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Lineage-specific morphogenesis in the developing pancreas: role of mesenchymal factors

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SUMMARY

Pancreatic organogenesis has been a classic example of epitheliomesenchymal interactions. The nature of this interaction, and the way in which endocrine, acinar and ductal cell lineages are generated from the embryonic foregut has not been determined. It has generally been thought that mesenchyme is necessary for all aspects of pancreatic development. In addition islets have been thought to derive, at least in part, from ducts. We microdissected 11-day embryonic mouse pancreas and developed several culture systems for assays of differentiation: (i) on transparent filters; (ii) suspended in a collagen I gel; (iii) suspended in a basement membrane rich gel; (iv) under the renal capsule of an adult mouse. Epithelia were grown either with or without mesenchyme, and then assayed histologically and immunohistochemically. Epithelium with its mesenchyme (growth systems i-iv) always grew into fully differentiated pancreas (acinar, endocrine, and ductal elements). In the basement membrane-rich gel, epithelium without mesenchyme formed ductal structures. Under the renal capsule of the adult mouse the epithelium without mesenchyme exclusively formed clusters of mature islets. These latter results represent the first demonstration of pure islets grown from early pancreatic precursor cells. In addition, these islets seemed not to have originated from ducts.

We propose that the default path for growth of embryonic pancreatic epithelium is to form islets. In the presence of basement membrane constituents, however, the pancreatic anlage epithelium appears to be programmed to form ducts. Mesenchyme seems not to be required for all aspects of pancreatic development, but rather only for the formation of acinar structures. In addition, the islets seem to form from early embryonic epithelium (which only express non-acinar genes). This formation occurs without any specific embryonic signals, and without any clear duct or acinus formation.

Key words: pancreatic development, mesenchyme, morphogenesis, epithelium, mouse

INTRODUCTION

The pancreas has long been thought to develop from epitheliomesenchymal interaction (Fell and Grobstein, 1968; Githens, 1986; Golosow and Grobstein, 1962; Wessels and Cohen, 1967) wherein undifferentiated epithelium in the duodenal anlage is stimulated by the overlying mesenchyme to grow and differentiate into mature pancreas with components of acinar, endocrine and ductal structures (Fell and Grobstein, 1968; Kallman and Grobstein, 1968; Wessels and Cohen, 1967). During the 1970s a search for the 'mesenchymal factor' in the embryonic pancreas was conducted without success (Filosa et al., 1975; Levine et al., 1973; Pictet and Rutter, 1972; Pictet et al., 1975; Ronzio and Rutter, 1973; Rutter et al., 1964). Since that time, it has been generally thought that pancreatic organogenesis is almost completely dependent on the mesenchyme, and that without the mesenchyme, only low levels of endocrine differentiation, without any apparent pancreatic morphogenesis, would occur. With the advent of transplantation of the islets of Langerhans as a potential treatment for diabetes mellitus, has come a renewed interest in the factors controlling pancreatic growth and differentiation (Dudek and Lawrence, 1988; Dudek et al., 1991; Gu and Sarvetnik, 1993; Hammer et al., 1987; Lim et al., 1992; Mullen et al., 1976, 1989; Noltorp et al., 1989; Ricordi et al., 1988; Sandler et al., 1989; Tuch et al., 1984; Voss et al., 1989). The islets themselves have been thought to originate from smaller pancreatic ducts (Dudek et al., 1991; Gu and Sarvetnik, 1993; Laguesse, 1894; Rao et al, 1990), rather than de novo.

Given the complex nature of the mature pancreas, it seemed plausible that the influence of the mesenchyme was through several pathways, each influencing the fate of the epithelium in a different way. In the salivary gland, for example, factors necessary for the production of branching morphogenesis have been determined and shown to be distinct from the mesenchymal inductive effect of salivary acinar differentiation (Bernfield and Banerjee, 1982; Kratochwil et al., 1986; Nakanishi and Ishii, 1989; Nogawa and Nakanishi, 1987; Takahashi and Nogawa, 1991). Kidney development also has been shown to be variable, with more or less tubular epithelium or glomeruli depending on variations in the nature of the mesenchyme (Weller et al., 1991).

Early work on the pancreas showed that in culture the epithelium was unable to thrive or grow without mesenchyme. During this time, however, there was a low level expression of endocrine genes (Clark and Rutter, 1972; De Gasparo et al., 1975; Filosa et al, 1975; Rall et al., 1973, 1977; Rutter et al., 1964). We hypothesized that the pancreatic epithelium may have a separable lineage-specific response to various signals generated by the mesenchyme. In order to study this possibility we grew early pancreatic epithelium in various culture environments with or without pancreatic mesenchyme. The environments were chosen to focus on different components of the mesenchymal milieu, such as the three dimensional scaffolding, the basement membrane, or the in vivo environment with its vascularization and humoral influences.

MATERIALS AND METHODS

Pancreas dissection

Overnight matings were performed between male and female B6D2 mice or between male and female C57 mice. A vaginal plug the next morning was indicative of pregnancy and noon of that day was treated as day 0.5 of gestation. Embryonic pancreata were harvested on day 11.5 of gestation and dissected as described previously (Gittes and Galante, 1993). Isolated epithelium was obtained by first

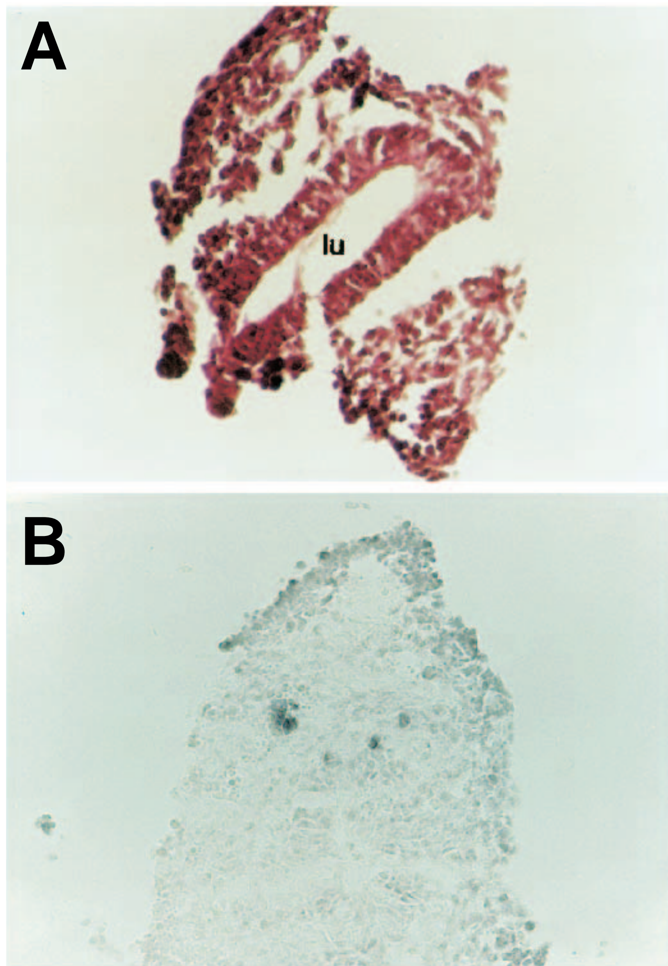

Fig. 1. Pancreas harvested from 11-day embryo and immediately processed for immunohistology, stained with hematoxylin and eosin (A) and immunostained for insulin (B). Note that there is only scattered insulin expression without evidence of any islets. The pancreas at this gestational age is only a simple unilobular structure with a single lumen (lu).

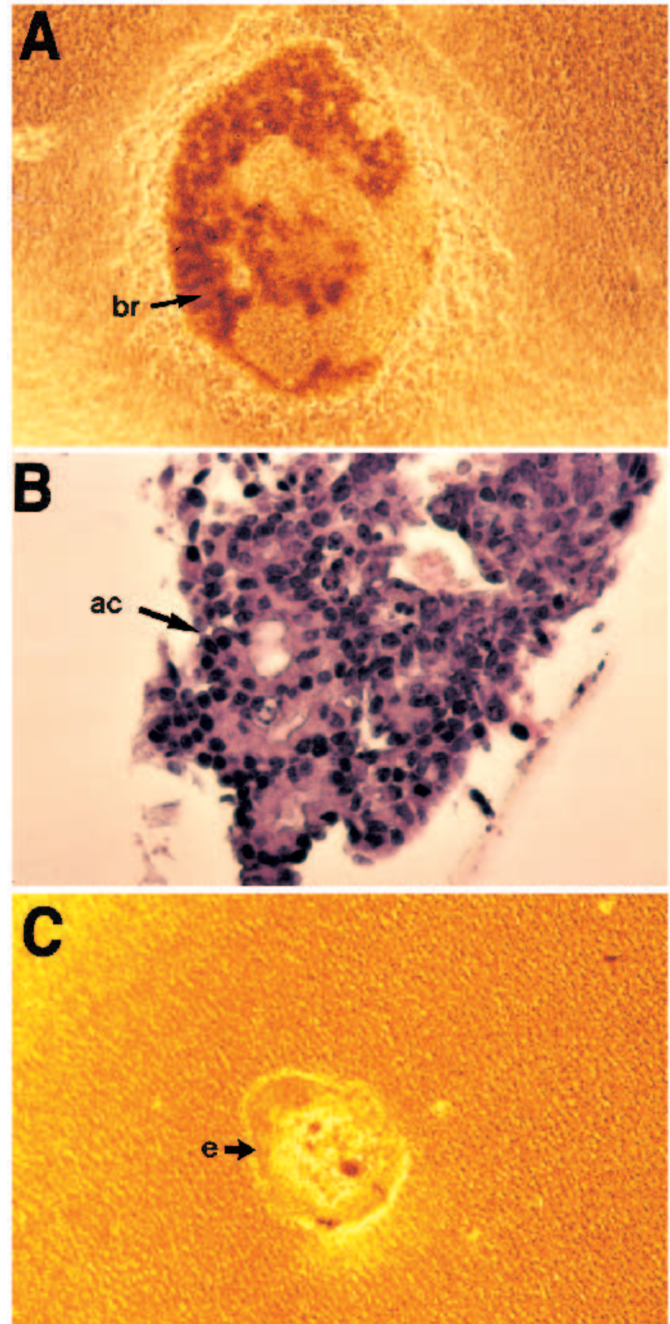

Fig. 2. Organ culture of 11-day embryonic mouse pancreas grown in a collagen I gel for 7 days. Diffferentiation of pancreatic epithelium occurred in the presence of mesenchyme, but not with epithelium alone (A,B) Whole pancreas (with mesenchyme) grown for 7 days as seen under phase contrast microscopy (A) and with hematoxylin/eosin staining (B). Branching morphogenesis and acini (ac) can be seen. (C) Isolated epithelium (e) seen under phase contrast microscopy. Cells are dispersing with no evidence of differentiation (immunohistology not shown).

mechanically removing most of the mesenchyme, and subsequently treating it with 1% bovine trypsin at 4ºC for 15-20 minutes. Trypsin was then reversed with 10% fetal calf serum in DME-H21 and the residual mesenchyme gently stripped from the epithelium under high power magnification in a dissecting microscope so that individual residual mesenchyme cells could be visualized and removed with precision.

Culture conditions

Epithelia, with or without the removed mesenchyme reapproximated, were transferred into culture conditions using sterile 'slick' pipette tips. Further manipulation of tissues in culture, such as opposing epithelium and mesenchyme, was done under direct microscopic examination. Tissues were grown in Millipore filter inserts by placing the inserts in standard 24-well plates with 350 µl of RPMI medium

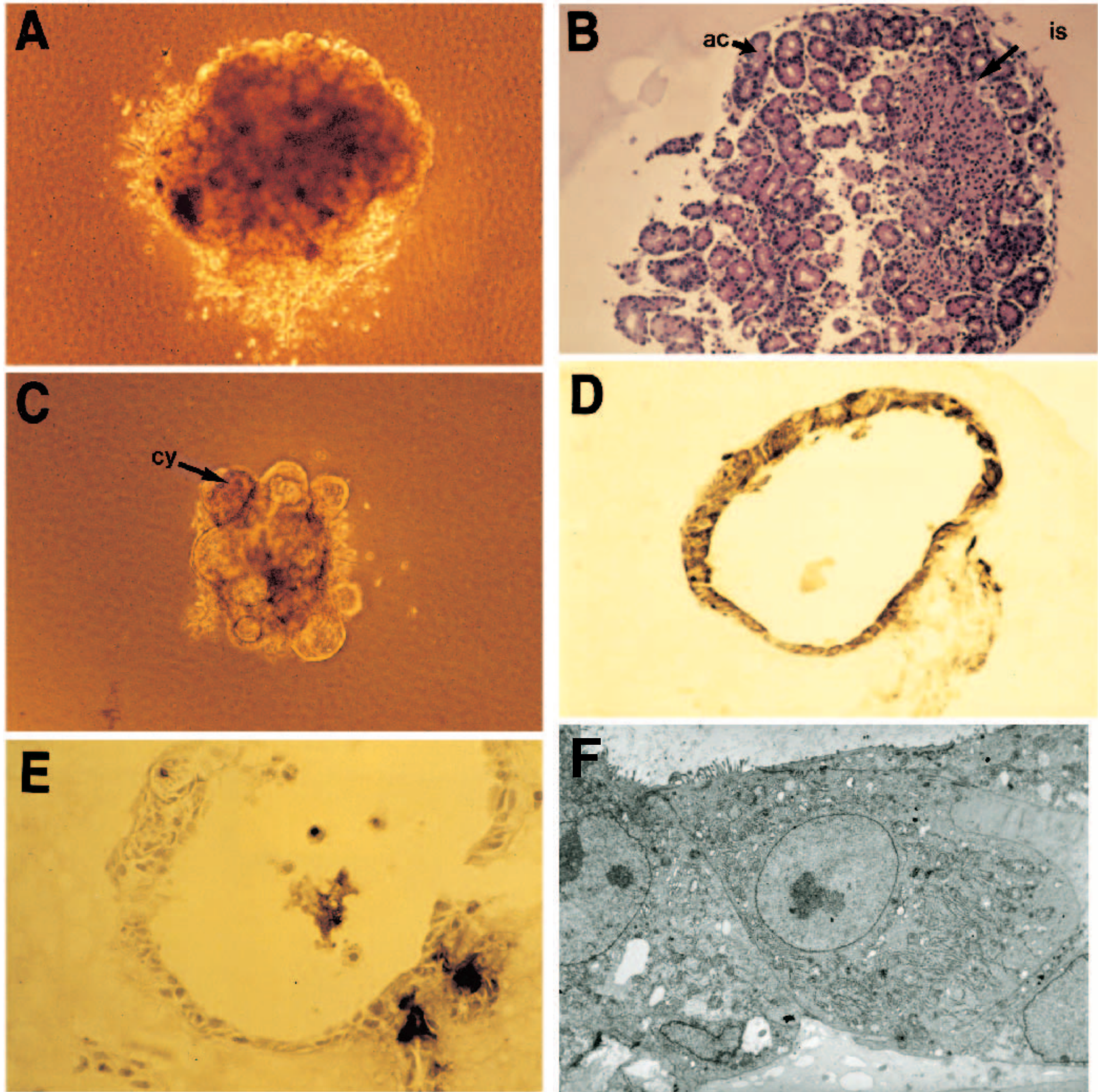

Fig. 3. 11-day embryonic mouse pancreas grown in Matrigel for 7 days. Isolated epithelium formed duct-like structures with scattered endocrine cells. With mesenchyme, full pancreatic diffferentiation was seen. (A,B) Whole pancreas as seen under phase contrastmicroscopy (A) and hematoxylin/eosin staining (B). Note the presence of acini (ac) and islet (is) structures. (C) Isolated epithelium grown in Matrigel form multiple cystic structures (cy), as seen here under phase contrast microscopy. (D,E) Immunohistochemistry of the cystic structures seen in C. Staining for cytokeratin 7 (ducts) in D and for insulin in E. Note the lack of exocrine tissue and the familiar 'islet around duct' orientation of mature pancreas. (F) Electron micrograph of duct-like cell lining the cystic structure. Note the polarization with tight junctions and apical microvilli.

with 10% fetal calf serum. When collagen I gel (Vitrogen, Celtrix Pharmaceuticals) or a basement membrane-rich gel (Matrigel, Collaborative Research) were used, 100 μl of the cold liquid gel was added to the filter system. The tissues were then placed on the filter or suspended in the cold liquid gel prior to gel coagulation. Tissues were cultured in 5% CO_2 at 37°C.

Subcapsular implants

Adult female C57 mice were anesthetized with 60 mg/kg pentobarbital. Through a small midline incision in the abdomen, both kidneys were exposed and a tear made in the renal capsule. Tissues were then grasped gently between the tips of fine forceps and placed on the bare area of the kidney with the aid of a microscope at ×6 magnification. The tissues were then gently pushed under the renal capsule and the abdominal wound closed using silk suture. Mice were allowed to awaken and given free access to food and water. After 5-14 days the mice were killed and the pancreas with surrounding kidney tissue was harvested.

Immunohistology

Harvested tissues were fixed in 4% paraformaldehyde for 4 hours, then cryoprotected overnight in 30% sucrose and embedded in Tissue Tek OCT compound and frozen in liquid nitrogen. Tissue sections of 10μm were quenched with 0.3% H_2O_2 in methanol. Slides were then blocked for 30 minutes in 5% normal goat serum (NGS) and 0.1% Triton X-100 in PBS. The sections were exposed overnight (12-16 hours) at 4°C to primary antibody. After washes, the slides were incubated with biotinylated secondary antibody for 1 hour at room temperature using a 1:200 dilution with 1% NGS. ABC reaction (Vectastain) was performed for 45 minutes at room temperature followed by exposure twice for 5 minutes to 0.25% diaminobenzidine for signal enhancement.

Antibodies: (1) Rabbit anti-rat carboxypeptidase A1 (Courtesy of W.J. Rutter laboratory); (2) Rabbit anti-glucagon (Linco 4030-01); (3) Guinea Pig anti-insulin (Linco 4011-01); (4) Rabbit anti-cytokeratin 7 (DAKO 061), specific for ducts within the pancreas.

RESULTS

In order to test how different components of the embryonic microenvironment may affect lineage-specific growth and morphogenesis, early embryonic pancreatic epithelia, with or without the surrounding embryonic pancreatic mesenchyme, were grown in various culture conditions. In general, results reported represent consistent findings in all successful (free of infection or technical problems) cultures performed (more than 10 cultures each for all groups).

In order to establish the baseline of differentiation and morphogenesis, we first investigated the histologic appearance and pancreas-specific gene expression in the pancreatic epithelium at the time of harvesting the pancreatic anlage from the embryos. We found only a scattered, low-level expression of endocrine (insulin and glucagon) genes in the epithelium, with no evidence of acinar enzyme (carboxypeptidase A) gene expression nor morphogenesis at this early time point (Fig. 1). Also at this early time point there was no evidence of morphological structures of the pancreas other than a single lumen communicating with the main lumen of the gut. These findings are consistent with previous findings of others (Teitelman et al., 1987a,b; Herrera et al., 1991; Han et al., 1986).

Initially, to study growth without a three-dimensional scaffolding, the epithelia were grown on filter inserts, situated at the air-fluid interface in an organ culture fashion. To study the effects of a three-dimensional environment, growth of epithelia in a collagen gel matrix was then studied. This gel is a simple polymer and lacks any complex biological structure or growth factors. To then assess the added effect of basement membrane, with potential growth factor activity, the epithelia were cultured in a basement membrane-rich gel (Matrigel). Finally, to assess the effect of the in vivo environment, with the effects of circulating hormones and of vascularization, epithelia were placed under the renal capsule of a syngeneic adult mouse.

Growth on transparent filters

In the presence of mesenchyme, pancreatic epithelia grown on transparent millipore filters at the air-medium interface developed acinar, ductal and endocrine structures.

In the absence of mesenchyme, however, no evidence of differentiation was seen by histological or immunohistochemical criteria. The epithelia did not appear to grow and the cells remained round with no evidence of duct (by cytokeratin 7 staining), acinar (by carboxypeptidase A staining), or islet (by insulin and glucagon staining) formation. These results are consistent with earlier studies on transfilter cultures (Gittes and Galante, 1993; Golosow and Grobstein, 1962; Wessels and Cohen, 1967).

Growth in three-dimensional gel

In the presence of mesenchyme, the pancreatic epithelia grown in either collagen I gel or basement membrane-rich gel grew and differentiated into endocrine, acinar and ductal structures within 7 days (Figs 2, 3). In the absence of mesenchyme, however, the isolated epithelia grown in a collagen I gel did not differentiate (and in fact, even the low level expression of endocrine genes was not seen). This collagen I data suggests that the mere presence of a three-dimensional scaffolding is not sufficient for differentiation. In contrast, the epithelia grown in basement membrane-rich gel formed multiple cystic structures (Fig. 3). By electron microscopy it became apparent that these cystic structures were lined by polarized, secretory epithelial cells. In addition, these cells stained positively for the pancreatic ductal antigen (cytokeratin 7). There were also endocrine cells loosely associated around the cystic structures. Acinar cells were never identified in these mesenchyme-free gel cultures, either by immunohistochemistry (carboxypeptidase A) or by electron microscopy.

Growth under renal capsule of a syngeneic adult mouse

In the presence of mesenchyme, pancreatic epithelia developed into mature pancreatic tissue with acini, ducts and mature islets showing peripheral glucagon expression and central insulin expression (Fig. 4). This full spectrum of differentiation was seen best after 5-10 days of growth under the renal capsule. At later time points (14 days or more), however, the acinar tissue began to undergo autodigestion and fibrosis.

The isolated pancreatic epithelia, grown under the renal capsule for 10 days, showed a dense aggregate of pure mature islets. No ductal structures, no acinar structures, and little or no connective tissue was observed. The islets appeared to be mature because they had the mature orientation of central insulin-expressing cells and peripheral glucagon-expressing cells. (Fig. 5). The possibility of early formation of acini and

ducts followed by autodigestion was essentially ruled out because at earlier time points (3 and 7 days) there were no such structures (data not shown), and additionally, at 10 days, no fibrosis (fibrosis being characteristic of autodigestion; Daikoku et al., 1990; Walker et al., 1992).

DISCUSSION

The control of pancreatic differentiation in the early embryo is poorly understood. Studies 20-30 years ago focused on the possible inductive interaction of the pancreatic mesenchyme with the undifferentiated pancreatic epithelium via a 'mesenchymal factor' (Filosa et al., 1975; Golosow and Grobstein, 1962; Levine et al., 1973; Pictet and Rutter, 1972; Pictet et al., 1975; Ronzio and Rutter, 1973; Rutter et al., 1964). Since then, little research has focused on the control of pancreatic growth and differentiation during development, especially with regard to epitheliomesenchymal interactions (Dudek and Lawrence, 1988; Dudek et al., 1991; Kramer et al., 1987; Spooner et al., 1977; Stein and Andrew, 1989).

The embryonic pancreas is known to pass through at least 3 stages of development (Pictet and Rutter, 1972). The first is an early, undifferentiated stage wherein the endoderm evaginates to initiate morphogenesis. Of the pancreatic cell differentiation genes, only insulin and glucagon are expressed during this phase (Alpert et al., 1988; Gittes and Rutter, 1992; Han et al., 1986; Herrera et al., 1991; Teitelman et al., 1987a,b). A second phase entails epithelial branching morphogenesis with the concomitant formation of primitive ducts. Here islet cells begin to differentiate as they break away from the epithelium and lose their attachment to the basement membrane (Argent et al., 1992; Cantenys et al., 1981; Gu and Sarvetnik, 1993; Pictet and Rutter, 1972). A third stage begins with the formation of acinar cells at the apices of the ductal structures, with development of enzyme-carrying zymogen granules. Acinar cells usually start secreting their enzymes shortly after birth (Chang and Jamieson, 1986; Doyle and Jamieson, 1978; Kolacek et al., 1990; Larose and Morisset, 1977; McEvoy et al., 1973; Oates and Morgan, 1989; Pavelka and Ellinger, 1987).

The default pathway for the epithelial primordium is formation of islets

Isolated pancreatic epithelium, in the absence of mesenchyme and grown under the renal capsule, formed pure clusters of mature islets, without any acinar nor ductal components. The formation of pure islets suggests that the developmental program necessary for the early pancreatic epithelial cells to form mature islets is already in place at this early stage in development, and that the default setting for this early epithelium may be to form islets. This pure islet growth probably does not represent regression of ductal and acinar cells, because at early time points there is no evidence of ducts nor acini. Autodigestion thus seems unlikely, especially since there is no evidence of fibrosis.

The purity of the islets suggests that the lineage of most or all of the original epithelial cells has been channeled toward the endocrine phenotype. This concept is consistent with previous data showing that at the time of harvesting the epithelium from the embryo the only cell differentiation genes expressed at a significant level are endocrine (Alpert et al., 1988; Gittes and Rutter, 1992; Teitelman et al., 1987a,b). We cannot, however, rule out the possibility that the subcapsular environment has selected for endocrine precursor cells and that other precursor cell types have involuted without autodigestion.

The results also show, for the first time, that islets can form in the absence of mature ducts, and suggests that neither ducts nor mesenchyme are required for the formation of islets. Previously, islets have generally been thought to arise from ducts, rather than de novo (Dudek et al., 1991; Gu and Sarvetnik, 1993; Laguesse, 1894; Rao et al., 1990).

Previously, mesenchyme has been thought to be important for <u>all</u> components of pancreatic differentiation and without mesenchyme, only a low level of endocrine gene expression, without any morphologic development, would occur (Rutter et al., 1964). Our results seem to refute this premise, given that we found that mature islets formed from early embryonic epithelium in the absence of mesenchyme.

The islets that formed under the renal capsule in our experiments appear to be mature, with no scattered (extra-islet) endocrine cells, and with a mature arrangement of peripheral glucagon (α-cells), and central insulin (β-cells) expression as seen in mature islets. Reapproximating mesenchyme to these mature islets did not induce differentiation of acinar or duct structures, suggesting that these islet cells are terminally differentiated and that there are no hidden acinar nor duct progenitors. The data suggest that any potential acinar or duct precursors either have been channeled away from acinar and ductal phenotypes, and toward the endocrine phenotype, or else that they have died.

A pure population of islets has not previously been generated from embryonic precursor cells. Since this unique growth could only be seen in vivo, we suspect that specific aspects of the milieu under the renal capsule, such as the nature of the structural scaffolding, the presence of diffusible mediators, or the potential for neovascularization, may be necessary for islet formation. However, it appears that <u>specific</u> embryonic mesenchymal signals are not necessary.

Basement membrane control of ductal differentiation

The formation of ductal structures from isolated epithelia grown in the basement-membrane rich gel suggests that basement membrane or components of basement membrane are important in determining duct cell cytodifferentiation and polarization and three-dimensional ductal morphogenesis. To support this possibility, there are multiple examples of similar inductive effects, including the effects of basement membrane in Matrigel on adult pancreas (Bendayan et al., 1986), mammary epithelium (Sakakura et al., 1979; Streuli et al., 1991), kidney epithelium (Weller et al., 1991), and vascular endothelium (Grant et al., 1989). Laminin seems to be the key molecule in many of these interactions (Grant et al., 1989; Schnaper et al., 1993; Schuger et al., 1990). Heimann showed that primary adult pancreatic duct cell cultures grew best on Matrigel, less well on collagen matrix, and very poorly on plastic alone (Heimann and Githens, 1991). Hootman showed that extracellular matrix protein was important in maintaining guinea pig pancreatic duct epithelium in a differentiated state in culture (Hootman and Logsdon, 1988). Ingber showed that an acinar cell carcinoma cell line could reestablish cell polarity

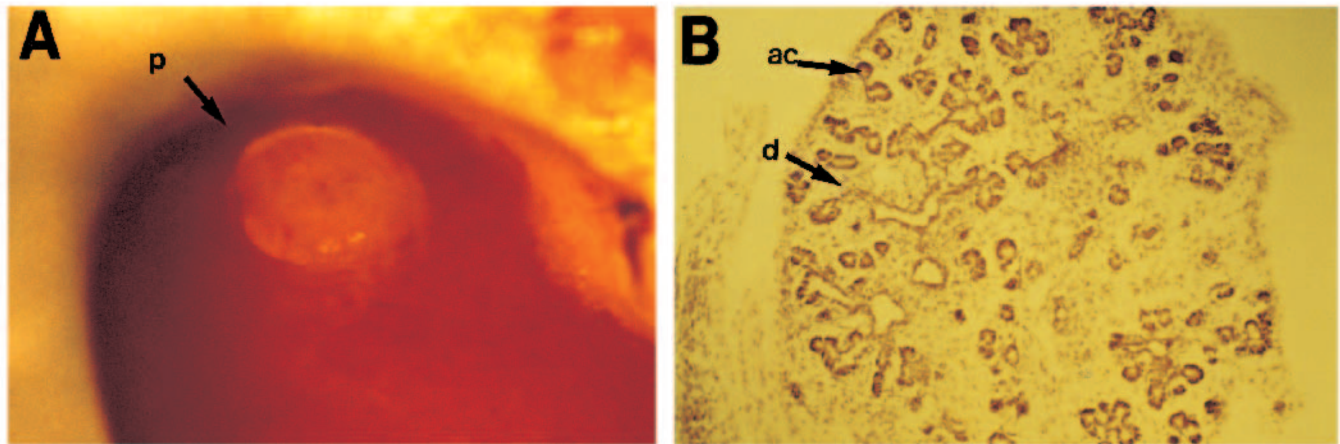

Fig. 4. Whole 11-day embryonic mouse pancreas grown under the renal capsule of a syngeneic adult mouse. Full pancreatic differentiation was seen: (A) viewed through the dissecting microscope, the pancreas (p) is seen with the kidney behind. (B) Staining shows acini (ac) and ducts (d).

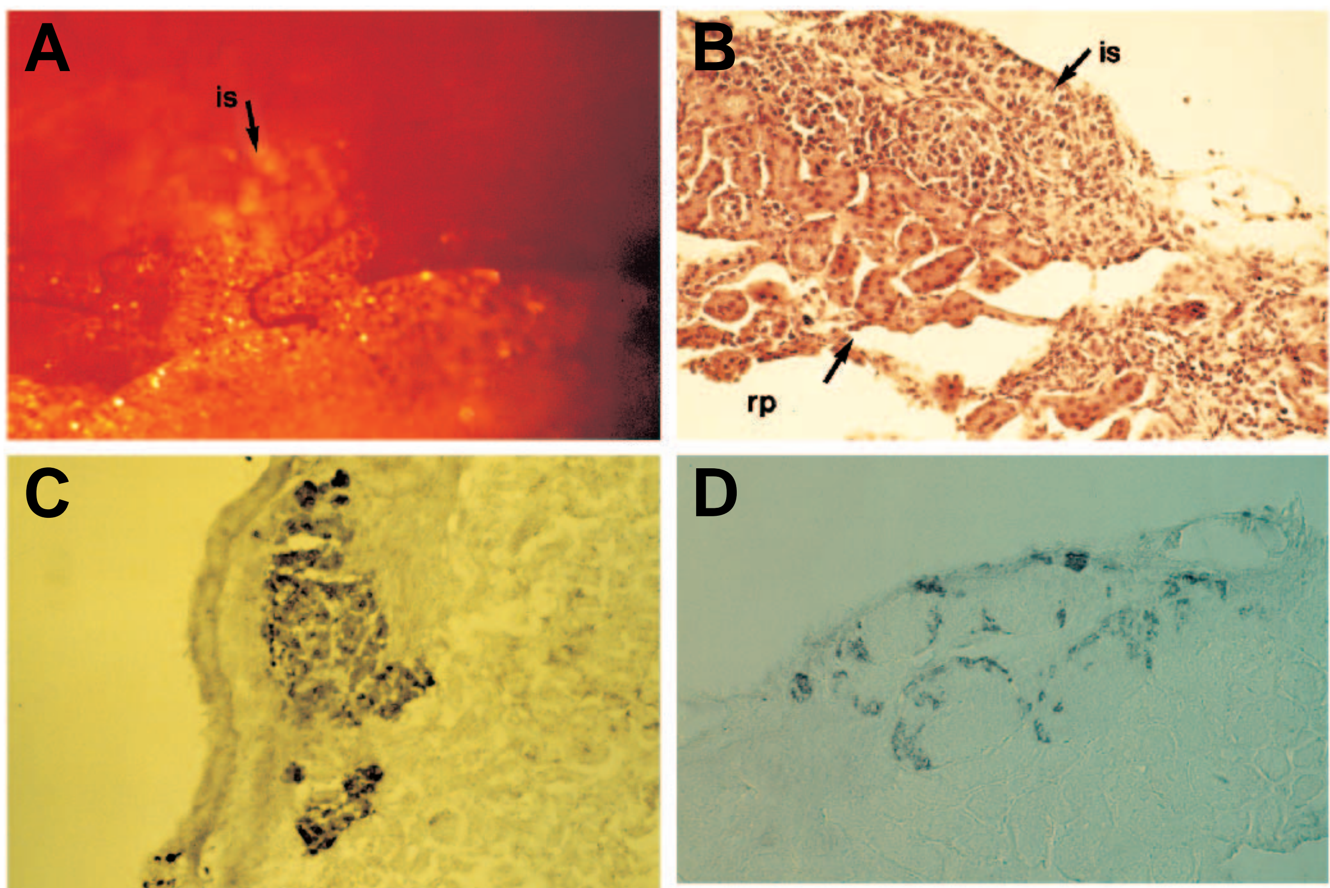

Fig. 5. Isolated 11-day embryonic mouse pancreatic epithelium grown in vivo under the renal capsule of an adult mouse for 7 days. Only pure clusters of islets were observed. (A) Viewed through the dissecting microscope. Note the clusters of white structures which are islets (is). (B) Hematoxylin/eosin staining shows the clusters of islets (is) on top of the renal parenchyma (rp). (C,D) Immunohistochemistry for insulin (C) and glucagon (D) confirm that the subcapsular epithelium has developed into pure clusters of mature islets with central insulin and peripheral glucagon.

if grown on basement membrane from amnion (Ingber et al., 1986). The basement membrane components responsible were laminin, and to a lesser extent, collagen IV.

Sanvito et al. recently showed that whole pancreas from 12.5 day embryonic mice grown in the presence of commercial basement-membrane gel formed cystic structures with little or no acinar nor endocrine cells (Sanvito et al., 1994). Additionally, with basement-membrane rich gel made in their own lab-

oratory these investigators found that the whole pancreatic rudiments formed predominantly endocrine cells and ducts. The differences in their data here are hard to interpret given the variable nature of the response (we observed full pancreatic differentiation of the whole pancreatic rudiment in similar conditions), but when considered in conjunction with our data, may imply some ability of labile diffusible factors in the basement-membrane gel to override the effects of the mesenchyme.

Mesenchyme is only required for acinar differentiation

Throughout all of our studies it appears that mesenchyme is required for acinar development, and possibly that basement membrane components in mesenchyme (e.g. laminin) are required for ductal development. This separation of inductive components in mesenchyme was first suggested in early studies by Rutter in which the effect of 'mesenchyme factor' was separable into a diffusible and a membrane bound component (Filosa et al., 1975; Levine et al., 1973; Pictet et al., 1975; Ronzio and Rutter, 1973). These early studies also showed that pancreatic epithelium without mesenchyme expressed insulin and glucagon at low levels, but did not express acinar genes (Clark and Rutter, 1972; Jin et al., 1992; Przybyla et al., 1979; Rall et al., 1977). It appears that the absence of mesenchyme or basement membrane-rich gel, as in the growth on filter inserts, leads to neither overt differentiation nor morphogenesis. This finding is in contrast to a previous study by Spooner in which acinar and endocrine differentiation could occur in isolated pancreatic epithelia grown alone in tissue culture on plastic (Spooner et al., 1977). The difference from our results may be a reflection of improved dissecting microscope optics and dissecting instruments which allow exclusion of the fine layer of mesenchyme that is otherwise left on the epithelium and which is difficult to see and remove, particularly at later gestational ages. With our techniques we have been unable to reproduce the results of Spooner.

The variable growth pattern and lineage selected by the early pancreatic epithelium is depicted schematically in Fig. 6. Lineage selection beyond the scattered endocrine cells present in the early pancreatic epithelium (Ohlsson et al., 1991, 1993; Wright et al., 1988) may be determined by the presence or absence of basement membrane (for duct formation), and by the presence or absence of 'mesenchymal factor(s)' (for acinus formation). Other than the isolated study by Spooner (Spooner et al., 1977), acinar differentiation and morphogenesis has never been found to occur in the absence of mesenchyme or mesenchymal extracts. The concentration of the necessary 'mesenchymal factor(s)' may determine the proportion of acinar differentiation, e.g. salivary gland mesenchyme, which has been shown to induce an overabundance of acinar tissue from pancreatic epithelium, may contain a greater amount of the mesenchymal factor (Fell and Grobstein, 1968; Golosow and Grobstein, 1962). In addition to the 'mesenchymal factor(s)', basement membrane components such as laminin or collagen IV (present in Matrigel), may be necessary determinants for ductal formation.

Thus development of the pancreatic anlage into the complex, multifaceted mature pancreas seems to entail a complex interplay of signals. Our results give insight into the specific mechanisms of this interplay including the forces controlling the extent and direction of pancreatic morphogenesis during development.

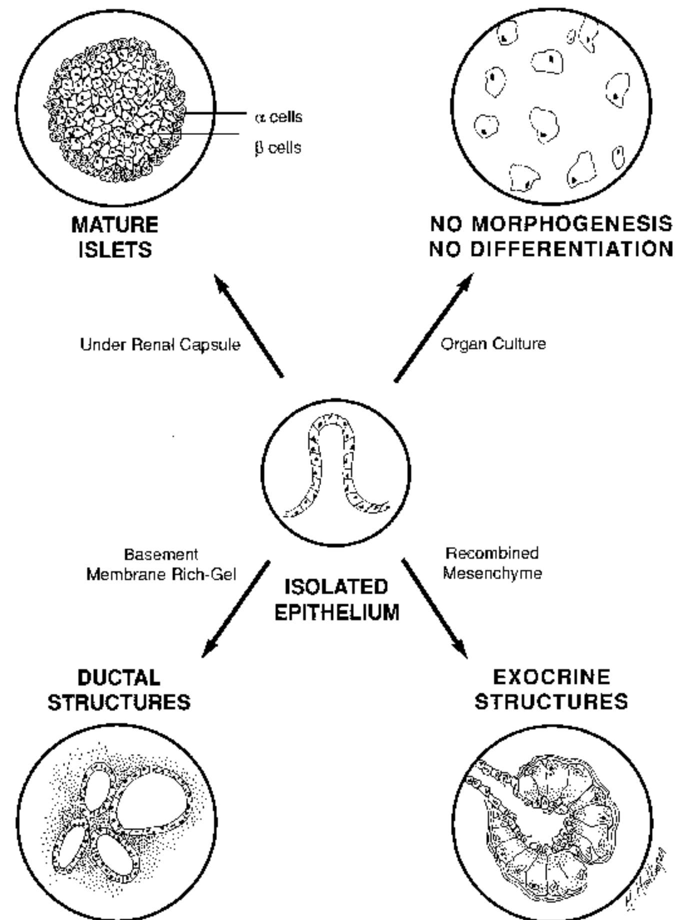

Fig. 6. Schematic illustrating the plasticity of the undifferentiated pancreatic epithelium, depending on its environment. Without a three-dimensional matrix or in a Collagen I gel matrix the epithelium shows no evidence of differentiation or morphogenesis. Under the renal capsule of a syngeneic adult mouse, however, there is full differentiation into clusters of pure mature islets, with no evidence of ductal or exocrine differentiation. In a basement membrane-rich gel (Matrigel), the pancreas differentiates into cystic structures representing pancreatic duct cells. In the presence of mesenchyme, and only in the presence of mesenchyme, do we see the development of exocrine structures.

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