The goal of this work is to identify genes that fashion the vertebrate gut and its derivative organs. We hope to identify signals that guide lineage assignment, promote region-specific tubular growth and budding, and maintain the differentiated state.

The principle cellular functions of the gut are the digestion and absorption of nutrients, the former dependent upon exocrine and the latter upon absorptive epithelial cells. These cells differ in specialized function between the anterior and posterior intestine. The hepatocytes of the liver are crucial for metabolism of a broad array of endogenous and exogenous substances and for excretion of bile. The pancreas is composed both of acini of exocrine cells, which secrete digestive enzymes such as lipases and peptidases, and the Islets of Langerhans, which secrete insulin and other peptide hormones.

The gut in higher vertebrates originates from the endoderm-lined anterior and posterior intestinal portals (AIP and PIP), which will give rise to the fore- and hindgut, respectively. Although the details of assembly vary among species, in general a continuous tube is generated from these pouches by endodermal growth and migration combined with ventral folding of the embryo (Lamers et al., 1987). The foregut becomes the esophagus, stomach, and duodenum. The hindgut becomes the distal large intestine, and the region in between, the midgut, becomes the intermediate zone of the small and large intestines. Growth of the gut is accommodated by looping and rotation as the absorptive surface is increased by villus formation. The pancreas and liver originate as endodermal buds from the foregut.

When the experiments reported here were initiated it was not clear how fruitful a genetic approach would be to a vertebrate organ system such as the gastrointestinal tract, because it develops relatively late and depends upon cascades of prior embryological decision-making. However, targeted mutation of known genes in mice has shown, for example, that absence of IPF-1 blocks development of pancreas (Jonsson et al., 1994), loss of neuronal nitric oxide synthase causes hypertrophy of the stomach (Huang et al., 1993), and mice homozygous for a null mutation in Bcl-2 show degeneration of intesti-
nal villi (Kamada et al., 1995). These phenotypes illustrate the potential power of genetics in dissecting the steps of organotypic decision-making, despite the inherent complexities of large multicellular tissue arrays.

**MATERIALS AND METHODS**

**Fish, embryos and larvae**

Fish were raised and handled as described by Westerfield (1993). Day 1 of development was considered to commence 24 hours after fertilization. The ENU mutagenesis screen is described in the accompanying paper (Driever et al., 1996). Progeny were screened for developmental defects involving the digestive organs on days 5 and 6 using a Wild M10 dissecting microscope. Complementation analyses were performed by pairwise matings between all members of groups assigned locus names, with the exception of no relief\(^{m726}\), which was not complemented to piebald\(^{m497}\). Three mutants (m73, m140, m750) were lost as a result of illness during the course of this screen, and are not available for further analysis.

**Histology, immunocytochemistry and electron microscopy**

Embryos and larvae were fixed in 4% paraformaldehyde, embedded in methacrylate (JB-4, Polysciences, Warrington PA) and 3-5 μm serial sections were stained with hematoxylin and eosin, or methylene blue-azure II. Labelling for glycogen was performed using periodic acid-Schiff staining with and without diastase. For immunocytochemistry, embryos and larvae were fixed in Bouin’s and embedded in paraffin. Five μm serial sections were stained with a guinea pig anti-porcine insulin antibody (Linco, Eureka MO), 1:100, overnight at 4°C, or rabbit anti-bovine carboxypeptidase A antibody (Rockland Inc., Gilbertsville PA), 1:1000, overnight at 4°C. Texas red-conjugated goat anti-guinea pig and FITC-conjugated goat anti-rabbit antibodies (Jackson Immuno Research, West Grove, PA.) were used as secondary antibodies, 1:100, for 2 hours at room temperature.

For anti-cytokeratin staining, larvae and adult tissue were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and treated with 0.2 mg/ml pronase XXV (Sigma) in PBS at 37°C for 30 minutes prior to application of the anti-cytokeratin antibody (AE1/AE3; Boehringer Mannheim) 1:100, 4°C overnight, followed by peroxidase-conjugated biotinylated goat anti-mouse secondary antibody (ABC Kit; VectorLabs). For laminin immunostaining, rabbit anti-laminin antibody (Sigma) 1:200 was used at 4°C overnight, followed by a biotinylated goat anti-rabbit antibody (ABC Kit, VectorLabs). For glucagon immunostaining, rabbit anti-glucagon antibody (generously provided by Dr Julia Polak, Royal Postgraduate Medical School, London, UK) was used 1:50, overnight at 4°C, followed by a FITC-conjugated goat anti-rabbit secondary antibody (Jackson Immuno Research, West Grove, PA.), 1:100 for 2 hours at room temperature.

Histochemical detection of leucine aminopeptidase, lipase and ATPase was accomplished using standard protocols (Bancroft and Stevens, 1990), as was alkaline phosphatase (Genius System, Boehringer Mannheim).

For electron microscopy, embryos and larvae were fixed in 2% glutaraldehyde for 1 hour at room temperature and postfixed in 1% osmium tetroxide, 1 hour, at 4°C. Specimens were dehydrated in ethanol, infiltrated with epoxy resin (Polysciences, Warrington PA), cut, mounted and stained following standard EM protocols and viewed with a Philips CM10 electron microscope.

Whole mount in situ hybridization was performed as described (Ostoby and Jowett, 1993), using an anti-sense riboprobe generated from a full-length zebrafish GATA-5 cDNA, a generous gift of Leonard Zon (Boston, MA), and were viewed and photographed using a Wild M10 dissecting microscope.

**RESULTS**

**Zebrafish gastrointestinal tract development**

The adult zebrafish digestive system is similar to that of other vertebrates (Fig. 1). The intestinal epithelium (Fig. 1A) includes most of the cell types observed in the small intestine of other vertebrates (absorptive, endocrine, goblet) but lacks crypts (Harder, 1975). Paneth cells have not been described in the fish intestine and an intestinal stem cell has not been identified in the teleost intestine, although there are differentiated proliferating cells in the base of the intestinal folds (Rombout et al., 1984). In Cyprinids, fat is absorbed in the proximal intestine and protein absorption occurs more distally (Rombout and Taverner-Thiele, 1984). A short terminal segment of the intestine is likely to be involved in ion transport and may be a homologue of the colon. As in other vertebrates, peristalsis is achieved by the contraction of an inner circular and outer longitudinal layer of smooth muscle, and is regulated by enteric nerves. The structure of the anterior end of the gut varies among vertebrates. As in all Cyprinids, the zebrafish is stomachless and the intestine is continuous with the pharynx through a short esophagus composed of a stratified squamous epithelium (Harder, 1975). The teleost liver (Fig. 1B)
Zebrafish digestive organ mutations

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The zebrafish digestive organ system resembles that of the mammal but lacks a lobular architecture with portal tracts (Harder, 1975). The hepatocytes are arranged as cords of cells bathed in sinusoidal portal blood. The intrahepatic bile ducts arise from bile canaliculi and are scattered throughout the liver parenchyma. As in other vertebrates, a gall bladder is present for the storage of bile. The adult zebrafish pancreas (Fig. 1C) is a less coherent structure than that of the mammal in that several prominent islets are located near the proximal end of the pancreatic duct and accessory islets and exocrine tissue are scattered along the intestinal mesentery in close proximity to the pancreatic ducts (M. Pack, personal observation).

Fate maps of the zebrafish blastula indicate that endodermal precursors originate from marginal blastomeres, which involute early in gastrulation (Warga and Kimmel, 1990). As shown in Fig. 2A, at 36 hours postfertilization (hpf) the developing zebrafish gut is a cord of radially aligned cells. The anus and esophagus have not yet developed. By 52 hpf, as shown in Fig. 2B, a lumen is evident in the rostral gut, although not yet connected to the pharynx. By 72 hpf (Fig. 2C), differentiation is evident. Goblet cells have appeared and proximal intestinal cells have begun to polarize. The developing esophagus joins the proximal intestine to the pharynx. By 4 days postfertilization (dpf) the exocrine cells of the pancreas have differentiated (arrowhead) at 4 dpf. The liver is rostral to the plane of section in A and B. e, exocrine pancreas; i, pancreatic islet; l, liver; n, notochord; y, yolk; * labels pneumatic duct of gas bladder. Scale bars, A and D, 25 μm; B, 20 μm; C, 30 μm.

Fig. 2. Histology of the digestive organs in the developing zebrafish. Cross sections of proximal gut. (A) A small lumen is visible in the developing intestine (arrow) of the 36 hour postfertilization (hpf) embryo. (B) The intestine has enlarged (arrow) and the pancreatic islet (i) and exocrine precursor cells (arrowhead) are visible in the 52 hpf embryo. (C) The intestinal epithelium (arrow) has begun to polarize in the 72 hpf larvae. Exocrine precursors surround the islet (arrowhead). (D) The intestinal epithelium (arrow) has fully polarized and the pancreatic exocrine cells have differentiated (arrowhead) at 4 dpf. The liver is rostral to the plane of section in A and B. e, exocrine pancreas; i, pancreatic islet; l, liver; n, notochord; y, yolk; * labels pneumatic duct of gas bladder. Scale bars, A and D, 25 μm; B, 20 μm; C, 30 μm.

Fig. 3. Mutations affecting the developing zebrafish intestine. (A,C,E,G,I) Lateral views of living 5 dpf larvae. (B,D,F,H,J) Corresponding histological cross-sections through the mid-intestine. Lumen size varies with position along the anterior-posterior axis and with muscular contractions. (A,B) Wild type. The intestinal epithelium (arrow) is highly folded, bile is visible in the lumen and the gas bladder is inflated. The intestinal epithelium is polarized with basal nuclei and a microvillus brush border. (C,D) slj m74, (E,F) m750, (G,H) sst m311, (J) pie m497. The intestinal wall of all mutants is thin, lacks folds and the epithelium is not fully polarized and shows signs of degeneration. Scale bars, B, F and H, 20 μm; D, 15 μm; J, 25 μm.
differentiated, and the intestinal epithelium is polarized along its entire length and has a prominent microvillus brush border (Fig. 2D). Intestinal folds have developed in the rostral and mid-gut and peristaltic contractions are obvious. At 5 dpf proximal-distal regionalization is demonstrable by staining for lipase in the proximal and aminopeptidase in the distal epithelium (data not shown).

By Nomarski optics, liver cells and bile are visible by 3 dpf and hepatic blood flow by 4 dpf. Hepatocytes are evident histologically by 36 hpf, the extrahepatic biliary duct by 52 hpf, and endothelial-lined sinusoids at 3 dpf. The pancreas is visible using Nomarski optics at 4 dpf.

We have developed a series of molecular markers that characterize specific cell types of the zebrafish gut, liver, and pancreas and which have been helpful for mutant analysis. These are outlined in Table 1.

### Mutations affecting digestive organs

The gastrointestinal tract is relatively obscured by overlying tissues. Peristalsis is slow and irregular, so cannot highlight defects as do contractions of the heart for the cardiovasculature. In addition, because the gut develops late relative to many developmental decisions, it is secondarily affected by mutations with effects elsewhere. We selected for further studies those with relatively gut-specific effects (Table 2).

### Mutations that affect intestinal epithelium

Seven mutants affecting intestinal development were isolated. In six (examples of four mutants shown in Fig. 3), the intestinal tube develops without evident abnormality until 4 dpf. At this time the wild-type intestine (Fig. 3A,B) is folded and contains bile, and the epithelial cells are polarized and have a microvillus brush border. By contrast, in *slim jim (slj)* (Fig. 3C,D), *m750* (Fig. 3E,F), *straight shot (sst)* (Fig. 3G,H), *piebald (pie)* (Fig. 3I,J) and *no relief* (not shown), the intestinal epithelium is thin and lacks folds and the cells have only few scattered microvilli and lack evident polarization. In each of these mutants the intestinal epithelium begins to degenerate by 4-5 dpf.

Limitation of the defect to specific regions is a feature of several mutants. Anterior intestine is selectively perturbed in *slj*, posterior intestine is selectively affected in *meltdown (mlt)* and the esophagus in *m140* embryos. In *slj* mutants (Fig. 3C,D), despite the apparent lack of polarization of the anterior intestine, some cellular differentiation does occur. As shown by electron microscopy at 4 dpf, adherens and tight junctions are clearly visible between cells in the anterior intestinal epithelium of wild type (Fig. 4A,C) and *slj* (Fig. 4B,D). In *slj* ...

### Table 1. Cell-specific markers for the digestive organs of zebrafish embryos and larvae

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA-5</td>
<td>Hepatocytes, exocrine cells of pancreas, intestinal epithelium</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>Biliary cells of liver, ductular cells of pancreas, intestinal epithelium</td>
</tr>
<tr>
<td>Lipase</td>
<td>Intestinal epithelium (proximal)</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>Intestinal epithelium (mid-distal)</td>
</tr>
<tr>
<td>Insulin</td>
<td>Pancreatic islet</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Pancreatic islet</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>Exocrine cells of pancreas</td>
</tr>
<tr>
<td>Laminin</td>
<td>Basement membrane of intestine</td>
</tr>
<tr>
<td>ATP-ase</td>
<td>Intestinal epithelium, endothelial cells of liver sinusoids</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Intestinal epithelium</td>
</tr>
</tbody>
</table>

**Fig. 4.** Electron micrographs showing regional intestinal differentiation. (A,C,E) wild type and (B,D,F) *sljm74*. (A) The wild-type anterior intestinal epithelium is columnar and has a prominent microvillus brush border (arrow). (B) *sljm74* anterior intestinal epithelial cells are cuboidal with few microvilli. (C,D) Adherens (arrowhead) and tight junctions (arrow) are present in the anterior intestine of both (C) wild type and (D) *sljm74*. (E,F) Posterior intestinal epithelium appears normal in both (E) wild type and (F) *sljm74*. * labels the intestinal lumen. Magnification: A, ×950; B, ×1100; C, ×22,000; D, ×28,500; E, ×2200; F, ×2950.
these cells are also immunoreactive for a cytokeratin marker, the expression of which normally appears contemporaneously with polarization, and they have apical alkaline phosphatase activity (data not shown). The slj posterior intestinal epithelium is normal, even as assessed by electron microscopy (Fig. 4F; wild type, Fig. 4E). In mlt, however, the mid- and distal intestinal epithelium is disorganized and the mesenchyme expanded while the anterior intestine, liver and pancreas develop normally (Fig. 5). In m140, the pharynx, proximal intestine and liver begin to develop normally, but remain unconnected due to failure of esophageal development (Fig. 6). Subsequently the pharyngeal and intestinal epithelium degenerate.

In the four other intestinal mutants, epithelial degeneration occurs throughout the intestine. In one of these examined by electron microscopy, pie (Fig. 3I,J), the epithelial cells become polarized and develop adherens and tight junctions before degenerating (data not shown). In all mutations affecting the intestine, pronephric and mesonephric epithelium develop normally, indicating that the epithelial abnormality is limited to the gastrointestinal system.

### Mutations that affect the liver

Two liver mutants were identified (Fig. 7). In m73 mutants (Fig. 7C,D), reddish brown pigmentation accumulates in the liver. This is evident by Nomarski optics by 4 dpf, although histologically it appears by 2 dpf. Hepatocellular degeneration is evident by histology (Fig. 7D), and the abnormal liver pigmentation appears to be due to blood pooling around the degenerating hepatocytes. Development of the intra- and extra-hepatic bile ducts appears to be normal, as indicated by the presence of bile in the 4 dpf m73 intestinal bulb and the presence of anti-cytokeratin immunoreactivity (data not shown). m73 embryos also show brain degeneration. In humans, glycogen storage diseases are known to affect liver and brain. In the absence of feeding, glycogen is depleted from wild-type embryos by 6 dpf (Fig. 7G) but persists in the liver of m73 embryos (Fig. 7H) at 6 dpf. This is compatible with impaired glycogen utilization in the m73 liver. In the beefeater (bef) liver mutant (Fig. 7E,F) the histology resembles m73, but there is no brain degeneration.

### Mutations that affect the pancreas

The relationship of the endocrine and exocrine lineages of the pancreas is uncertain. It has been proposed that they share a common precursor (Alpert et al., 1988; Teitelman et al., 1987; Gu et al., 1994; Guz et al., 1995), or that endocrine cells derive from the neural crest (Alpert et al., 1988). Therefore, it is of interest that four mutants develop islets in the essential absence of pancreatic acini.

Normally, the exocrine pancreas is first evident by the end of 3 dpf, a time which precedes overt intestinal differentiation, and appears fully developed by 4 dpf (Fig. 8A). In slj (Fig. 8B) and pie mutants, the islet is evident at 4 dpf, and expresses immunoreactive insulin (Fig. 8D) as does the wild type (Fig. 8C). slj islets are essentially devoid of surrounding cells (Fig. 8B), and the few there are lack zymogen granules. The exocrine cells of wild-type larvae at this stage express GATA-
and immunoreactive carboxypeptidase A (Fig. 8E,G). In slj, carboxypeptidase A immunoreactivity is absent from the pancreas (Fig. 8F). This failure of exocrine cell development is also seen using a GATA-5 probe, a marker of early gut development (Laverriere et al., 1994). Normally, all exocrine cells of the exocrine pancreas express GATA-5 at 4 dpf (Fig. 8G). slj mutants lack GATA-5 expression in this location (Fig. 8H) but retain it in other locations such as the heart, liver and intestine. However, at 3 dpf there is a normal pattern of GATA-5 expression in the developing pancreas of all embryos of heterozygous slj matings. This suggests that in the slj pancreas there may be a transient population of exocrine precursors which do not survive. In pie, cellular exocrine differentiation seems to proceed further. Even though mature acini do not develop, a few cells express GATA-5 and carboxypeptidase A in a ring around the islet at 4-5 dpf, in a pattern that resembles the 3 dpf islet. This selective failure of pancreatic exocrine development, with normal growth of islets, is also seen in sst and nor mutants. In m750 embryos, the exocrine pancreas develops but is smaller than the wild type.

**DISCUSSION**

**Screening for gastrointestinal mutations**

We report here nine mutations that perturb gastrointestinal development. This is from a total of about 500 lines screened specifically for digestive organ defects at 5-6 dpf. It does not include mutations with generalized retardation or markedly pleotropic effects. The pancreas could not be identified reliably by this type of visual screen independently of the gut, but was involved in several foregut defects. Additional screens undoubtedly might enhance sensitivity by the use of lineage- or organ-specific markers. For example, cytokeratin and digestive enzyme markers distinguish the early stages of intestinal epithelial differentiation; the pancreatic exocrine cells express GATA-5 and then carboxypeptidase; the pancreatic islets synthesize insulin and glucagon. Peristalsis can be rendered visible with dyes, and biliary excretion marked with phenol red (M. Pack, personal observation).

**Fig. 6.** In m140 the esophagus fails to develop normally. (A) Wild type, sagittal section, 4 dpf. The intestine (arrow) is joined to the pharynx by the esophagus (*). (B) m140, 4 dpf. There is no connection (large arrow) between the intestine (small arrow) and pharynx, both of which have begun to degenerate. p, pharynx; g, glomerulus; y, yolk. Scale bars: A, 40 μm; B, 30 μm.

**Fig. 7.** Liver mutants. (A,BG) Wild type. (C,D,H) m73. (E,F) bef m362. (A) Lateral view of wild-type 5 dpf larva with bile (b) in intestinal lumen; arrow points to liver. (B) Cross section of wild-type liver 5 dpf with erythrocytes in the sinusoids (*) and hepatocytes (h). (C) The day-4 m73 liver (arrow) has a gray-brown hue. (D) m73 hepatocytes are degenerating and erythrocytes (arrow) are pooling in the sinusoids. (E) bef m362 liver (arrow) has a red-brown hue. (F) bef m362 hepatocytes also degenerate and compress the sinusoids. (G) Wild-type liver 6 dpf does not stain with PAS. (H) m73 liver 6 dpf stains for PAS. Scale bar, 15 μm.
**Patternning and differentiation**

Specialization along the length of the gut characterizes all vertebrate digestive systems, and establishment of axial polarity is assumed to be a key component of its organogenesis, although it has been without molecular underpinning. Several mutations disrupt the early stages of gut development in a region-specific manner. *m140* prevents formation of the esophagus, of interest because poor development of this gut region is responsible for a set of congenital human disorders referred to as esophageal atresia, and because intestinal metaplasia in the esophagus, which is considered a forerunner of esophageal cancer, may result from disordered cell renewal in this organ. *slj* has a selective effect upon the anterior intestine and *mlt* upon the posterior intestine. These regions have their origins in the endoderm of the foregut and hindgut, respectively, and remain different in function throughout life. The molecular underpinning of this regionalization is unknown but several homeobox (Yokouchi et al., 1995; Gaunt et al., 1990; Geada et al., 1992) and forkhead (Monagan et al., 1993; Sasaki and Hogan, 1993) genes are expressed in the developing gut in a region-specific manner and have been proposed to play such patterning roles. *Xlhbbox8* (the *Xenopus* IPF-1 homologue; Wright et al., 1988), has specifically been proposed in this regard for anterior gut.

The signals that regulate the differentiation, death, and renewal of gut epithelial cells are largely unknown. Perhaps because of the high rate of proliferation, gut epithelium is an affected site in mutations of *N-myc* (Stanton et al., 1992) and *Bcl-2* (Kamada et al., 1995), genes which affect the balance of cell proliferation and cell death. In explant culture, mesenchyme of developing gut induces epithelial development (reviewed in Haffen et al., 1987), and, in some cases, can cause a heterotopic epithelium to adopt a new fate concordant with that of the region from which the mesenchyme was derived (Haffen et al., 1987; Mizuno and Yasugi, 1990).

In *slj* and most of the other gut mutants, differentiation proceeds through the original stages of organotypic assembly, with enzyme synthesis and epithelial polarization, and thereafter the intestine begins to degenerate. One explanation could be that a requirement for trophic support is initiated after the onset of differentiation, and that elements of this process, which may result from epithelial-mesenchymal interactions, are perturbed by the mutations. Precursor and differentiated cells certainly may have different trophic dependencies. Along these lines, EGF receptor mutant mice can generate an intestine, but villus development is impaired and intestinal architecture becomes disorganized and degenerates soon after birth (Threadgill et al., 1995; Miettinen et al., 1995).

**Lineage**

The lineage relationship of pancreatic endocrine and exocrine cells is of especial interest, partly because there is no significant renewal of either cell type in the adult, which has important therapeutic implications for diabetes and chronic

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**Fig. 8.** Pancreas mutants. Pancreas development in (A,C,E,G) wild type and (B,D,F,H) *slj* embryos. (A) Cross-section of the pancreas at 4 dpf showing islet (i) and exocrine (e) cells. (B) In *slj* the islet appears normal but the exocrine cells have not developed. (C) Immunoreactive insulin in the wild-type islet. (D) There is little if any immunoreactive insulin in the *slj* pancreas at 4 dpf. Arrowheads mark the position of islet cells immunoreactive for insulin. Arrow, background staining in intestine. (E) Immunoreactive carboxypeptidase A in the wild-type exocrine pancreas. (F) There is little if any immunoreactive carboxypeptidase A in the *slj* pancreas at 4 dpf. Arrowheads mark the position of islet cells immunoreactive for insulin. Arrow, background staining in intestine. (G) *GATA-5* in situ hybridization in the wild type shows expression in the exocrine pancreas (arrows). (H) *slj* does not express *GATA-5* in the exocrine pancreas. Scale bars, A,B,D and F, 15 µm; C,E, 20 µm.
pancreatitis. Whether there is a common precursor for the two cell types is controversial. Most cells express exocrine or endocrine markers alone, but recent reports suggest that there may be a few cells expressing both (Guz et al., 1995; Gu et al., 1994). The IPF-1 gene may be a marker of the cells in the duodenum and pancreas with this bipotentiality (Guz et al., 1995). Compatible with this suggestion is the observation that, in the absence of the IPF-1 gene, neither cell type is generated in the mouse. In slj mutants, islet cells can develop in the absence of differentiated pancreatic exocrine cells. It is possible that the GATA-5 expressing population, which transiently surrounds the developing islet at 3 dpf in mutants and wild type, represents an undifferentiated exocrine-endocrine precursor, one which could give rise to islet precursors before being lost by 4 dpf. Transplantation may help to determine whether the defect affects the exocrine cells in a cell-autonomous manner.

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