10% fetal bovine serum, as described in the methods section. However, these cells will survive in growth media containing 2% fetal bovine serum for several days. We typically do not use low serum media until at least 24 hours after isolation. Once portal fibroblasts undergo myofibroblastic differentiation, they can be passaged several times and kept as frozen stocks of myofibroblasts.

## Disclosures

No conflicts of interest declared.

## Acknowledgements

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