

# Follistatin regulates the relative proportions of endocrine versus exocrine tissue during pancreatic development

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## SUMMARY

In this study, we have investigated the role of the embryonic mesenchyme in the development of the pancreas. We have compared the development *in vitro* of E12.5 rat pancreatic rudiments grown in the presence or absence of mesenchyme. When the E12.5 pancreatic epithelial rudiment is cultured in the presence of its surrounding mesenchyme, both morphogenesis and cytodifferentiation of the exocrine component of the pancreas are completely achieved, while only a few immature endocrine cells develop. The pancreatic rudiments grown in the absence of mesenchyme develop in a completely different way; the exocrine tissue develops poorly and fails to undergo acinar morphogenesis, while the endocrine tissue develops actively. Four times more insulin-positive cells develop after removal of the mesenchyme than in the cultures performed in the presence of mesenchyme. Moreover, the insulin-expressing cells developed in the mesenchyme-depleted

rudiments appear mature since they do not coexpress glucagon, express the glucose transporter Glut-2 and express Rab3A, a molecule associated with the secretory granules. Moreover, these endocrine cells are able to associate and form true islets. Both the inductive effect of the mesenchyme on the proper development of the exocrine tissue and its repressive effect on the development of the endocrine cells are mediated by soluble factors. Follistatin, which is expressed by E12.5 pancreatic mesenchyme, can mimic both inductive and repressive effects of the mesenchyme. Follistatin could thus represent one of the mesenchymal factors required for the development of the exocrine tissue while exerting a repressive role on the differentiation of the endocrine cells.

Key words: Pancreatic development, Follistatin, Mesenchyme,  $\beta$ -cells, Exocrine, Endocrine, Insulin, Rat

## INTRODUCTION

Several studies have pointed out that epithelial-mesenchymal interactions are essential for both morphogenesis and differentiation of the pancreas (Golosow and Grobstein, 1962; Kallman and Grobstein, 1968; Wessels and Cohen, 1967; Wessels and Evans, 1968). *In vitro*, it has been shown that, while the entire E11 mouse pancreatic rudiment can develop, a pancreatic rudiment devoid of mesenchyme fails to grow and shows only very low levels of differentiation (Golosow and Grobstein, 1962). The same authors also showed that the mesenchyme can induce the differentiation of the pancreatic epithelium across a Millipore filter, suggesting that the mesenchyme secretes soluble factors that are necessary for pancreatic development (Golosow and Grobstein, 1962). However, the identity of the soluble factor(s) remains unknown.

Although the studies mentioned above have shown that pancreatic rudiments can develop *in vitro* and that pancreatic differentiation is dependent on epithelial-mesenchymal interactions, they focused mainly on the exocrine component of the pancreas (Pictet and Rutter, 1972; Wessels and Cohen, 1967). Information concerning the role of the mesenchyme in

pancreatic endocrine differentiation is more scarce with some degree of contradiction. While, in some studies, it was shown that the epithelium was unable to differentiate into endocrine tissue in the absence of mesenchyme (Andrew et al., 1994; Wessels and Cohen, 1967), other studies pointed out that endocrine cell development was facilitated in the absence of mesenchyme (Gittes et al., 1996; Rutter et al., 1978). In a recent study, Gittes et al. (1996) have shown that, when pancreatic rudiments were grafted under the kidney capsule in association with their surrounding mesenchyme, they developed into mature pancreatic tissue, forming acini, ducts and endocrine islets. On the contrary, the isolated epithelia (without mesenchyme) grown under the kidney capsule developed, forming dense aggregates of pure islets, with neither acinar nor ductal tissue being found in these aggregates. In the opinion of the authors, these results suggest that specific embryonic mesenchymal factors are necessary for the differentiation of the exocrine component of the pancreas but not for the differentiation of the endocrine part. Based on these observations, it was proposed that the default pathway for the pancreatic epithelial primordium was the formation of endocrine islets.

In the present work, we have studied *in organoculture* the

implication of the mesenchyme in the development of E12.5 rat pancreas. For that purpose we have (i) quantified the number of endocrine and exocrine cells that developed in culture from pancreatic rudiments grown in the presence or in the absence of mesenchyme; (ii) studied the implication of the mesenchyme in the maturation of the cells and (iii) searched for factors that could mimic the effect of the mesenchyme.

Our data demonstrate that, at E12.5, while the mesenchyme is necessary for the proper development of the exocrine pancreas, it represses the development of the endocrine pancreas. Both the stimulatory effect of the mesenchyme on the development of the exocrine pancreas and its inhibitory effect on the development of the endocrine pancreas can be mimicked by follistatin, a known inhibitor of factors implicated in cell differentiation (Nakamura et al., 1990; Ueno et al., 1987; Yamashita et al., 1995). Moreover, follistatin is expressed by the E12.5 rat pancreatic mesenchyme.

## MATERIALS AND METHODS

### Animals and pancreatic rudiments dissection

Pregnant Wistar rats were purchased from Janvier breeding center (Le Genet, France). The morning of the discovery of the vaginal plug was designated as embryonic day 0.5 (E0.5). Pregnant rats at 12.5 days of gestation were killed by cervical dislocation. The embryos were removed from the uterus. Their whole digestive tract was isolated and used either to dissect the dorsal pancreatic primordium (Gittes and Galante, 1993) or to separate the mesenchyme from the dorsal pancreatic epithelium. For that purpose, the dorsal pancreatic rudiment was incubated in a 0.03% collagenase A (Boehringer-Mannheim) solution in RPMI 1640 synthetic medium (Gibco BRL) for 1 hour at 37°C (Kedinger et al., 1981). The enzymatic digestion was stopped by a 30 minutes incubation in a 1:1 mixture of RPMI 1640 medium and fetal calf serum. This treatment provokes the disruption of the basal membrane lining between the epithelial and mesenchymal tissue. Thereafter, the dorsal pancreatic epithelial bulge can be easily freed of its surrounding mesenchyme using tungsten needles.

### Culture of the dorsal pancreatic rudiments in three-dimensional collagen gels

Dorsal pancreatic rudiments with (M+) or without (M-) their mesenchyme were grown into three-dimensional collagen gels in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin as described by Montesano et al. (1983). In some experiments, recombinant human follistatin (obtained from the National Hormone and Pituitary Program of the NIDDK) was added to the culture medium. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Medium was replaced every 48 hours. At the indicated times, the rudiments were photographed and fixed for immunohistology as described below.

### Immunohistology

The pancreatic rudiments were fixed at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 hours, cryoprotected overnight at 4°C in 30% sucrose and frozen. Consecutive sections (6 µm thick) were cut and collected on gelatinized glass slides. For immunostaining, the sections were first incubated for 30 minutes in PBS containing 3% bovine serum albumin. Subsequently the sections were incubated for 2 hours at room temperature (or overnight at 4°C) with the primary antibodies. After washing in PBST (PBS containing

0.5% Tween-20), the sections were incubated with the appropriate fluorescent secondary antibodies. Finally the sections were extensively washed in PBST and mounted with a fluorescence protecting medium (Vectashield, Vector Laboratories, Inc.). The sections were examined and photographed with a Leitz DMRD microscope. Colocalization of the endocrine hormones was determined by double-labeling immunofluorescence. For this purpose, antibodies prepared in two different species were used and revealed using anti-species antibodies labeled with two different fluorochromes. Single-labeled sections incubated with mismatched secondary antibodies showed no immunostaining, confirming the specificity of the secondary antisera.

The antisera employed in this study were used at the following dilutions: guinea pig anti-porcine insulin (Dako) 1:500, mouse monoclonal anti-porcine glucagon (Sigma) 1:2000, rabbit anti-human amylase (Sigma) 1:2000, mouse monoclonal anti-porcine vimentin (clone: V9, Dako) 1:30, rabbit anti-bovine keratins (Dako) 1:200, mouse monoclonal anti-human mitotic proteins (Clone: MPM-2, Dako) 1:30, rabbit anti-rat Glut-2 (East-Acres Biologicals, Southbridge, MA) 1:500, rabbit anti-human somatostatin (Dako) 1:1000, mouse monoclonal anti-human insulin (Sigma) 1:2000 and rabbit anti-human Rab3A (a gift from Dr F. Darchen) 1:500, mouse monoclonal anti-human syntaxin (Sigma) 1:200 and mouse monoclonal anti-rat SNAP25 (Sternberger Monoclonals) 1:200.

The fluorescent secondary antibodies were: fluorescein anti-guinea pig antibodies (Dako) 1:500, and the following secondary antibodies from Jackson Immunoresearch: fluorescein anti-rabbit antibodies 1:200, fluorescein anti-mouse antibodies 1:200, Texas-red anti-rabbit antibodies 1:500 and Texas-red anti-mouse antibodies 1:200.

### Quantitative analysis

To determine the number of each cell type per pancreatic rudiment, serial 6 µm sections were cut and collected on multiwell glass slides. One out of two consecutive sections (that is sections separated by 12 µm) was analyzed by immunocytochemistry for a given antigen in order to avoid counting the same cell twice. 50±5 sections were analyzed per rudiment. Only the immunoreactive cells for which a nucleus was clearly visible were counted. The results of each experimental point were obtained by quantifying the absolute number of each pancreatic cell type in at least 5 pancreatic rudiments. Data are presented as mean ± s.e.m. Statistical analysis was performed using Student's *t*-test.

### RNA extraction and analysis

Total RNA was extracted from pancreatic rudiments (Chomczynski and Sacchi, 1987) and used either for northern blot analysis or for RT-PCR. For northern blot analysis, total RNA corresponding to 10 pancreatic M+ or M- rudiments, were used. RNA were analyzed by electrophoresis in a 1% agarose-formaldehyde gel and transferred to Hybond-N nylon membrane (Amersham). Membranes were cross-linked by exposure to UV illumination. Hybridization was carried out according to the method of Church and Gilbert (Church and Gilbert, 1984), as previously described, (Atouf et al., 1997), using probes labeled by random priming.

For RT-PCR analysis, RNA was reverse transcribed as previously described (Atouf et al., 1997). The oligonucleotides used for amplification were:

Follistatin (sense) 5'-CCGAGGAGGATGTGAACGAC-3'  
 Follistatin (antisense) 5'-CACCACACAAGTGGAGCTGC-3'  
 Cyclophilin (sense) 5'-ATGGTCAACCCACCGTGT-3';  
 Cyclophilin (antisense) 5'-CGTGTGAAGTCAACCACCCT-3'.

30 cycles of amplification were performed. Amplification parameters included a 1 minute denaturation step at 94°C, a 1 minute annealing step at 57°C, and a 30 second extension step at 72°C. The products of amplification were separated on a 2% agarose gel.

## RESULTS

### Morphological and immunohistological development of pancreatic rudiments depleted or not of their mesenchymes

At E12.5, the dorsal pancreatic primordium consists of a central epithelial bud surrounded by a considerable amount of mesenchymal tissue (Fig. 1A). The E12.5 epithelium was separated from its surrounding mesenchyme using collagenase. This procedure allowed removal of the majority of the mesenchymal tissue. However, because at that stage the external surface of the epithelial bud is very irregular and some lobulation is already visible, few mesenchymal cells (vimentin-positive cells) remained attached to the epithelium (Fig. 1B). This residual mass of mesenchyme was very low in the depleted rudiments (M<sup>-</sup> rudiments), when compared to the mass found in the non-depleted pancreatic rudiment (M<sup>+</sup> rudiments) (compare the vimentin staining before (Fig. 1A) and after depletion (Fig. 1B)).

We next compared the development *in vitro* of M<sup>+</sup> and M<sup>-</sup> rudiments. When E12.5 rat pancreatic M<sup>+</sup> rudiments were cultured into a three-dimensional collagen gel, during the first 3 days of culture, the epithelial tissue spread into the mesenchyme and lobulated. After 7 days of culture, dark spots appeared throughout the epithelium. These spots represent areas where zymogen granules accumulate (differentiated acinar cells) (Fig. 1, compare panel C M<sup>+</sup> dorsal rudiments before culture and E after 7 days in culture). On the contrary, M<sup>-</sup> rudiments acquired a spherical shape and no lobulation was observed throughout the culture period. After 7 days *in vitro*, the primordium appeared as small dark spheres surrounded by a variable number of translucent buds (Fig. 1, compare D M<sup>-</sup> dorsal rudiments before culture and F after 7 days in culture).

The quantitative analysis, performed after immunohistochemistry, showed that before culture, the E12.5 dorsal pancreatic rudiments contained  $170 \pm 10$  cells expressing glucagon and  $50 \pm 13$  cells expressing insulin (mean  $\pm$  s.e.m.;  $n=10$ ). Cells expressing somatostatin or amylase were not detected at that stage. After 7 days in culture, the dorsal pancreatic rudiment grown with its mesenchyme (M<sup>+</sup>) had developed mainly into exocrine tissue.  $4500 \pm 220$  amylase-positive cells could be found per pancreatic rudiment. These amylase-positive cells were organized into acinar structures (Fig. 2A). On the contrary, the development of M<sup>-</sup> rudiments into exocrine tissue was poor and only  $900 \pm 150$  amylase-positive cells had developed after 7 days in culture. Moreover, these amylase-positive cells were not organized into acini (Fig. 2B). Thus, the development of the exocrine tissue is favored in the presence of mesenchyme when compared to the absence of mesenchyme (Fig. 3A for the quantitative analysis).

On the contrary, the mesenchyme has a repressive effect on the development of the endocrine tissue. Indeed, four times more insulin-positive cells had developed from M<sup>-</sup> rudiments compared to M<sup>+</sup> rudiments (Fig. 2, compare C and D, and Fig. 3A for quantification). The numbers of glucagon- and somatostatin-positive cells developing *in vitro* during 7 days from M<sup>-</sup> rudiments were also higher than those obtained from M<sup>+</sup> rudiments ( $450 \pm 29$  versus  $250 \pm 20$  for glucagon-positive cells and  $125 \pm 8$  versus  $25 \pm 5$  for somatostatin-positive cells) (Fig. 2C,D).

Northern blot analysis further confirmed these results. As shown in Fig. 3B, the steady-state levels of insulin and glucagon mRNA per rudiment were higher in M<sup>-</sup> rudiments grown during 7 days in culture, when compared to M<sup>+</sup> rudiments grown in the same conditions. Inversely, the amylase mRNA steady-state level detected in M<sup>+</sup> rudiments was higher than those detected in M<sup>-</sup> rudiments.

The mesenchyme is thus necessary for the proper development of the exocrine pancreatic tissue, while it represses the development of the endocrine tissue.

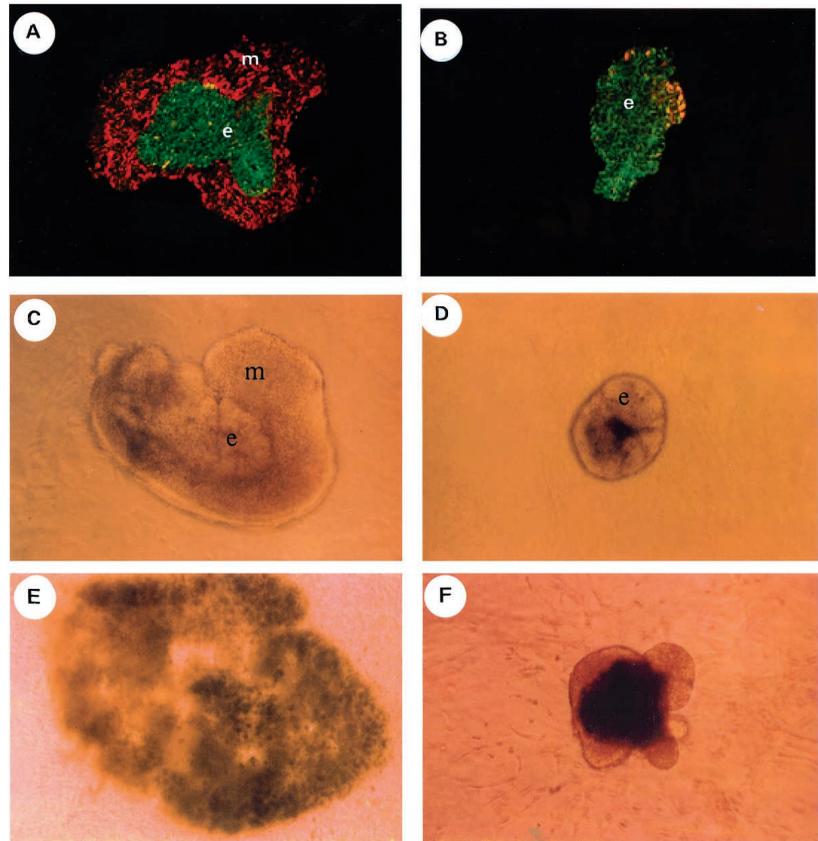
The time course of development of the endocrine mass from M<sup>+</sup> and M<sup>-</sup> rudiments over the 7-day culture period was compared. For this purpose, the number of cells expressing insulin or glucagon at days 0, 1, 3, 5 or 7 of the culture was determined. During the first 2 days of culture, the number of glucagon-expressing cells appeared similar in M<sup>+</sup> and M<sup>-</sup> rudiments. In both cases, the number of glucagon-positive cells increased during the first 24 hours of culture. During the following days, in M<sup>+</sup> rudiments, the number of glucagon-positive cells decreased progressively reaching a mean number of  $250 \pm 20$  at the end of the culture. On the contrary, in M<sup>-</sup> rudiments, the number of glucagon cells remained stable at a mean number of  $450 \pm 29$  glucagon-positive cells per rudiment.

In M<sup>+</sup> rudiments, during the first day *in vitro*, the number of insulin-positive cells was multiplied by 2 ( $50 \pm 13$  before culture versus  $120 \pm 10$  one day later), while a 4.8-fold increase was observed in the number of insulin-positive cells in M<sup>-</sup> rudiments during the same period ( $50 \pm 13$  insulin-expressing cells before culture versus  $220 \pm 14$  1 day later). The spectacular increase in the number of insulin-positive cells observed in M<sup>-</sup> rudiments (4.8-fold increase during the first day in culture) suggested that these endocrine cells arose by differentiation from precursor cells rather than by proliferation of preexisting insulin-positive cells. To further confirm this point, the number of proliferating insulin-expressing cells was determined by quantifying the number of insulin-positive cells expressing the M-phase-specific antigen recognized by the anti-mpm-2 antibody (Westendorf et al., 1994). After 1 day in culture, we found that only 2 % of the insulin-positive cells developed from M<sup>-</sup> rudiments stain positive for both insulin and mpm-2. Thus, the rapid increase in the  $\beta$ -cell mass observed in M<sup>-</sup> rudiments cannot be attributed to the proliferation of preexisting  $\beta$ -cells but rather to the differentiation of precursor cells.

### Characterization of the $\beta$ -cells developed *in vitro*

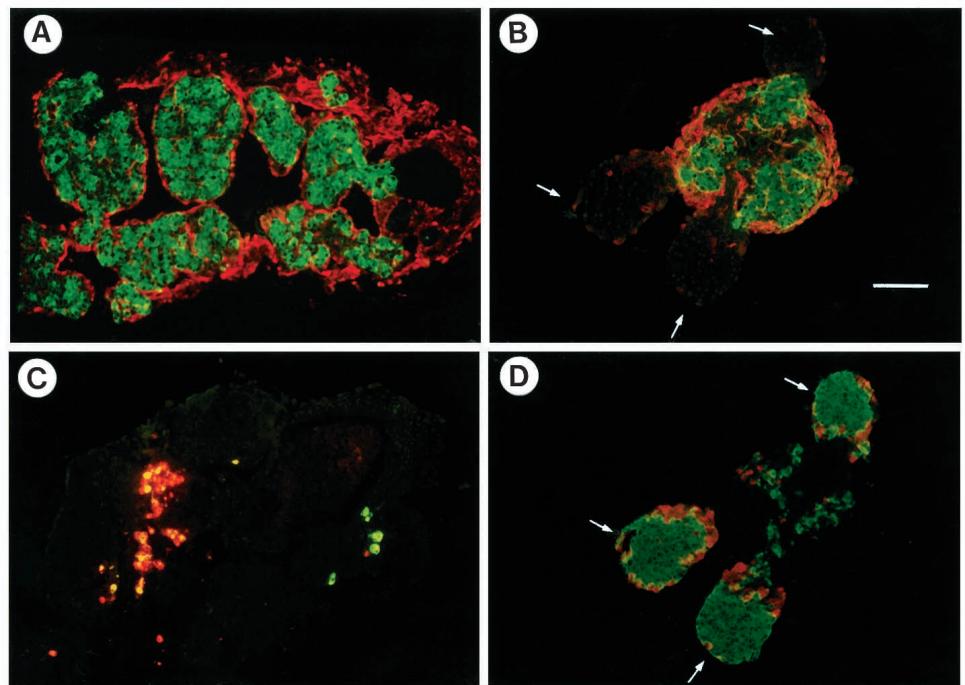
Four different parameters, which are related to  $\beta$ -cells maturity, were chosen to compare the insulin-positive cells developed in M<sup>+</sup> and M<sup>-</sup> rudiments: their property to express only insulin without glucagon coexpression, their capacity to express the glucose transporter Glut-2, their ability to recreate normal islet morphogenesis and their expression of proteins associated with the secretory granules.

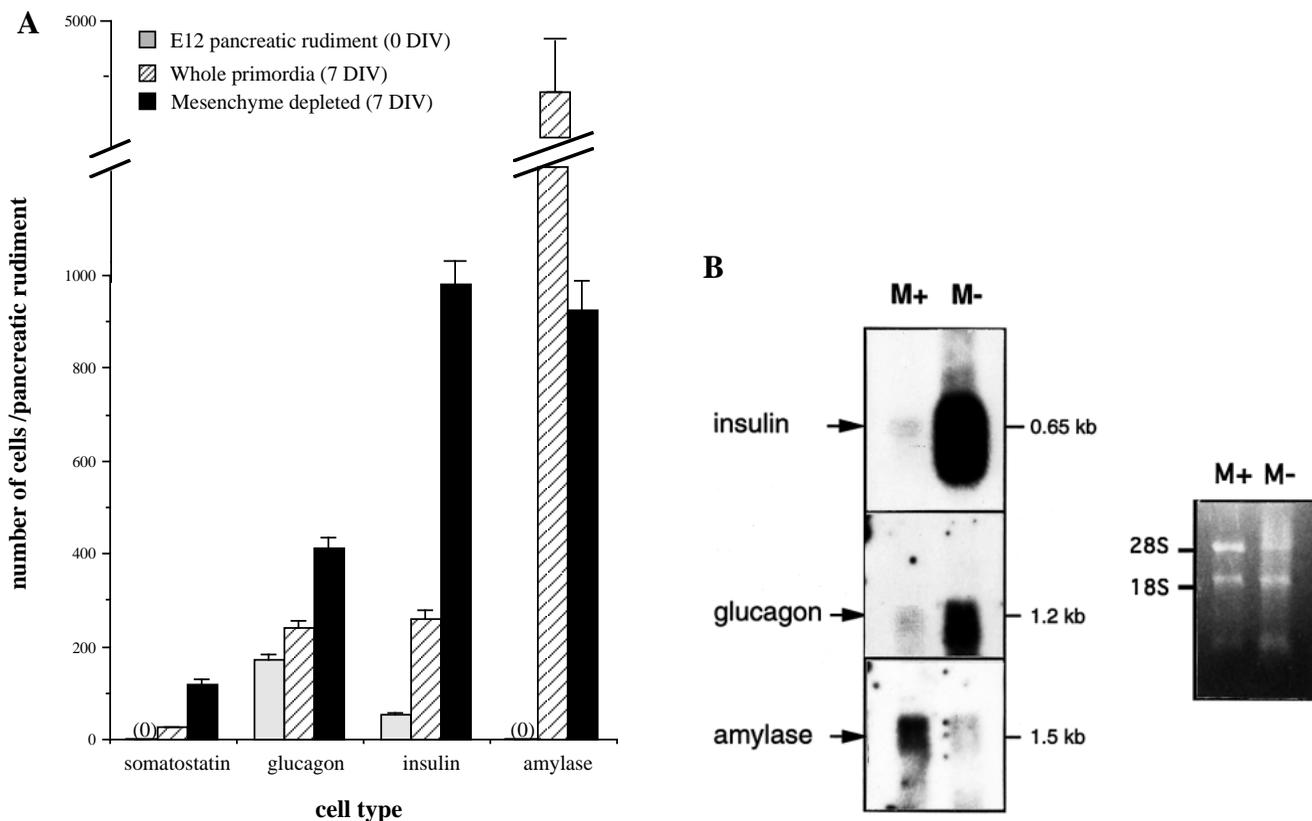
Our immunohistological study revealed that, at E12.5, before culture, all the insulin-positive cells coexpressed glucagon. After 7 days in culture, while in M<sup>+</sup> rudiments 50% of the insulin-expressing cells coexpressed glucagon, almost all the insulin-expressing cells developed in the M<sup>-</sup> rudiments stained negative for glucagon (compare Fig. 2C and D). Islet morphogenesis was next analyzed. As shown Fig. 2C, while, in M<sup>+</sup> rudiments, endocrine cells formed either small clusters, or were dispersed as single cells, in M<sup>-</sup> rudiments, glucagon-positive cells



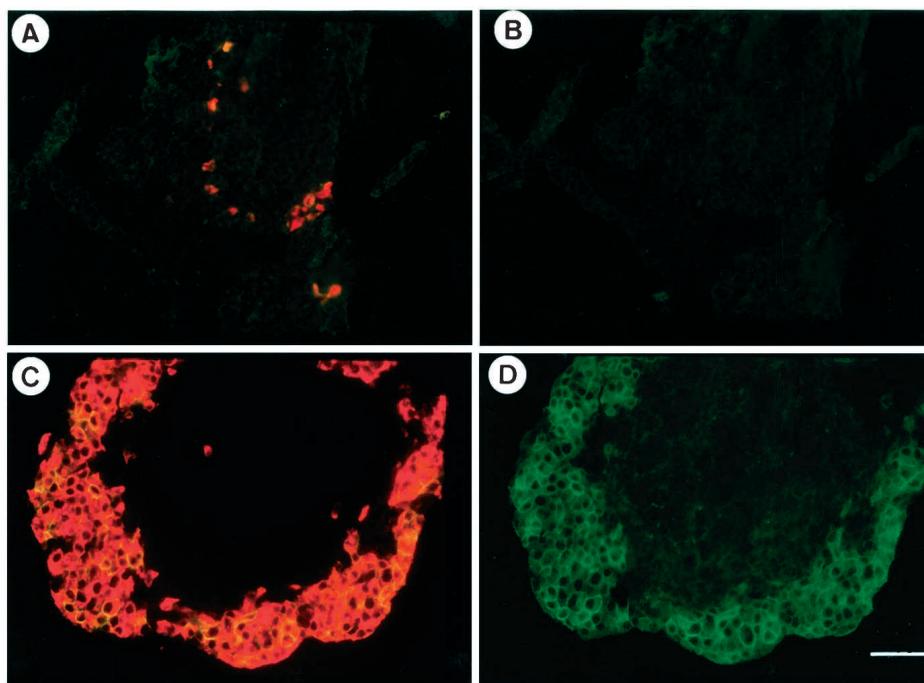
**Fig. 1.** The E12.5 rat dorsal pancreatic rudiment grown into collagen gel. (A) At E12.5, the dorsal pancreatic rudiment is composed of a bulb of epithelial tissue, labeled using an anti-pan cytokeratin antibody (green fluorescence), surrounded by a mesenchymal cap revealed using an anti-vimentin antibody (red fluorescence). (B) After depletion of the mesenchymal cap, while the vast majority of the mesenchymal tissue has been removed, a few mesenchymal cells (red fluorescence) closely associated with the epithelial bulb, are still detectable. (C,E) Isolated E12.5 dorsal pancreatic rudiment (mesenchyme + epithelium) after 1 (C) and 7 days (E) in collagen; (D,F) Isolated E12.5 dorsal pancreatic rudiment depleted of its mesenchyme after 1 (D) and 7 (F) days in collagen. e, epithelial bulb; m, mesenchymal tissue. Magnification: A,B,  $\times 280$ ; C-F,  $\times 200$ .

**Fig. 2.** Immunohistological analysis of pancreatic rudiments grown in vitro during 7 days in the presence (M+) or in the absence (M-) of mesenchyme. E12.5 rat dorsal pancreatic rudiments were grown for 7 days into collagen gel in the presence (M+) or in the absence (M-) of mesenchyme. The development of the exocrine and endocrine tissues were analyzed by immunohistochemistry. (A,B) The exocrine cells and the mesenchymal tissue were evaluated, respectively, after anti-amylase and anti-vimentin staining. (A) In M+ rudiments, a considerable number of amylase-positive cells were detected. These cells were associated forming acinar structures (arrows) dispersed into the mesenchyme. (B) In M- rudiments, acinar cells are confined to a central sphere surrounded by a thin cap of mesenchymal tissue. Moreover, the amylase-positive cells present in M- rudiments do not form acinar structures. (C,D) The development of the endocrine tissue was evaluated after anti-insulin (green) and anti-glucagon (red) staining. (C) In M+ rudiments, glucagon-positive cells form small clusters (red). Few cells express only insulin (green). Cells coexpressing insulin and glucagon appear in yellow. (D) In M- rudiments, the majority of endocrine cells are organized into round structures (arrows), which appear to bud from the central mass of the rudiment. Observe how glucagon-positive cells surround a core of insulin-positive cells like in an islet of Langerhans. Bar, A-D, 100  $\mu$ m





**Fig. 3.** Quantitative analysis of pancreatic development in vitro. (A) The absolute number of somatostatin-, glucagon-, insulin- and amylase-expressing cells was analyzed in M+ and M- rudiments. Gray bars: E12.5 rudiment before culture; hatched bar: M+ rudiments after 7 days in vitro; black bars: M- rudiments after 7 days in culture. Note that after 7 days in culture more endocrine cells have developed from M- rudiments than from M+ rudiments grown in the same conditions. On the contrary, the exocrine tissue developed more from M+ rudiments than from M- rudiments. Each data point represents the mean  $\pm$  s.e.m. of 10 pancreatic rudiments. (B) Northern blot analysis of insulin, glucagon and amylase mRNA steady-state levels in M+ and M- rudiments after 7 days in culture. Total RNA were prepared from 10 pancreatic rudiments grown for 7 days in the presence or absence of mesenchyme and hybridized using insulin, glucagon and amylase probes. Ethidium bromide picture corresponding to the quantity of RNA present in each lane is also presented



**Fig. 4.** Expression of Glut-2 by insulin-positive cells in pancreatic rudiments developed in vitro. Sections of M+ (A,B) and M- rudiments (C,D) grown 7 days in culture were stained for both insulin (red) and Glut-2 (green). Note that, while in M+ rudiments insulin-positive cells do not express Glut-2, in M- rudiments, the vast majority of the insulin-immunoreactive cells stain positive for Glut-2. Bar, 50  $\mu$ m.

accumulate at the periphery of large clusters of insulin-positive cells (Fig. 2D). Thus, structures with the typical organization of islets of Langerhans are formed in M<sup>-</sup> rudiments. We next analyzed the expression of Glut-2 in the insulin-expressing cells developed in vitro. While in M<sup>+</sup> rudiments, Glut-2 expression was not detected in insulin-expressing cells (Fig. 4A,B), almost all the insulin-positive cells developed in M<sup>-</sup> rudiments stained positive for Glut-2 (Fig. 4C,D). Finally, we studied the expression of Rab3A, a protein associated with the secretory granules. In vivo, Rab3A is not expressed by insulin-positive cells present at E12.5 but is found in insulin-positive cells present in the adult pancreas. Rab3A expression was found in insulin-positive cells developed from M<sup>-</sup> rudiments, but not in insulin-positive cells developed from M<sup>+</sup> rudiments (data not shown). Identical data were obtained when the expression of SNAP25 or syntaxin, other proteins associated with the secretory granules, was tested (data not shown).

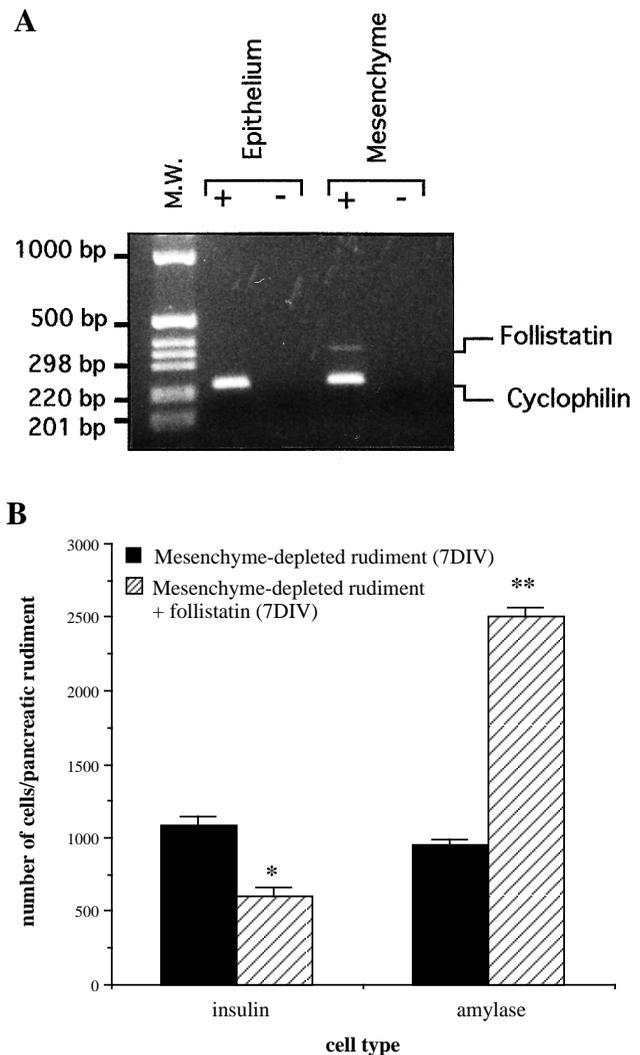
### Reversion of the relative proportions of endocrine versus exocrine tissue after reassociation and upon treatment with follistatin

To evaluate whether the effect of the mesenchyme on pancreatic development was dependent or not on direct contact between the epithelium and the mesenchyme, transfilter experiments were performed. For this purpose, the epithelial rudiment was grown on one side of a transwell filter, while the mesenchyme was placed in the other side of the filter. After 7 days in culture, the epithelial tissue had differentiated similarly to the whole pancreatic primordium (data not shown). This suggest that the mesenchyme can secrete soluble factors that regulate the developmental fate of the pancreatic epithelium.

We next asked whether follistatin could represent one of the factors produced by the embryonic mesenchyme, and thus be able to mimic its effect. By RT-PCR, we demonstrate that follistatin mRNA can be amplified from RNA prepared from E12.5 pancreatic mesenchyme, but not from mRNA prepared from the epithelium at the same stage (Fig. 5A). We next added follistatin (100 nM) to M<sup>-</sup> rudiments and quantified the number of insulin- and amylase-expressing cells developed after 7 days of treatment. As shown in Fig. 5B, in the presence of follistatin the number of insulin-positive cells is decreased by a factor 2 when compared to untreated M<sup>-</sup> rudiments, while the number of amylase-positive cells is increased by almost 3-fold. Thus, follistatin mimics, at least in part, the effect of the mesenchyme: it represses the development of the cells of the endocrine pancreas and induces at the same time the development of the cells of the exocrine pancreas.

## DISCUSSION

Here we demonstrate that, in vitro, the E12.5 rat dorsal pancreatic rudiment surrounded by its mesenchymal cap (referred here as M<sup>+</sup> rudiment) develops in a way that favors essentially the differentiation of the exocrine component of the pancreas. On the contrary, when the mesenchyme is removed (M<sup>-</sup> rudiments), the differentiation of the endocrine cells is largely favored. We also demonstrate that follistatin is produced by the embryonic mesenchyme and mimics both the inductive effect of the mesenchyme on the development of the cells of the exocrine pancreas and the repressive effect of the mesenchyme on the development of the cells of the endocrine pancreas.



**Fig. 5.** Expression of follistatin mRNA by E12.5 rat pancreatic mesenchyme and effect of exogenous follistatin on the in vitro development of M<sup>-</sup> rudiments. (A) Coamplification of follistatin and cyclophilin from RNA prepared from either E12.5 pancreatic epithelium or from E12.5 pancreatic mesenchyme and reverse transcribed in the presence (+) or in the absence (-) of reverse transcriptase. (B) Effect of follistatin treatment on the number of insulin- and amylase-positive cells developed from M<sup>-</sup> rudiments over a 7-day culture period. Each bar represents the mean ± s.e.m. of 6 pancreatic rudiments. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ .

It is well established that the exocrine component of the pancreas can differentiate from embryonic pancreatic rudiments grown in vitro (Golosow and Grobstein, 1962; Pictet and Rutter, 1972). We have confirmed this observation. This proper development is mesenchyme-dependent. Indeed, in M<sup>-</sup> rudiment, five times less amylase-positive cells develop when compared to M<sup>+</sup> rudiments. While the development of the exocrine tissue is severely impaired when the mesenchyme is removed, it is not totally abolished in our experimental conditions. This is at variance with other studies performed in mice showing that, at E11, pancreatic rudiments devoid of mesenchyme do not differentiate into exocrine tissue (Ahlgren et al., 1996; Gittes et al., 1996). The apparent difference between

these studies and ours could depend on the age of the pancreatic rudiment. When E11 rat pancreatic rudiments were depleted of their mesenchyme and grown in culture, no sign of differentiation into exocrine tissue was observed (data not shown).

The major information of the present study concerns the repressive effect of the mesenchyme on the differentiation of the  $\beta$ -cells. Indeed four times more insulin-positive cells developed in the absence of mesenchyme. This repressive effect of the mesenchyme partly contrasts with a previous work demonstrating that the mesenchyme has a permissive effect on the development of the endocrine pancreas (Dudek et al., 1991). It is however difficult to compare this work with our own data, since in the work of Dudek et al. (1991) (i) the fetal mesenchyme was not derived from pancreas but from stomach and (ii) the pancreatic epithelium was not from embryos but from adult rats.

The increase in  $\beta$ -cell mass observed in M<sup>-</sup> rudiments is probably the result of an increase in the number of precursor cells that differentiate into  $\beta$ -cells. This assertion is sustained by the time-course study of the  $\beta$ -cell mass in M<sup>-</sup> rudiments and by the analysis of the proliferative capacity of insulin-positive cells. Indeed, the absolute number of  $\beta$ -cells developed from M<sup>-</sup> rudiments is multiplied by 4.8 during the first 24 hours in culture. If this increase was due to the proliferation of preexisting  $\beta$ -cells, all the  $\beta$ -cells should divide every 5 hours. This is unlikely since our own analysis of the proliferative capacity of insulin-positive cells in M<sup>-</sup> rudiments, demonstrates their very low capacity to proliferate, which is in agreement with previously published observation during late fetal life (Hellerström and Swenne, 1985). Thus, the increase in the number of  $\beta$ -cells developed in the absence of mesenchyme is mainly due to differentiation.

In M<sup>-</sup> rudiments, not only are  $\beta$ -cells more abundant, but they are, according to different criteria, more mature than  $\beta$ -cells generated from M<sup>+</sup> rudiments. Indeed, after 7 days in culture, many of the insulin-expressing cells (50%) developed from M<sup>+</sup> rudiments coexpress glucagon, as insulin-positive cells found in vivo around E12 (Alpert et al., 1988; Jackerott et al., 1996; Pang et al., 1994; Pictet and Rutter, 1972). On the contrary, insulin-positive cells developed from M<sup>-</sup> rudiments like insulin-positive cells found in the adult pancreas, do not express glucagon. A second criterium of maturity is represented by the expression of Glut-2, a high capacity glucose transporter (Thorens et al., 1988). Pang et al. (1994) have shown that, while in vivo before E15 insulin-positive cells do not express Glut-2, the ones developed later do express Glut-2. Our data clearly demonstrate that, while none of the insulin-positive cells developed from (M<sup>+</sup>)rudiments express Glut-2, nearly all the insulin-expressing cells developed in the absence of mesenchyme coexpress Glut-2. The ability of endocrine cells to associate and form insular structures can also be considered as a criterium of maturity. In this sense, although some small clusters of endocrine cells were observed in (M<sup>+</sup>)rudiments cultured for 7 days, no true islets were found. On the contrary in M<sup>-</sup> rudiments, after culture, the large majority of clusters of insulin-positive cells were surrounded by glucagon-positive cells (see Fig. 3F). This suggests that, in M<sup>-</sup> rudiments, the endocrine cells have acquired the capacity to distribute in the way characteristic of the islets of Langerhans. Finally, it is established that insulin-positive cells found in the adult rat pancreas do express molecules associated with the secretory granules such as Rab3A (Regazzi et al., 1996), while insulin-positive cells found at

E12.5, which are known to be devoid of  $\beta$  granules (Pictet and Rutter, 1972), stain negative for Rab3A (the present study). We demonstrate here that, while insulin-positive cells developed in the presence of mesenchyme stain negative for Rab3A, the ones developed in the absence of mesenchyme stain positive for Rab3A. Taken together our results demonstrate that  $\beta$ -cells developed from M<sup>-</sup> rudiments resemble mature  $\beta$ -cells.

Our results together with those of other groups reinforce the idea that, in rodents, insulin-positive cells develop in two waves as proposed by Pictet and Rutter (1972). During the first wave, between E10 and E12.5, a few insulin-containing cells develop, which coexpress glucagon, do not associate to form islets, and do not express either Glut-2 or molecules associated with the secretory granules (Gittes et al., 1996; Pang et al., 1994; Pictet and Rutter, 1972). This phase would be mesenchyme dependent since when the mesenchyme is depleted at E11, both the endocrine and exocrine tissue fail to develop in vitro (Andrew et al., 1994; Wessels and Cohen, 1967 and the present study). This wave is followed by a 3-day period during which the absolute number of insulin-positive cells increases poorly (Herrera et al., 1991; Pang et al., 1994). During the second wave starting at E15.5, a large number of  $\beta$ -cells would differentiate from precursor cells. These new insulin-containing cells do not coexpress glucagon, are Glut-2 positive, express molecules associated with the secretory granules and can associate into islets. When the dorsal pancreatic rudiment is grown in vitro with its mesenchyme, a few insulin-positive cells develop that resemble, at the quantitative and qualitative level, the cells developing in the first wave. On the contrary, after removal of the mesenchyme, insulin-positive cells develop that resemble those developing in vivo during the second wave (the present study). Therefore, according to this scheme, the development of  $\beta$ -cells in the absence of the surrounding mesenchyme resembles what occurs in vivo during the secondary transition, starting at E15.

In different models, an external signal controls the choice of a common precursor between two cell fates. In the peripheral nervous system, for example, the neuregulins have been shown to induce the commitment of multipotent precursor cells derived from the neural crest to a glial cell fate (Schwann cells) with repression of the neural lineage (Dong et al., 1995; Shah et al., 1994). It has been shown that the bone morphogenetic proteins (BMPs), which are members of the TGF- $\beta$  factors superfamily, induce, in a dose-dependent manner, the selective differentiation of the multipotent progenitor cells of the subventricular zone into astroglia, while suppressing the neuronal and oligodendroglial lineages (Gross et al., 1996; Shah et al., 1994). The existence of a common precursor for both endocrine and exocrine lineages has been previously proposed (Gu et al., 1994; LeDouarin, 1988; Pictet and Rutter, 1972). The present study strongly suggests that a mesenchymal factor(s) regulates the differentiation of such a common precursor by inducing its differentiation into an exocrine fate. Our data suggest that follistatin could represent one of these mesenchymal factors. Follistatin is a soluble factor originally purified from ovarian fluid (Nakamura et al., 1990). It is known to bind factors important for cell differentiation that belong to the transforming growth factor superfamily such as activin or BMP7. When follistatin binds these factors, it inhibits their actions (Nakamura et al., 1990; Ueno et al., 1987; Yamashita et al., 1995). Follistatin had been previously shown to be

expressed in the pancreas in development (Ritvos et al., 1995). We demonstrate here that, at E12.5, the pancreatic mesenchyme produces follistatin. We also demonstrate that follistatin can mimic both the repressive effect of the mesenchyme on the development of the endocrine cell mass and its inductive effect of the exocrine cell mass. We thus propose that follistatin produced by the embryonic pancreatic mesenchyme could bind activin or BMP7 produced by the embryonic epithelium and inhibit a possible inductive effect of activin and BMP7 on the development of the endocrine pancreas. This hypothesis is reinforced by recent data demonstrating that both activin and BMP7 are expressed by pancreatic epithelial cells during early development (Furukawa et al., 1995; Lyons et al., 1995) and that members of the TGF- $\beta$  superfamily influence the development of the pancreatic endocrine tissue (Henry et al., 1996; Mashima et al., 1996; Sanvito et al., 1995).

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## REFERENCES

- Ahlgren, U., Jonsson, J. and Edlund, H. (1996). The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. *Development* **122**, 1409-1416.
- Alpert, S., Hanahan, D. and Teitelman, G. (1988). Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. *Cell* **53**, 295-308.
- Andrew, A., Rawdon, B. and Alison, B. (1994). Failure of insulin cells to develop in cultured embryonic chick pancreas: a model system for the detection of factors supporting insulin cell differentiation. *In vitro cell dev biol* **30A**, 664-670.
- Atouf, F., Czernichow, P. and Scharfmann, R. (1997). Expression of neuronal traits in pancreatic beta cells: implication of NRSF/REST, a neuron-restrictive silencer. *J. Biol. Chem* **272**, 1929-1934.
- Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156-159.
- Church, G. and Gilbert, W. (1984). Genomic sequencing. *Proc Natl Acad Sci USA* **81**, 1991-1995.
- Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R. and Janssen, K. (1995). Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. *Neuron* **15**, 585-596.
- Dudek, R., Lawrence, L., Ronald, J., Hill, S. and Johnson, R. (1991). Induction of islet cytodifferentiation by fetal mesenchyme in adult pancreatic ductal epithelium. *Diabetes* **40**.
- Furukawa, M., Eto, Y. and Kojima, I. (1995). Expression of immunoreactive activin A in fetal rat pancreas. *Endocrine Journal* **42**, 63-68.
- Gittes, G. and Galante, P. (1993). A culture system for the study of pancreatic organogenesis. *J Tiss Cult Meth* **15**, 23-28.
- Gittes, G., Galante, P., Hanahan, D., Rutter, W. and Debas, H. (1996). Lineage specific morphogenesis in the developing pancreas: role of mesenchymal factors. *Development* **122**, 439-447.
- Golosow, N. and Grobstein, C. (1962). Epitheliomesenchymal interaction in pancreatic morphogenesis. *Dev Biol* **4**, 242-255.
- Gross, R., Mehler, M., Mabie, P., Zang, Z., Santschi, L. and Kessler, J. (1996). Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. *Neuron* **17**, 595-606.
- Gu, D., Lee, M.-S., Krahl, T. and Sarvetnick, N. (1994). Transitional cells in the regeneration pancreas. *Development* **120**, 1873-1881.
- Hellerström, C. and Swenne, I. (1985). Growth pattern of pancreatic islets in animals. In *The diabetic pancreas* (Ed., Wolk, B. and Arquilla, E.), pp. 53-79. Plenum Publishing Corporation.
- Henry, G., Brivalou, I., Kessler, J., Hemmati-Brivalou, A. and Melton, D. (1996). TGF- $\beta$  signals and a prepattern in *Xenopus laevis* endoderm development. *Development* **122**, 1007-1015.
- Herrera, P., Huarte, J., Sanvito, F., Meda, P., Orci, L. and Vassali, J. (1991). Embryogenesis of the murine pancreas; early expression of pancreatic polypeptide gene. *development* **113**, 1257-1265.
- Jackerott, M., Oster, A. and Larsson, L. (1996). PYY in developing murine islet cells: comparisons to development of islet hormones, NPY, and BrdU incorporation. *J Histochem Cytochem* **44**, 809-817.
- Kallman, F. and Grobstein, C. (1968). Fine structure of differentiating mouse pancreatic exocrine cells in transfilter culture. *J Cell Biol* **20**, 399-413.
- Kedinger, M., Simon, P., Grenier, J. and Haffen, K. (1981). Role of epithelial-mesenchymal interactions in the ontogenesis of intestinal brush border enzymes. *Dev Biol* **86**, 339-347.
- LeDouarin, N. (1988). On the origin of pancreatic endocrine cells. *Cell* **53**, 169-171.
- Lyons, K., Hogan, B. and Robertson, E. (1995). Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during mouse development. *Mech Dev* **50**, 71-83.
- Mashima, H., Ohnishi, H., Wakabayashi, K., Mine, T., Miyagawa, J., Hanafusa, T., Seno, M., Yamada, H. and Kojima, I. (1996). Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J into insulin-secreting cells. *J Clin Invest* **97**, 1647-1654.
- Montesano, R., Mouron, P., Amherdt, M. and Orci, L. (1983). Collagen matrix promotes reorganization of pancreatic endocrine cell monolayers into islet-like organoids. *J Cell Biol* **97**, 935-939.
- Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K. and Sugino, H. (1990). Activin-binding protein from rat ovary is follistatin. *Science* **247**, 836-838.
- Pang, K., Mukonoweshuro, C. and Wong, G. (1994). Beta cells arise from glucose transporter type 2 (Glut2)-expressing epithelial cells of the developing pancreas. *Proc Natl Acad Sci USA* **91**, 9559-9563.
- Pictet, R. and Rutter, W. (1972). Development of the embryonic pancreas. In *Handbook of Physiology* (Ed., DF, S. and N, F.), pp. 25-66, Washington.
- Regazzi, R., Ravazzola, M., Lezzi, M., Lang, J., Zahraoui, A., Anderreggen, E., Morel, P., Takai, Y. and Wollheim, C. (1996). Expression, localization and functional role of small GTPases of the Rab3 family in insulin-secreting cells. *J Cell Sci* **109**, 2265-2273.
- Ritvos, O., Tuuri, T., Eramaa, M., Sainio, K., Hildén, K., Saxén, L. and Gilbert, S. (1995). Activin disrupts epithelial branching morphogenesis in developing glandular organs of the mouse. *Mechanisms of Development* **50**, 229-245.
- Rutter, W., Pictet, R., Harding, J., Chirgwin, J., MacDonald, R. and Przybyla, A. (1978). An analysis of pancreatic development: role of mesenchymal factor and other extracellular factors. *Symp Soc Dev Biol* **35**, 205-227.
- Sanvito, F., Nichols, A., Herrera, P., Huarte, J., Wohlyvend, A., Vassali, J. and Orci, L. (1995). TGF- $\beta$ 1 overexpression in murine pancreas induces chronic pancreatitis and, together with TNF- $\alpha$ , triggers insulin-dependent diabetes. *Biochem Biophys Res Commun* **217**, 1279-1286.
- Shah, N., Marchionni, M., Isaacs, I., Stroobant, P. and Anderson, D. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. *Cell* **77**, 349-360.
- Thorens, B., Sarkar, H., Kaback, H. and Lodish, H. (1988). Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney, and  $\beta$ -pancreatic islet cells. *Cell* **55**, 281-290.
- Ueno, N., Ling, N., Ying, S., Esch, F., Shimazaki, S. and Guillemain, R. (1987). Isolation and partial characterization of follistatin: a novel Mr 35,000 monomeric protein that inhibits the release of follicle stimulating hormone. *Proc Natl Acad Sci USA* **84**, 8282-8286.
- Wessels, N. and Cohen, J. (1967). Early pancreas organogenesis: morphogenesis, tissue interactions, and mass effects. *Dev Biol* **15**, 237-270.
- Wessels, N. and Evans, J. (1968). Ultrastructural studies of early morphogenesis and cytodifferentiation in the embryonic mammalian pancreas. *Dev Biol* **17**, 413-446.
- Westendorf, J., Rao, P. and Gerace, L. (1994). Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope. *Proc Natl Acad Sci USA* **91**, 714-718.
- Yamashita, H., Dijke, P. t., Huylebroeck, D., Sampath, T. K., Andries, M., Smith, J., Heldin, C. and Miyazono, K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J Cell Biol* **130**, 217-226.