Protocol

Creation of Primary Cell Lines from Lineage-Labeled Mouse Models of Cancer

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Frequently, it is necessary to isolate pure populations of cancer cells for downstream assays, such as transcriptional analysis, signaling studies, and the creation of noncontaminated primary cell lines. Genetic lineage labeling with fluorescent reporter alleles allows for the identification of epithelial-derived cells within tumors. This protocol describes a method to isolate lineage-labeled pancreatic epithelial cells for ex vivo analysis, but it can be adapted for any type of lineage-labeled tumor.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Collagenase with protease inhibitors (Sigma-Aldrich C6079)

Prepare at a concentration of 1 mg/mL in DMEM/F12 (Life Technologies 11330-032).

DAPI (4',6-diamido-2-phenylindole) (3 mM)

Dulbecco's modified Eagle's medium (DMEM)/F12, prechilled to 4°C

DMEM/F12 containing 10% fetal calf serum (FCS), prechilled to 4°C

Mice with cells/tissues that have been labeled using any fluorescent fluorophore

This protocol describes how to isolate yellow fluorescent protein (YFP)-labeled pancreatic epithelial cells from mice. The mice can be genetically engineered to develop tumors (e.g., Pdx-Cre; LSL-Kras^{G12D/+}; p53^{fl/+}; Rosa26^{LSL-YFP}).

Pancreatic ductal epithelial cell (PDEC) medium <R>

Trypsin-EDTA <R>

Equipment

Cell strainer (40 µm; BD Biosciences 352340)

Cell strainer tube (contains 40-µm strainer in the cap; BD Biosciences 352235)

Conical centrifuge tubes (50 mL)

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Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot078287

Dissecting instruments (sterile)
Fluorescence-activated cell sorting (FACS) instrument
Hemocytometer or other cell counter
Needles (16–18 gauge)
Syringes (5 mL)
Tissue-culture dishes
Tissue-culture incubator (37°C and 5% CO₂)
Tissue-culture plates (six well)

METHOD

1. Kill a mouse. Dissect the pancreas quickly.
2. Place the pancreas in a 50-mL beaker with ~5–10 mL of cold DMEM/F12. Swirl and decant.
3. Wash the pancreas twice more, each time with 5–10 mL of cold DMEM/F12. After the third wash, pour off as much of the media as possible.
4. Mince the pancreas with sterilized scissors into small pieces of roughly ~1 mm in length. Approximately 100 chops will do. Larger tumors will require approximately 200 chops.
5. Add 10 mL of 1 mg/mL collagenase/protease inhibitors in DMEM/F12 to the minced pancreas. Transfer to a 50-mL tube and vortex.
6. Incubate for 20 min at 37°C. Vortex every 5 min.
7. After 20 min, vortex and pour the mixture into a 40-µm cell strainer that has been seated in a 50-mL centrifuge tube.
8. Dilute the filtrate with cold DMEM/F12 to 50 mL. Cap and centrifuge at 900g for 5 min at ~10°C.
9. Decant and resuspend the cell pellet in 25 mL of cold DMEM/F12 containing 10% FCS. Place on ice.
10. Remove the cell strainer and place upside down in a culture dish. Use a scalpel to cut the filter from the plastic. Using sterile forceps remove the filter and place it in a culture dish with the tissue facing up. Immerse the tissue in 0.05% trypsin-EDTA prewarmed to 37°C.
11. Transfer the contents of the dish to a 50-mL tube and vortex. Incubate for 5 min in a 37°C water bath, vortexing every 2 min.
12. Place a 40-µm strainer over the 50-mL centrifuge tube containing collagenase-released cells (from Step 9). Pour the trypsin-treated tissue into the strainer. Discard the strainer and fill the tube with DMEM/F12 to 50 mL.
See Troubleshooting.
13. Centrifuge at 900g for 5 min at ~10°C. Decant, and resuspend the cells in 50 mL of DMEM/F12.
14. Repeat Step 13 two more times, but after the final wash, resuspend the cells in 1 mL of cold DMEM/F12 containing 10% FCS.
15. Remove an aliquot and use to determine the cell concentration.
16. Add 1 µL of 3 nM DAPI to the cells, mix by pipetting, and incubate on ice for 10 min.
17. Centrifuge at 900g for 5 min at 10°C.
18. Decant, and add DMEM/F12 containing 10% FCS to the cell pellet to a final concentration of 1×10^6 – 1×10^7 cells/mL.
19. Aspirate the cell suspension into a 5-mL syringe with a 16- to 18-gauge needle. Remove the needle.
20. Affix the syringe to a cell strainer FACS tube and transfer the cell suspension over 20–30 sec.

21. Sort YFP⁺ cells into a 5-mL tube containing 2 mL of cold DMEM/F12 containing 10% FCS.
Make sure that the sort tubes are chilled before beginning the cell sorting.
See Troubleshooting.
22. Centrifuge the YFP⁺ tube at 900g for 5 min at ~10°C.
23. Decant and resuspend the cell pellet in 1.2 mL of PDEC medium. Transfer the cells to a six-well plate (200 μ L/well). Place the plate in a tissue-culture incubator at 37°C, 5% CO₂. Allow the cells to settle for 30 min.
24. Flood each well with 3 mL of 37°C PDEC medium and return the plate to the incubator. Incubate the cells for 24 h.
25. Remove the medium (and dead cells). Refeed with 5 mL of 37°C PDEC medium per well.
26. Refeed with 37°C PDEC medium every 2–3 d and passage with 0.05% trypsin-EDTA when the cells are 40%–50% confluent. Thereafter, passage when the cells are 80% confluent.
See Troubleshooting.

TROUBLESHOOTING

Problem (Step 12): A large amount of tumor tissue remains on the cell strainer after trypsin treatment.

Solution: Tumors rich in stroma and fibrosis are difficult to digest. However, one must weigh the yield of additional digestion with negative effects on cell viability. This protocol has been optimized to yield viable cells but with incomplete tumor digestion.

Problem (Step 21): The number of live YFP⁺ cells is low.

Solution: The yield for viable YFP⁺ primary tumor cells varies with tissue type. For pancreatic tumors, yield is typically low, possibly because of autodigestion by pancreatic digestive enzymes. Keep cells on ice and in the dark as much as possible. Perform this procedure quickly, including transferring the sorted cells to culture. We take ~1.25 h to prepare cells for sorting.

Problem (Step 26): Cells are not growing.

Solution: If cells are not 40%–50% confluent after 1 wk, then lift the cells with trypsin-EDTA and transfer them to a new six-well plate.

DISCUSSION

This protocol, which is adapted from Rhim et al. (2012), has been used extensively by our laboratory to establish pure epithelial primary tumor lines from lineage-labeled mouse models. This approach ensures that fibroblast contamination, a frequent problem in the generation of primary cancer cell lines, does not occur. This protocol can be used for any type of lineage-labeled tumor, although it has been optimized for pancreatic cancer.

The major drawback to this protocol is potential bias introduced in creating the cell lines. Multiple steps in this protocol are likely to lead to some degree of artificial selection of cells for culture (e.g., extended periods of time in which cells are in suspension and the high-pressure forces applied to cells during flow sorting). Further, YFP labeling can be heterogeneous within tumor cells that can provide a challenge in creating gates on FACS.

Because this protocol generates a single-cell suspension of primary tumor cells, this approach lends itself to several modifications. It is possible to stain cells for various surface epitopes for subgroup analysis. We have established cultures of subsets of tumor cells that contain various putative cancer stem cell markers using this approach with success. In addition, a portion of sorted cells can be dedicated for RNA and DNA extraction. Finally, depending on the flow cytometer available, single cells can be sorted directly into ultralow attachment plates for assessment of clonal growth and self-renewal (Rovira et al. 2010).

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Genetic lineage labeling allows for the creation of “pure” primary cancer cell lines, and therefore a more accurate assessment of cancer cells in vitro. As downstream analyses of primary murine cell lines continue to evolve, including genomic and clonal analyses, it will be vital to ensure the purity of the material being analyzed.

RECIPES

Pancreatic Ductal Epithelial Cell Medium

Reagent	Amount
DMEM/F12 (Life Technologies 11330-032)	500 mL
D-Glucose (Sigma-Aldrich G5400)	2.5 g
Nicotinamide (Sigma-Aldrich N3376)	0.61 g
Soybean trypsin inhibitor (type 1; Sigma-Aldrich T6522)	50 mg
ITS+ Premix (BD Biosciences 354352)	2.5 mL
Bovine pituitary extract (BD Biosciences 354123; 3 mg/mL in DMEM/F12)	4.2 mL
3,3',5-Triiodo-L-thyronine (T3; 50 nM) ^a	50 µL
Dexamethasone (Sigma-Aldrich D1756; 100 mM in DMSO)	5 µL
Nu-serum IV (BD Biosciences 355104)	25 mL
Cholera toxin (Sigma-Aldrich C8052; 1 mg/mL in H ₂ O)	50 µL
Penicillin-streptomycin (100×; Life Technologies 15140-122)	5 mL
Fungizone	2.5 mL

^aTo prepare a 50 nM T3 stock solution, dissolve 250 mg of T3 (Sigma-Aldrich T2877) in 10 mL of DMSO and then dilute 1.5 µL of the T3 solution in 1 mL of DMEM/F12. Store at −20°C.

Trypsin-EDTA

0.05% trypsin
0.5 mM EDTA (pH 8.0)

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (DK088945), the Penn Center for Molecular Studies in Digestive and Liver Diseases (P30-DK050306), the National Pancreas Foundation, and the American Gastroenterological Association (Fellow to Faculty award).

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Cold Spring Harbor Protocols

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot078287

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