THE PANCREAPEDIA
Exocrine Pancreas Knowledge Base
www.pancreapedia.org

METHODS PAGE

Isolation of Quiescent Pancreatic Stellate Cells from Rat and Human Pancreas

Minoti Apte
Pancreatic Research Group, South Western Sydney Clinical School
University of New South Wales, Australia
e-mail: m.apte@unsw.edu.au

Version 1.0, April 8, 2011 [DOI: 10.3998/panc.2011.10]

1. Introduction

Prominent fibrosis is a characteristic histological feature of two major diseases of the pancreas, chronic pancreatitis and pancreatic cancer (1). The study of pancreatic fibrogenesis had been a long neglected area in the field of pancreatology. However, this situation changed significantly just over a decade ago, when methods were described to successfully isolate and culture pancreatic stellate cell (PSCs) (2, 3), the cells that are now well established as playing a critical role in pancreatic fibrosis (4).

PSCs are resident cells of the pancreas, located close to the basolateral aspect of pancreatic acinar cells. They have a central cell body and long cytoplasmic projections that encircle the basal aspect of adjacent acinar cells. In health, the cells are in their quiescent phase, with abundant vitamin A containing lipid droplets in their cytoplasm. They play a role in normal extracellular matrix remodelling in the gland via the synthesis and secretion of matrix degrading enzymes and their inhibitors. During pancreatic injury, the cells are activated by several factors such as cytokines, growth factors and oxidant stress. Activated PSCs lose their cytoplasmic lipid droplets, transform into a myofibroblast-like phenotype, proliferate and synthesise excessive amounts of extracellular matrix proteins that comprise fibrous tissue.

The isolation of quiescent PSCs from rat pancreas, using a density gradient centrifugation method, was first developed by Apte and colleagues and reported in a paper published in Gut in 1998 (2). This was followed in the same year, by a publication by Bachem et al (3) describing the isolation of pre-activated human PSCs from resected pancreatic tissue, using the outgrowth method. More recently, Vonlaufen et al (5) have reported the isolation of quiescent human PSCs from resected normal human pancreatic tissue.

This Chapter will detail the methodology for isolation of quiescent pancreatic stellate cells from both rat and human pancreas.

2. Isolation and culture of rat pancreatic stellate cells

a) Reagents

1. Gey's Balanced Salt Solution

[GBSS; prepared with sodium chloride (GBSS + NaCl) and without sodium chloride (GBSS – NaCl) sodium chloride]

Magnesium chloride ($MgCl_2.6H_2O$)	0.21 g/l
Magnesium sulfate ($MgSO_4$; anhydrous)	0.0342 g/l
Potassium chloride (KCl)	0.37 g/l
Potassium dihydrogen phosphate (KH_2PO_4; anhydrous)	0.03 g/l
Sodium bicarbonate ($NaHCO_3$)	2.27 g/l
Sodium chloride (NaCl)	7 g/l
Sodium hydrogen phosphate (Na_2HPO_4; anhydrous)	0.112 g/l
Calcium chloride ($CaCl_2$)	0.225 g/l

Preparation of GBSS with and without salt

We usually make 500 mL fresh GBSS (with and without salt) at a time. The solutions are stable for up to two weeks.

Steps

a. Label 2 beakers: i) GBSS + SALT; ii) GBSS-SALT.
b. Weigh the following chemicals out on the analytical balance and add to beaker 1 and beaker 2 :

$MgCl_2 \cdot 6H_2O$	0.105 g
$MgSO_4$ (anhydrous)	0.0171 g
KCl	0.185 g
KH_2PO_4 (anhydrous)	0.015 g
Na_2HPO_4 (anhydrous)	0.0598 g
$NaHCO_3$	1.135 g
Glucose	0.5 g

c. Weigh out NaCl 3.5 g and ONLY add to beaker with GBSS + NaCl
d. Add 450 mL of MilliQ water into both beakers and stir (on a magnetic stirring plate) until all the salts dissolved.
e. Weigh out Calcium Chloride 0.1126 g and dissolve in 2 ml of Milli-Q water.
f. Add the calcium chloride solution *dropwise* into two beakers (keep on stirring) using a transfer pipette.
   Note: must add very slowly or $CaCl_2$ will precipitate.
g. Make up to 500mL with MilliQ water.
h. Filter to sterilize promptly.
i. Store at room temperature.

2. Enzyme solution in GBSS + NaCl

Collagenase P (Roche Diagnostics Cat # 11213873001)	1.3 mg/ml
Protease (Sigma Aldrich, Cat # p5147)	1 mg/ml
Deoxyribonuclease (Roche Diagnostics, Australia)	0.01 mg/ml

3. Culture Medium (Invitrogen, Carlsbad, CA, USA)

Iscove's modified Dulbecco's medium (IMDM), containing:

Foetal bovine serum	10 %
Glutamine	4 mM
Penicillin	100 U/ml
Streptomycin	100 μg/ml

4. Sterile PBS (Invitrogen, Carlsbad, CA, USA)

5. Hanks Balanced Salt Solution (HBSS)

Potassium chloride (KCl)	0.4 g/l
Potassium dihydrogen phosphate monobasic (KH_2PO_4, anhydrous)	0.06 g/l
Sodium chloride (NaCl)	8 g/l
Sodium hydrogen phosphate dibasic (Na_2HPO_4, anhydrous)	0.0447 g/l
D-glucose	1 g/l
Phenol red	0.01 g/l
Sodium bicarbonate ($NaHCO_3$)	0.35g/l

HBSS is purchased from Sigma (Cat. # H2387) as a powder and then made up with MilliQ water. The solution is then sterilised before use.

6. 0.3% Bovine serum albumin (BSA; Pierce, Cat # A9418) 0.15 g BSA / 50ml GBSS + NaCl

7. Nycodenz (Nycomed Pharma, Oslo, Norway)

2.87 g Nycodenz / 10 ml GBSS – NaCl

8. Trypsin (0.05%) – 0.2 g/L Ethylene diamine tetraacetate [EDTA] (Invitrogen, Cat. # 25300)

9. Culture flasks/plates (Techno Plastic Products AG, Trasadingen Switzerland) TPP® T75 Flask #90076; T25 flask 90026; 60 mm plate #93060

b) Method

Usually, pancreas from a single male Sprague-Dawley rat (150-200 g) yields an adequate number of PSCs (approximately 3 million/g pancreas) for in vitro studies. Animals are sacrificed by decapitation (see Note 1). After the rat is killed, the abdomen is opened by a mid-line incision and two horizontal incisions. The pancreas is dissected, removed, placed into a 50 mL glass beaker containing an ice-cold solution of 0.9% NaCl, trimmed of adipose and connective tissue as well as of large blood vessels and moved into a PC-2 biological safety cabinet. It is then transferred to a 60 mm plastic Petri dish.

Using an insulin syringe, the pancreas is injected with sufficient enzyme solution (10mL collagenase P, protease, Dnase solution; approximately 300 µL injected per injection site) until all pancreatic lobules are well separated (see Note 2). The pancreas is then transferred into a conical (Erlenmeyer) flask, to which another 10 ml of enzyme solution (total 20 mL) is added. The tissue is incubated at 37°C for 7 min in a shaking water bath [high speed (240 cycles/min) for 4 min and low speed (120 cycles/min) for 3 min]. The partially digested pancreas is then transferred back into a 60 mm plastic Petri dish, finely minced using curved scissors and any remaining adipose, connective or vascular tissue is removed (see Note 3). This is followed by a second incubation for 7 min at 37°C in a shaking water bath at 120 cycles/min.

The digested tissue is then transferred into a Falcon 50 mL tube and pipetted through successively narrower orifices using 5 mL and 1 mL Gilson pipette tips followed by filtration through a 250 µm nylon mesh, to obtain a suspension of dispersed cells. The cell suspension is then centrifuged at 450 g for 10 min at 4°C, using a Beckman Avanti J-E centrifuge. The cell pellet is washed in GBSS + NaCl containing 0.3% bovine serum albumin (BSA) and then centrifuged as described above. The pellet is then resuspended in 9.5 mL GBSS + NaCl containing 0.3% BSA, to which 8 mL of a 28.7% solution of Nycodenz in GBSS – NaCl (final Nycodenz concentration 11.4%) are added and mixed well. Six ml of GBSS + NaCl with 0.3% BSA are placed into a round-bottom polycarbonate centrifuge tube and the cell suspension in Nycodenz is gently layered underneath using a tubing of a polyvinyl pipette attached to a 30 ml syringe, taking care not to disrupt the interface (see Note 4). The sample is then centrifuged at 1400 g for 20 min at 4°C. PSCs separate into a fuzzy band just above the interface (i.e. between the BSA aqueous solution and Nycodenz). This band is harvested using a sterile glass transfer pipette without disturbing the density gradient layers. Cells are then washed in GBSS + NaCl with 0.3% BSA, centrifuged at 450 g for 10 min, resuspended in 1ml IMDM culture medium and counted. As noted earlier, the yield of PSCs using the above method is routinely **3 million cells per g pancreas**. Isolated cells can then be plated into 75 cm^2 culture flasks (if yield is > 2 million cells) or 25cm^2 culture flasks (if yield is < 2 million cells) and grown to confluence in a humidified atmosphere of 5% CO_2 / 95% air at 37°C.

The preparation of PSCs obtained using the above technique is usually devoid of contamination by acinar cells, macrophages and endothelial cell as assessed by phase contrast microscopy and negative immunostaining for the markers ED1/CD68 (macrophages) and factor

VIII. Purity of the preparation can also be assessed by a) fluorescence microscopy for transient blue green autofluorescence at 320nm, characteristic of vitamin A; and b) immunocytochemical staining for the PSC selective markers i) desmin (although it is to be noted that not all PSCs stain positive for this cytoskeletal protein) or ii) glial fibrillary acidic protein (GFAP, a more consistently expressed protein in PSCs), using an aliquot of cells cultured on coverslips. Culture medium is changed on the day following the isolation and from then on twice weekly. Cells are passaged and replated at required seeding densities (usually 100,000 cells per well in a six well plate) for further experiments. It is recommended that cells are used at early passages (≤ 3) for in vitro studies.

3. Isolation and culture of normal human pancreatic stellate cells

We have established this method using pancreatic tissue (0.2 – 0.8 g) obtained from patients undergoing a pancreatic resection for benign pancreatic conditions. Resected tissue is collected in ice-cold phosphate-buffer saline (PBS) containing penicillin 100 U/mL and streptomycin 100 µg/mL. Part of the specimen is fixed in 4% paraformaldehyde and subjected to H and E staining to confirm that the pancreas is histologically normal. PSCs are isolated from the adjacent tissue.

a) Reagents

These are as outlined for rat PSCs, with a minor modification in the culture medium :

Iscove's modified Dulbecco's medium (IMDM), containing:

Foetal bovine serum	20%
Glutamine	4 mM
Penicillin	100 U/ml
Streptomycin	100 µg/ml

b) Method

The method used for isolation of normal human pancreatic stellate cells is similar to that described for rat PSCs, with some modifications as detailed below.

Briefly, the piece of pancreatic tissue is trimmed of adipose and connective tissue, transferred to a 60 mm plastic Petri dish and weighed. The concentration of the enzyme is adjusted to the weight of the piece of tissue (e.g. for a piece of tissue weighing 0.5 g the concentrations of collagenase P and protease are half those used for 1 g of rat pancreas). Enzyme concentrations have been optimised in preliminary studies to ensure that the tissue is not overdigested. Using a 1 ml insulin syringe, the pancreas is injected with 10 ml of enzyme solution, such that small volumes of the enzyme solution are injected at multiple sites in the tissue. Whenever possible vascular and ductal structures should be cannulated and injected with enzymes as well. The pancreas is then transferred into a conical (Erlenmeyer) flask and incubated in 20 mL of enyzme solution at 37°C for 5 min in a shaking water bath [high speed (240 cycles/min) for 3 min and low speed (120 cycles/min) for 2 min]. The pancreas is then transferred back into a 60 mm plastic Petri dish, finely minced using curved scissors and any remaining adipose, connective or vascular tissue is removed. This is followed by a second incubation for 5 min at 37°C in a shaking water bath at a speed of 120 cycles/min. The remainder of the procedure is as described for rat PSCs.

It is to be noted that the yield of cells can vary considerably between preparations. This contrasts with the constant yield (approximately 3 x 10^6 cells) obtained with preparations of rat PSCs. Also, as opposed to rat PSCs which attach to culture flasks within 8 to 12 h, human PSCs display a longer settling time ranging from 8 h to 4 days (5). A major difference between rat and human pancreas relates to the tissue source. Whereas rat pancreas come from a largely

homogenous population of animals (Sprague-Dawley rats bred for laboratory purposes), human pancreas is obtained from a diverse group of individuals with possibly differing underlying morbidities.

Depending on the cell yield of the preparation, PSCs are plated into one or multiple wells of a 6-well dish and grown to confluence in a humidified atmosphere of 5% CO_2 / 95% air at 37°C. Culture medium is replaced for the first time between 48 and 72 h after isolation (depending on the time point of attachment which often varies between different preparations) and from then on twice weekly.

Purity of the cells is usually assessed at first passage by immunocytochemistry for stellate cell selective markers (GFAP, α smooth muscle actin). Normal human PSCs exhibit abundant lipid droplets in the cytoplasm upon isolation and in early culture**.** However, it is interesting to note that even after 72 h in culture on plastic (a time when rat PSCs uniformly lose their lipid droplets) 10-20% of normal human PSCs still retain their lipid droplets. The reasons for this difference are not immediately apparent but could imply that the human PSC isolate is comprised of different subpopulations of PSCs which may differ in their response to culture-activation on a plastic surface. However, all human PSCs lose their vitamin A droplets after trypsinisation at the first passage.

Cells are passaged and counted by standard methods. Following estimation of the total cell number, cells are plated at equal seeding densities ranging from 10^4 to 3 x 10^4 cells into 6-well culture plates, 10^5 and 3 x 10^5 in 60 and 100 mm Petri dishes respectively. In our laboratory, cells from each individual experiment are used between the first and the seventh passages.

4. Notes

1) Decapitation ensures adequate ex-sanguination and minimises congestion of the pancreas thereby aiding subsequent tissue digestion by proteases. However, other laboratories have also used CO_2 to euthanize animals and report good PSC yields.

2) Adequate injection of pancreatic tissue with the enzyme solution so as to maximise enzymatic digestion of tissue is very important for successful isolation of PSCs. This process should take about 5-7 min.

3) The tissue mincing step is also a critical process for successful PSC isolation and should take about 5-7 min. Observe the viscosity of the digested tissue; if it appears ‘sticky’ add 5-7.5 µL Dnase.

4) It is important to ensure that there are no air bubbles when the cell suspension in Nycodenz is drawn up into the 30 ml syringe with the tubing, so that there is no disruption of the interface when layering the cell suspension underneath the aqueous layer. Good separation of PSCs is highly dependent on an undisturbed interface.

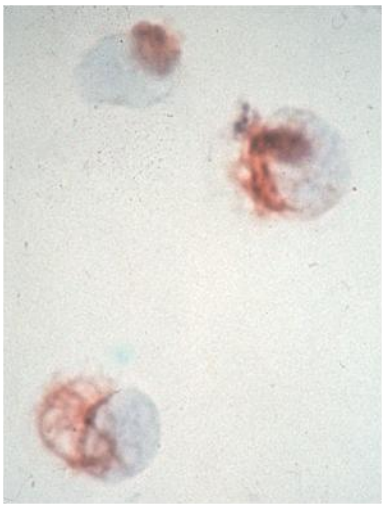

Fig. 1A

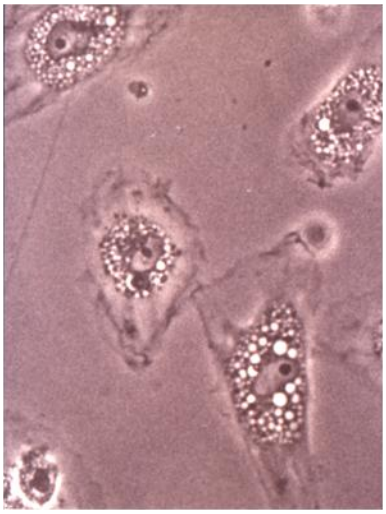

Fig. 1B

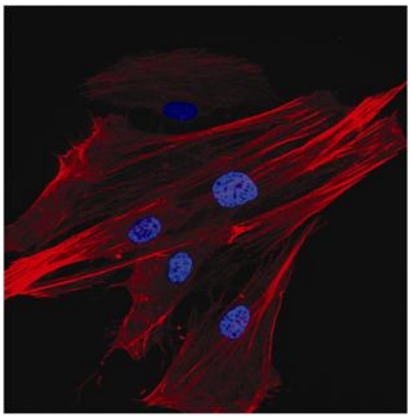

Fig. 1C

Figure 1 depicts A) cytospin preparation of freshly isolated rat PSCs stained for desmin; **B)** a phase contrast micrograph of rat PSCs in early culture showing abundant cytoplasmic lipid droplets; and **C)** human activated PSCs stained for α smooth muscle actin (a marker of cell activation).

5. References

1. **Apte MV, Pirola RC, Wilson JS.** Battle-scarred pancreas: role of alcohol and pancreatic stellate cells in pancreatic fibrosis. J Gastroenterol Hepatol Suppl 3:S97-S101, 2006. PMID: 16958684
2. **Apte MV, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, et al.** Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. Gut 43(1):128-33, 1998. PMID: 9771417
3. **Bachem MG, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, et al.** Identification, culture, and characterization of pancreatic stellate cells in rats and humans. Gastroenterology 115(2):421-32, 1998. PMID: 9679048
4. **Apte M, Pirola R, Wilson J.** New insights into alcoholic pancreatitis and pancreatic cancer. J Gastroenterol Hepatol Suppl 3:S51-6, 2009. PMID: 19799699
5. **Vonlaufen A, Phillips PA, Xu ZH, Yang L, Fiala-Beer E, Pirola R, et al.** Isolation of quiescent human pancreatic stellate cells; a useful in vitro tool to study hPSC biology. Pancreatology:10;434-443, 2010. PMID: 20733342