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Primary Explant Cultures of Adult and Embryonic Pancreas

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Summary

The developmental plasticity of adult pancreas is evidenced by the ability to undergo conversion between different epithelial cell types. Specific examples of such conversions include acinar to ductal metaplasia, ductal to islet metaplasia, and generation of ductal structures within islets. Although 90% of human pancreatic cancers are classified as ductal adenocarcinoma, markers of all pancreatic epithelial cell types (acini, ductal, and endocrine) as well as markers of gastric and intestinal lineages can be detected in these tumors. In recent years considerable knowledge has been gained regarding regulation of cellular differentiation and various signaling pathways involved in normal and neoplastic pancreas through studies of pancreatic cancer and immortalized ductal cell lines. However, these studies provide little insight into the context of normal developmental cues, the disruption of which leads to organ pathology. Here we have described a detailed method for preparation, maintenance, and manipulation of adult and embryonic mouse pancreas. These methods may be utilized in studies involving normal epithelial differentiation, contributing to improved understanding of pancreatic development and disease.

Key Words: Acini; adenovirus; cancer; collagen; development; dorsal bud; embryo; explant; metaplasia; mouse; pancreas; transdifferentiation.

1. Introduction

Although significant progress has been made in identifying genetic changes underlying pancreatic cancer, little is known regarding how these changes affect normal pancreatic epithelium, or how changes in epithelial differentiation may contribute to this disease. In addition to influencing the phenotype and biologic behavior of fully developed pancreatic cancer, it is intriguing to consider that early changes in epithelial differentiation may also contribute to initiation of pancreatic cancer precursor lesions. In this regard, acinar-to-ductal metaplasia has frequently been proposed as an initiating mechanism for pancreatic cancer (reviewed in ***1***). Patients with acinar-to-ductal metaplasia arising in the

From: *Methods in Molecular Medicine, Vol. 103: Pancreatic Cancer: Methods and Protocols*
Edited by: G. Su © Humana Press Inc., Totowa, NJ

setting of chronic pancreatitis carry a 16-fold increase in relative risk for pancreatic ductal adenocarcinoma ***(2)***, increasing to 50-fold in patients with familial chronic pancreatitis ***(3)***. Evidence that this alteration in epithelial differentiation may indeed be involved in pancreatic cancer initiation is provided by a mouse model of pancreatic ductal metaplasia initiated by chronic overexpression of transforming growth factor-α (TGF-α), in which metaplastic ductal epithelium progresses to pancreatic intraepithelial neoplasia (PanIN) and invasive pancreatic cancer ***(4)***.

The ability of adult pancreas to undergo metaplastic conversion between epithelial cell types emphasizes the high level of developmental plasticity displayed by this tissue. Specific examples of developmental plasticity in adult pancreas include ductal-to-islet metaplasia (islet neogenesis; ***5–10***), duct generation within islets ***(11–13)***, and acinar-to-ductal metaplasia ***(14–16)***. Presumably, these profound changes in pancreatic epithelial differentiation reflect the common origin of islet, acinar, and ductal cell types from a shared precursor pool within embryonic pancreatic epithelium. The multipotent differentiation capacity of pancreatic epithelium is further exemplified by examination of lineage markers in human pancreatic cancer. Although 90% of all pancreatic neoplasms may be classified as ductal adenocarcinoma, these tumors typically express combinations of ductal, islet, acinar, and/or markers of gastric and intestinal epithelium ***(17,18)***.

Although some insight regarding regulation of differentiation in normal and neoplastic pancreas has been gained from investigations utilizing pancreatic cancer cell lines and/or immortalized pancreatic ductal epithelial cells, it might be argued that changes in differentiation are best studied within the context of an intact epithelium. To this end, a number of primary explant culture systems have been developed for the in vitro study of pancreatic tissue ***(11,14,15,19,20)***. These systems have previously been valuable in addressing a variety of scientific questions, including studies of epithelial–mesenchymal signaling, transdifferentiation, islet neogenesis, and in vitro carcinogenesis. In this chapter, we provide detailed methodologies for the preparation, maintenance, and genetic manipulation of explant cultures from both adult and embryonic pancreas. Although these techniques may be applicable to establishing explant cultures of pancreatic epithelium from a variety of species, we primarily employ mouse pancreas in order to utilize tissue from targeted mouse strains.

2. Materials

2.1. Preparation and Culture of Adult Pancreas

1. Rat tail collagen-type I (Collaborative Biomedical Products, cat. no. 354236).
2. Waymouth's MB 752/1 media (Gibco BRL, cat. no. 51400-026).

3. Fetal bovine serum (FBS; Sigma).
4. 0.34 *N* NaOH.
5. Collagenase P (Roche no. 1249002).
6. Hank's balanced salt solution (HBSS) (Gibco BRL) 14025-084.
7. Soybean trypsin inhibitor (Sigma T-9003).
8. Dexamethasone (Sigma D-2915).
9. 24-Well tissue culture plates.
10. Polypropylene mesh (105 µm and 500 µm) (Spectrum Laboratories Inc., Spectra-Mesh-autoclaved 7.5 cm^2).

2.2. Adenoviral Infection

1. High-titer stock of adenovirus containing the gene of interest (10^9–10^{11} pfu/mL).
2. Cell counter (Fisher Scientific no. 0267110).
3. 14-mL Falcon culture tube (Becton Dickinson, cat. no. 2059).
4. 1X Waymouth's MB 752/1 media (Gibco BRL, cat. no. 51400-026).

2.3. Preparation and Culture of Embryonic Pancreas

1. 1X Phosphate-buffered saline (PBS) (Bufluids, cat. no. P315-500).
2. Medium 199 (BioWhittaker, cat. no. 12-119F).
3. Fungizone (Invitrogen, cat. no. 15295-017).
4. Penicillin G-streptomycin (Invitrogen, cat. no. 15140-122).
5. FBS (Invitrogen, cat. no. 16140-071).
6. 24-Well plates (BD Falcon, cat. no. 3047).
7. 60 × 15 mm tissue culture dish (BD Falcon, cat. no. 3002).
8. 0.4-µm Millicell-CM culture inserts (Millipore, cat. no. PICM 01250).
9. Microdissecting forceps (Roboz, cat. no. RS-4903 and RS-4974).
10. Microdissecting scissors (Roboz, cat. no. RS-5850).
11. Mouth pipet.

3. Methods

3.1. Explant Cultures of Adult Pancreas

3.1.1. Preparation of Collagen Plates (for One Mouse Pancreas)

1. On ice, in tissue culture hood, mix 9 mL of rat tail collagen, 900 µL of unsupplemented 10X Waymouth's media (filtered), 600 µL of 0.34 *N* NaOH (filtered). This is just an example. You need only 200 µL per well, the number of wells depending on the experiment you are doing.
2. Place two 24-well plates on ice. Then pipet 200 µL of collagen gel mixture into each well, ensuring that the gel covers the bottom of each well. Cover and place in incubator to solidify until ready to use. (It is easiest to get total coverage on the bottom of the well if you pipet into the center of each well.) Now place three 100 × 15 mm tubes in ice and fill each with 10 mL of HBSS. (These will be used to do a series of washes on the resected pancreas.)

3.1.2. Harvesting Adult Pancreas

1. Anesthetize the mouse with isofluorane (*see* **Note 1**).
2. Place the animal in a supine position and tape down limbs, wipe abdomen with betadine or 70% ETOH, and make midline abdominal incision.
3. Mobilize and resect the pancreas from tail to head with sterile instruments.
4. Place the resected pancreas into 10 mL of HBSS on ice.
5. In the hood, transfer the pancreas to fresh 10 mL of cold HBSS twice.
6. During the final wash, mince pancreas in HBSS into 1- to 5-mm pieces using sterile scissors.
7. Centrifuge at 720*g* for 2 min at 4°C.

3.1.3. Isolation of Adult Exocrine Epithelium (on Ice)

1. Aspirate the supernatant.
2. Resuspend pancreas in 5 mL of cold HBSS containing 1 mg of collagenase P (freshly made and filtered).
3. Shake gently at 37°C for 10 min. Wrap the tube top in parafilm (to prevent leaking in water bath). After shaking, rinse tube in 70% EtOH.
4. Stop reaction by placing on ice and adding 5 mL of HBSS with 5% FBS.
5. Centrifuge at 720*g* for 2 min at 4°C, remove the supernatant, and resuspend the pellet with 10 mL of cold HBSS with 5% FBS. Centrifuge at 400*g* for 2 min at 4°C (repeat two times).
6. After the final wash, resuspend the pellet in 5 mL HBSS with 5% FBS.
7. Pipet 5 mL of cell suspension through a sterile 500-μm mesh.
8. Rinse mesh with 5 mL of cold HBSS with 5% FBS.
9. Pipet a 10-mL cell suspension through a 105-μm mesh.
10. Add 10 mL of cell suspension carefully (use a 5-mL pipet) to the top of 20 mL of HBSS with 30% FBS.
11. Centrifuge at 180*g* for 2 min at 4°C.
12. Aspirate the supernatant.
13. Resuspend the pellet in 10 mL of cold Waymouth's media with 10% FBS, 1% penicillin/streptomycin, 100 μg/mL of trypsin inhibitor, and 1 μg/mL of dexamethasone (*see* **Note 2**).
14. Prepare the collagen gel suspension with 12 mL of rat tail collagen, 1200 μL of 10X media, and 800 μL of 0.34 *N* NaOH.
15. Mix the cell suspension and the collagen gel mixture together (equal volumes, depending on recovery).
16. Remove the 24-well collagen-coated gel plates from the incubator and place on ice.
17. Pipet 500 μL of the collagen gel/cell suspension mixture into each coated well.
18. Place the 24-well plates back into the 37°C incubator.
19. After 30 min the gel should be solidified. At room temperature add 0.5 mL of media with 10% FBS and 1% penicillin/streptomycin to each well. Media should be at room temperature or 37°C. At this stage the healthy epithelium consists primarily

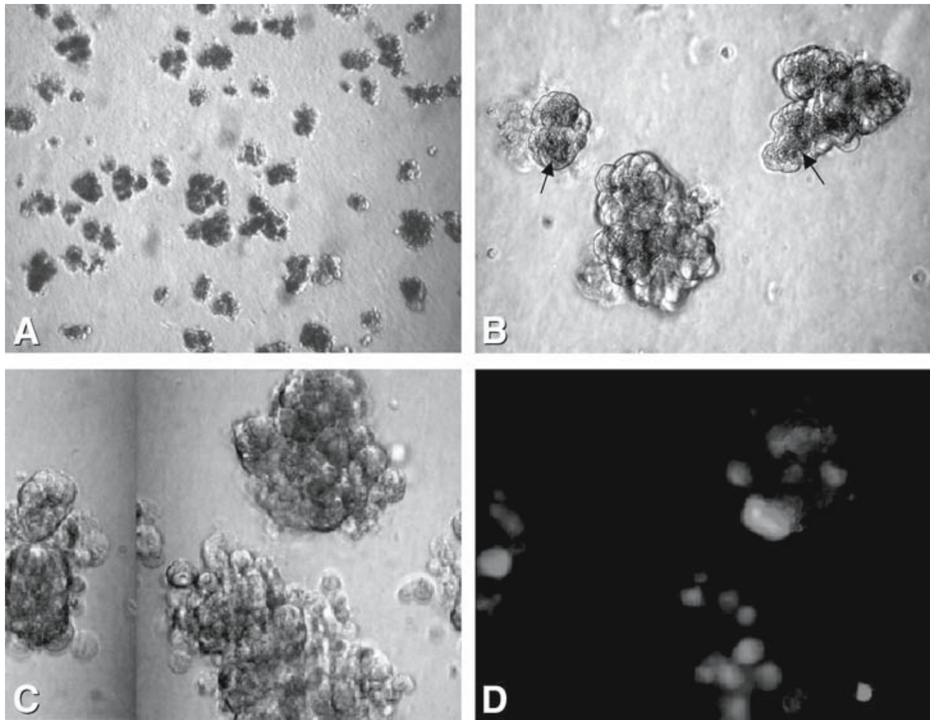


Fig. 1. Freshly harvested explants of mouse exocrine pancreas. Note predominance of acinar cells localized in intact acinar units, and near complete elimination large interlobular ducts and islet elements (**A**, ×100; **B**, ×400). *Arrows* indicate dark zymogen granules, characteristic of acinar cells. Phase-contrast photomicrograph of adenoviral-GFP infected acini preparation after 24 h of infection **(C)** with corresponding GFP expression **(D)**.

of intact exocrine acini, visualized as clumps with dark brown zymogen granules under a phase-contrast microscope (**Fig. 1A**).

20. Add growth factors/inhibitors, and so forth at this time (*see* **Note 3**).
21. Return the plates to the 37°C incubator.

3.1.4. Extended Culture of Adult Epithelium

We usually allow our cultures to continue for 5 d, but longer periods are possible. After 7–8 d, the cultures become acidic and the collagen begins to break down, requiring passage of explants into fresh collagen gels. For longer culture periods, transfer to fresh collagen gels is required.

1. Prepare the collagen gel as above. Coat bottom of 60 × 15 mm culture dish with 1.5 mL of collagen gel, making sure entire surface is covered. Allow solidifying for 30 min at 37°C.

2. Using sterile (bleached) Gelman forceps, place four gel discs into each culture disc, not allowing each other to touch.
3. Overlay with 1.5 mL of collagen gel mixture. Allow 30 min to solidify.
4. Overlay with 2 mL of media with appropriate supplements, growth factors, and so forth.

3.1.5. Adenoviral Infection of Adult Pancreatic Epithelium

Methods for high-titer adenoviral preparation have been described elsewhere ***(21)***. A series of preliminary infections should be performed to determine the multiplicity of infection (MOI), ideally resulting in greather than 50% transduction efficiency with acceptable cytotoxicity as determined by morphological comparison with mock-infected cells.

1. Prior to suspending the epithelium in the collagen gel mixture, reserve an aliquot to be trypsinized and counted to determine cell number for MOI calculations.
2. Mix the suspended epithelial structures with predetermined MOI of adenovirus containing gene of interest in 1X Waymouth's media in a 15-mL Falcon culture tube.
3. Incubate at 37°C for 1 h at CO_2 incubator at 4°C (*see* **Note 4**).
4. Centrifuge the epithelium at 400*g* for 2 min.
5. Aspirate the supernatant and resuspend the cells with appropriate 1X Waymouth's media.
6. Follow **step 13** onward as described in **Subheading 3.3.**
7. Check by immunofluorescence or other reporter assay after 24–48 h for successful infection (**Fig. 1B,C**).

3.2. Explant Cultures of Embryonic Pancreas

3.2.1. Culturing Pancreatic Explants Derived from E9.5 (approx 20-Somite) Mouse Embryos

Before culturing, prepare the pancreas culture media (PCM) with Medium 199, 10% FBS, 50 U/mL of penicillin G–streptomycin, and 1.25 µg/mL of Fungizone. This solution may be stored at 4°C up to 1 mo. It is recommended to warm the media to 37°C prior to use. The procedures described here for isolating and culturing pancreatic anlagen has been modified from original method described by others ***(19,22,23)***.

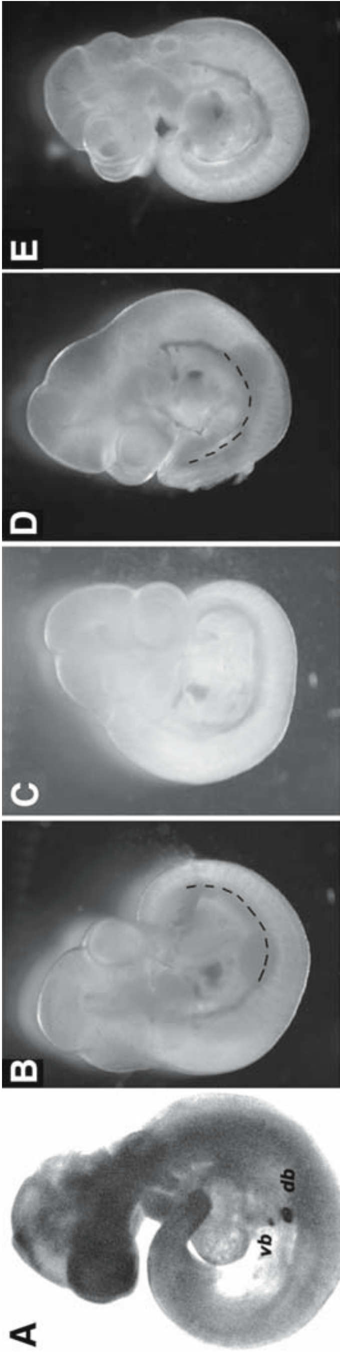
1. Add 400 µL of PCM in each well before placing the Millicell-CM culture insert into the well.
2. Kill the pregnant female(s) and transfer the uteri to sterile ice-cold PBS.
3. Transfer the uteri to a fresh dish containing ice-cold PBS in order to wash out blood and debris.
4. Isolate the embryos and transfer to a fresh dish.

5. Be sure the embryos are covered by PBS and kept on ice. Transfer one embryo at time to a fresh dish and perform the dissection.
6. Lay down the embryo on bottom of the dish with its right side facing you as shown in **Fig. 2B**.
7. By using forceps, carefully cut and remove the limb buds and lateral body wall along the rostral–caudal axis. This will allow visualization of the inner organs (**Fig. 2B,C**).
8. Flip the embryo vertically and repeat the same procedure on the other side (**Fig. 2D,E**). In the beginning it may be difficult to localize the pancreatic buds. To do this, try to follow the gut along the caudal–rostral axis, and you will see that at a certain point it becomes broader. That is the beginning of the stomach, and the pancreatic anlagen are located just below that on each side of the gut (**Fig. 2A,F**). The next step is to separate the gut from the neural tube.
9. Hold the embryo by one pair of forceps and insert the tips of another pair between the gut and the neural tube rostral to the stomach.
10. Carefully open and close the forceps to separate the tissues from each other, and move caudally along the axis by opening and closing the tweezers repeatedly (**Fig. 2F**).
11. Detach the gut from the rest of the embryo (**Fig. 2G**). Before transferring the gut to the membrane try to remove as much of the heart tissues connected to the gut tube as possible (**Fig. 2H,I**).
12. Transfer the gut by mouth pipet and place it on the MilliCell-CM insert.
13. Maintain the cultures for 7 d in a humidified incubator at 37°C with 5–10% CO_2.
14. Change the medium every other day by transferring the inserts to the next well (**Fig. 3**).

3.2.2. Isolating and Culturing Pancreatic Rudiments Derived from E10.5 Mouse Embryos

Isolating and culturing segments of the gut containing pancreatic rudiments from E10.5 embryos is similar to the procedures described above. In addition, at this stage we are also able to perform a more defined dissection by isolating only the pancreatic rudiments (both the dorsal and the ventral rudiments) without associated foregut structures. The culturing conditions are the same as for E9.5 explants. Moreover, it is also possible to separate the pancreatic epithelium from its surrounding mesenchyme to culture the epithelium alone or to recombine the mesenchyme with epithelium from other explants.

1. Perform the dissection as described in **steps 1–11** in **Subheading 3.2.1.** The isolated gut should be similar to the one shown in **Fig. 4**.
2. To isolate the intact dorsal pancreatic rudiment pinch the area indicated by arrows in **Fig. 4**. This will disconnect the pancreatic bud from the gut. Next try to cut the mesenchyme by pinching along the indicated line in **Fig. 4**. Now the intact dorsal pancreatic bud is isolated. If you wish to culture the intact bud, go directly to **step 4**.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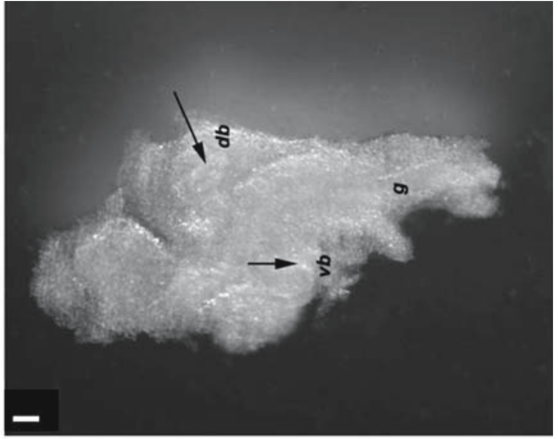
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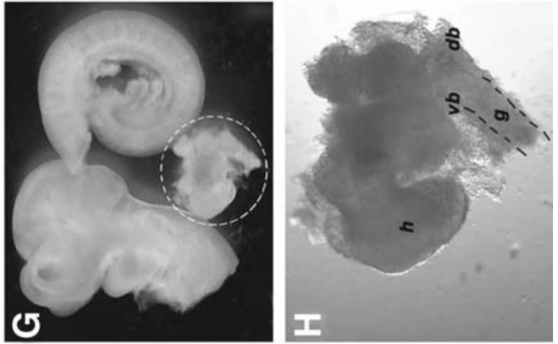
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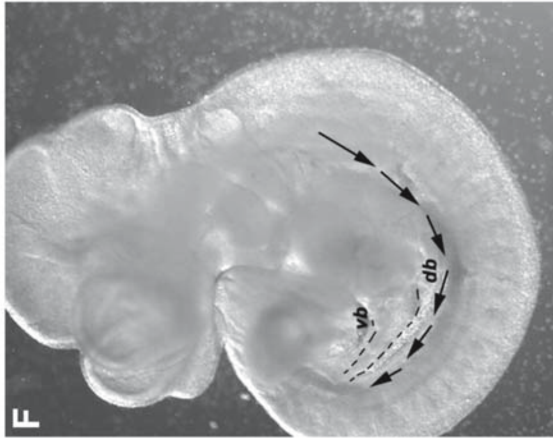
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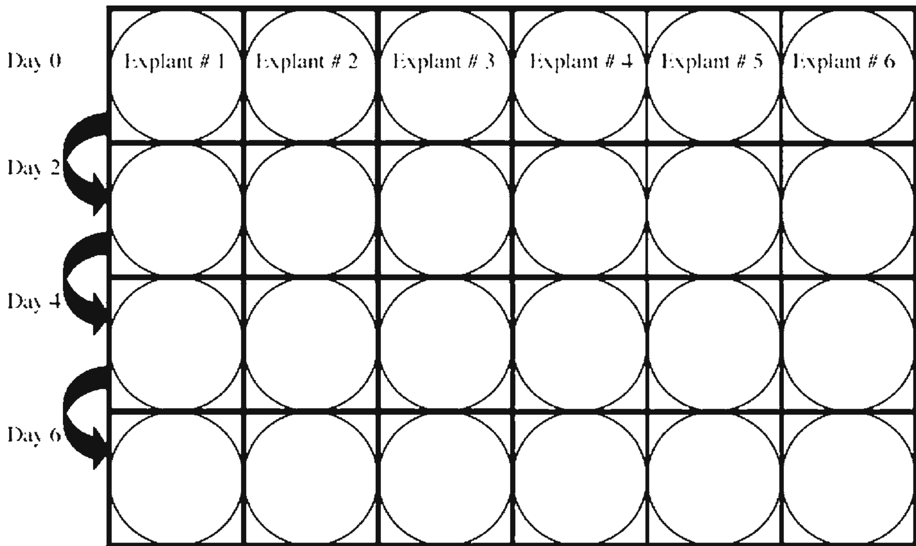

Fig. 3. Simplified method for changing explant medium every other day. Transferring the inserts to the next well minimizes risk of dislodging or disrupting the explant during aspiration and replacement of media.

3. To separate the epithelium from the surrounding mesenchyme, try to grip the intact bud with one pair of tweezers and carefully remove the mesenchyme with another pair of tweezers. Broken lines in **Fig. 4** indicate the border between epithelium and mesenchyme.
4. Transfer the tissue by mouth pipet and put it on the MilliCell-CM insert. If heterologous recombination of epithelium and mesenchyme from different genetic backgrounds is desired, at this point add the heterologous mesenchyme by mouth pipet and put it for example on top of a naked epithelium. To isolate the ventral pancreatic rudiment, follow the same procedure described above.

3.2.3. Adenoviral Infection of Embryonic Pancreatic Explants

We have developed a method for infection of pancreatic buds by recombinant adenoviruses. By infecting the intact bud, only the surrounding mesenchyme is efficiently transduced to express adenoviral genes. This provides the opportunity to selectively manipulate the mesenchyme and characterize the

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Fig. 2. (*Opposite page*) Dissection of E9.5 foregut segment containing embryonic pancreatic anlagen. Whole mount immunohistochemistry, using antibodies for detection of Pdx-1 in E9.5 mouse embryo (***20***). **(A)** Different steps of procedures described in **Subheading 3.2.1. (B–I)**. vb, Ventral bud; db, dorsal bud; h, heart; g, gut.

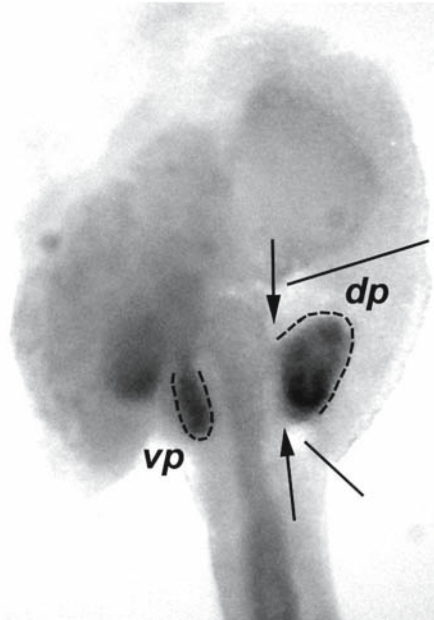

Fig. 4. Whole mount immunohistochemistry of E10.5 foregut segment containing pancreatic anlagen, using antibodies against Pdx-1.

subsequent effects on the epithelium. However, it is also possible to isolate the epithelium from its mesenchyme, infect it, and then culture it with or without mesenchymal recombination (**Fig. 5**). These two approaches thus offer complementary techniques for selective genetic manipulation of either epithelium or mesenchyme.

1. Transfer the intact or naked pancreatic bud onto MilliCell inserts as described in **Subheading 3.2.2.**
2. Add recombinant adenovirus to PCM to obtain 1×10^7 fp/mL of diluted viral stock. Two hundred microliters is required for each bud.
3. After mixing add 200 µL into the MilliCell insert, and incubate for 24 h at 37°C with 5–10% CO_2. Make sure that the tissue is submerged in the mixture.
4. After 24 h, carefully remove the infecting mixture by pipet, and transfer the membrane to a new well and culture as usual. If you are performing a heterologous recombination on the infected naked epithelium, follow the procedures described in **Subheading 3.2.2.**

4. Notes

1. Male mice between ages 4–8 wk give the best results. Prep should be done in the morning. The animal should be killed by 10 AM).

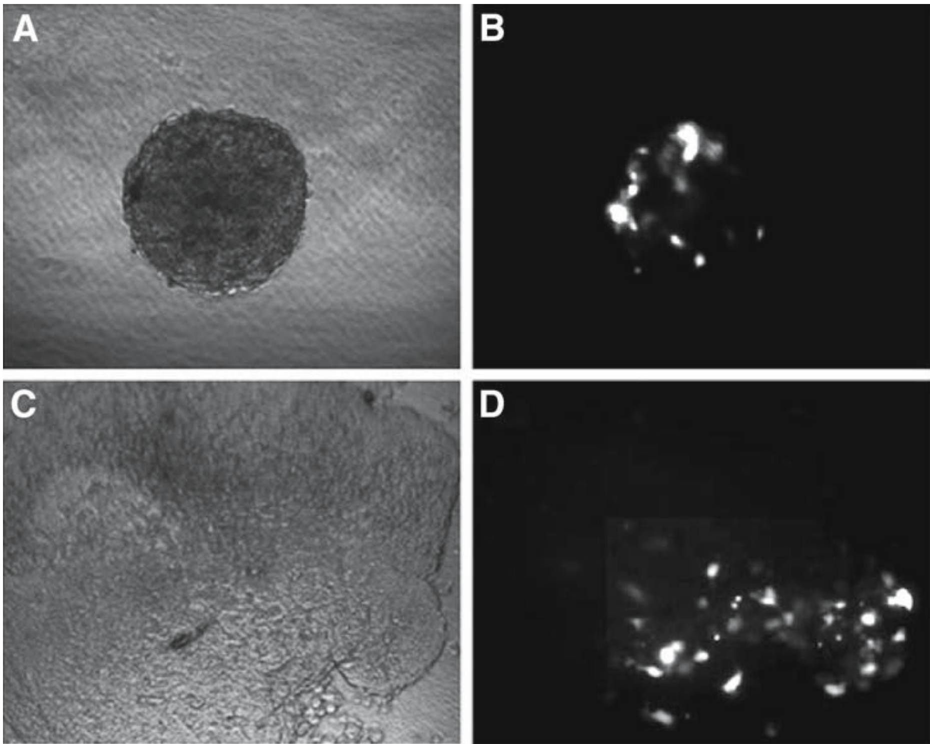

Fig. 5. Ad-GFP infection of E10.5 dorsal pancreatic epithelium. Phase-contrast **(A,C)** images of pancreatic epithelium expressing GFP **(B,D)** with **(C,D)** or without **(A,B)** mesenchyme recombination. Adenoviral infection performed with 1×10^7 pfu/mL for 24 h. GFP expression was detected on d 2 after infection.

2. The serum itself will induce a slight phenotypic switch secondary to the growth factors within the serum. If you wish, you can do this experiment without serum in the media. The results are still quite good.
3. We change the media and add new growth factor on the day following the prep. We then change the media every other day and add new growth factor, 0.1 mg/mL of trypsin inhibitor, and 1 μg/mL of dexamethasone, and so forth.
4. During incubation vortex very gently three or four times at regular interval to prevent cell aggregation at the bottom of the tube.

Acknowledgments

This work was supported by NIH Grant DK56211-01 (to S. D. L.). Dr. Leach is also supported by the Paul K. Neumann Professorship in Pancreatic Cancer at Johns Hopkins University. The authors wish to thank Dr. Ingrid Meszoely and Dr. Anna Means for their assistance in developing these techniques.

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