Transforming growth factor (TGF)β, fibroblast growth factor (FGF) and retinoid signalling pathways promote pancreatic exocrine gene expression in mouse embryonic stem cells

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Extracellular signalling cues play a major role in the activation of differentiation programmes. Mouse embryonic stem (ES) cells are pluripotent and can differentiate into a wide variety of specialized cells. Recently, protocols designed to induce endocrine pancreatic differentiation *in vitro* have been designed but little information is currently available concerning the potential of ES cells to differentiate into acinar pancreatic cells. By using conditioned media of cultured foetal pancreatic rudiments, we demonstrate that ES cells can respond *in vitro* to signalling pathways involved in exocrine development and differentiation. In particular, modulation of the hedgehog, transforming growth factor β, retinoid, and fibroblast growth factor pathways in ES cell-derived embryoid bodies (EB) resulted in increased levels of transcripts encoding pancreatic transcription factors and cytodifferentiation markers, as demonstrated by RT-PCR. In EB undergoing spontaneous differentiation, expression of the majority of the acinar genes (i.e. amylase, carboxypeptidase A and elastase) was induced after the expression of endocrine genes, as occurs *in vivo* during development. These data indicate that ES cells can undergo exocrine pancreatic differentiation with a kinetic pattern of expression reminiscent of pancreas development *in vivo* and that ES cells can be coaxed to express an acinar phenotype by activation of signalling pathways known to play a role in pancreatic development and differentiation.

Key words: acinar pancreas, differentiation pathway, embryonic stem (ES) cell, endocrine, transcription factor.

INTRODUCTION

Mouse embryonic stem (ES) cells can be propagated indefinitely in the undifferentiated state when cultured in the presence of leukaemia inhibitory factor (LIF) and, upon aggregation, they retain the ability to differentiate into all cell types of ectodermal, mesodermal and endodermal lineages. Clustering of cells leads to the formation of three-dimensional structures, called embryoid bodies (EB), from which cells differentiate in a process that mimics gastrulation and early organogenesis (reviewed in [1–4]). The molecular mechanisms promoting the commitment of stem cells into a specific lineage remain largely unknown. Recently, ES cells adopting an endocrine pancreatic phenotype have been described on the basis of their functional properties [5–8]. However, many important questions remain to be answered including the detailed time-course of pancreatic marker expression during spontaneous differentiation, the precise proportion of ES cells undergoing pancreatic differentiation (a recent re-examination of published data led to the conclusion that as few as $1/10^5$ cells are insulin-producing β-cells [9]), and the degree of functional maturity achieved. In any case, using currently available strategies, the ability of ES cells to differentiate along the various pancreatic lineages is dramatically low. Indeed, their potentiality to differentiate into exocrine cells has only been reported on the basis of amylase and carboxypeptidase transcript expression [10,11], suggesting that a very small number of cells is competent to adopt an acinar fate. This could result from two main limitations. The first one may rely on the appearance of a relatively small fraction of cells within the nascent embryoid bodies spontaneously adopting an endodermal fate, compared with much higher proportions of cells adopting neurectodermal or mesodermal fates [2,3]. Secondly, it is well known that the specification of pancreatic endoderm *in vivo*, takes place in only a subset of gut endoderm cells which both receive an inductive signal from the notochord and aorta, and escape from an inhibitory signal originating from the cardiac mesoderm (reviewed in [12–14]). The occurrence of these processes within the developing EBs remains largely uncontrolled.

Advances in the knowledge of the factors involved in pancreatic development and differentiation are thought to be essential to develop strategies to activate the pancreatic differentiation program from ES cells [15]. Both endocrine and exocrine pancreas originate from a domain of the foregut endoderm [16,17], which expresses at early developmental stages the transcription factor Pdx-1. In the mouse, targeted inactivation of this gene leads to an apancreatic phenotype [18,19], demonstrating its major role in both exocrine and endocrine pancreatic development. Moreover, overexpression of a modified Pdx-1 carrying the VP16 activation domain in other endodermal territories, such as the liver, leads to ectopic pancreas formation [20]. Soluble factors such as activin B and fibroblast growth factor-2 (FGF2), secreted by the chick notochord, repress expression of Sonic hedgehog (Shh) and Indian

Abbreviations used: E8.5 etc., 8.5 days after fertilization etc.; EB, embryoid body; ES, embryonic stem; FBS, fetal-bovine serum; FGF, fibroblast growth factor; HH, hedgehog; Shh, Sonic hedgehog; Ihh, Indian hedgehog; LIF, leukaemia inhibitory factor; PP, pancreatic polypeptide; RA, retinoic acid; RT, reverse transcriptase; TGF, transforming growth factor.

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hedgehog (Ihh), a prerequisite to pancreatic lineage specification, and trigger the emergence of pancreatic precursors expressing Pdx-1 [21]. Most evidence suggests that the endocrine phenotype is activated by default in pancreatic precursors, whereas exocrine differentiation requires the participation of extracellular matrix and mesenchymal cells (reviewed in [12,15,22]). Indeed, there is strong evidence that signalling induced by soluble factors is of crucial importance for cell differentiation during pancreatic development. We have analysed whether growth factors and compounds that can affect biochemical routes important for pancreatic development can also impact on the differentiation of ES cells towards the pancreatic lineage, and more specifically into acinar cells. We show that signalling pathways involved in early pancreas development also regulate exocrine pancreatic gene expression in differentiating ES cells and that the observed temporal patterns of pancreatic gene expression are reminiscent of *in vivo* development. These data, therefore, may be useful for the development of new strategies directing ES cell differentiation towards the pancreatic exocrine lineage *in vitro*.

MATERIALS AND METHODS

Cell culture and reagents

Undifferentiated ES cells (ENS cell line [23]) were cultured at 37 °C in a 7.5 % CO_2 atmosphere on mitomycin-treated mouse embryonic fibroblasts in Glasgow's modified Eagle's medium (GMEM, Gibco) supplemented with 10 % foetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1 % non-essential amino acids (Gibco), 2 mM glutamine, 1 % penicillin–streptomycin, and 1000 units/ml LIF. CGR8, a feeder-independent ES cell line, was also used [24]. AR42J (rat) and 266-5 (mouse) cells display some exocrine-like features and were cultured in Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with 10 % FBS, 2 mM glutamine, 1 mM sodium pyruvate, 1 % non-essential amino acids, and 1 % penicillin–streptomycin.

Recombinant human activin A was obtained through the National Hormone and Pituitary Program, (NIDDK, Bethesda, MD, U.S.A.; from Dr A. F. Parlow). Cyclopamine was a gift from Dr W. Gaffield (U.S. Department of Agriculture, Albany, CA, U.S.A.). All-*trans* retinoic acid (RA) and FGF7 were obtained from Sigma and R & D Systems (Europe Ltd., Oxford, U.K.) respectively.

The following rabbit antisera were used: anti-rat p48 cross-reactive with human p48 [25], anti-amylase (Sigma); anti-frog Pdx-1 cross-reactive with mouse Pdx-1 [kindly provided by Dr C. Wright (Nashville, TN, U.S.A.)]. Peroxidase, biotin or EnVision-labelled goat anti-rabbit Ig (Dako, Carpinteria, CA, U.S.A.) were used as secondary antibodies.

In vitro differentiation of ES cells

ES cells were grown in suspension (5×10^3 cells/ml) in non-adherent Petri dishes to allow formation of EB. FBS concentration was reduced to 3 % and LIF was withdrawn as indicated. In some experiments, EB formation was initiated in hanging drops (10^3 cells in 25 μl). After 2 days, EB were pooled and cultured for 2 to 5 days in Petri dishes. Then EB were allowed to adhere to gelatin-coated cell culture dishes and grown further in ES cell medium supplemented with the indicated factors.

Generation of foetal pancreatic culture supernatants

Foetal pancreas was dissected from mouse embryos at various developmental stages. The pancreatic rudiments ($n = 8$) were cultured in 5 ml of ES cell medium supplemented with 3 % FBS for 7 days. Conditioned medium was then collected, filtered, and stored at − 80 °C. A pool of supernatants was used for each experiment after dilution (1:1) in ES cell medium.

Immunocytochemistry

Sections of frozen EB pools were fixed in 2–4 % paraformaldehyde for 10 min and washed with PBS. For immunocytochemistry, sections were incubated for 10 min in H_2O_2 and levamisole (Sigma) to block endogenous peroxidase and alkaline phosphatase activities, followed by several washes in PBS. To improve the detection of nuclear antigens, sections were treated with 1 % SDS in PBS for 5 min, washed in PBS, and incubated in the blocking solution of the Tyramide-Signal Amplification kit (NEN™, Boston, MA, U.S.A.). Primary antibodies were added for 16 h at 4 °C. After washing in PBS, sections were incubated with peroxidase-coupled anti-Ig for 1 h, then with biotin-tyramide for 5 min, as indicated by the manufacturer. Sections were extensively washed with 20 % (v/v) DMSO, incubated with phosphatase-conjugated streptavidin (2 μg/ml) (Pierce, Rockford, IL, U.S.A.) for 25 min, and further washed in PBS. Reactions were developed using Fast Red as a chromogen. Alternatively, an EnVision-based amplification method (Dako) was used and reactions were developed with diaminobenzidine (DAB).

RT (reverse transcriptase)-PCR analysis

RNA was isolated from EB cells as described by Chomczynski and Sacchi [26] then treated with Dnase I to eliminate any contaminating DNA. From 0.5 to 1 μg of total RNA was reversed-transcribed and cDNAs were amplified using the Superscript one step RT-PCR kit (Gibco). Reactions (25 μl) containing mRNA-specific primers (50 pmoles each) were incubated at optimal annealing temperatures (as described below) and subjected to 35–40 cycles of amplification, except for β-actin cDNA which was amplified for 20 cycles. The PCR products were separated using 2 % agarose gels and stained with ethidium bromide. Their identity was confirmed by restriction endonuclease mapping. RNA from mouse pancreas or from the mouse pancreatic cell line βTC3 were used as positive controls; PCR products generated in the absence of RNA template were used as negative control. The oligonucleotide pairs used for PCR and the size of the amplified products were as follows: Pax4 (60 °C) 5′-CAGCAGGACGGACTCAGCAG-3′ sense and 5′-CAGGAAGGAGGGATTGGCAGT-3′ antisense (870 bp); carboxypeptidase A (58 °C) 5′-GAAATCGCAGGCAGGCACAG-3′ sense and 5′-TGGTGGTTAGGAGGCAGTTTG-3′ antisense (653 bp); Pax6 (55 °C) 5′-AGAAGATCGTAGAGCTAGCT-3′ sense and 5′-TACTGGGCTATTTTGCTTACA-3′ antisense (231 bp); pancreatic polypeptide (PP) (55 °C) 5′-CTGCCTCTCCCTGTTTCTCGT-3′ sense and 5′-GGCCTGGTCAGTGTGTTGATG-3′ antisense (179 bp); Nkx6.1 (55 °C) 5′-TCTTCTGGCCNGGGGTGATG-3′ sense and 5′-CCTGCTTCTTCTTGGCGGTG-3′ antisense (311 bp). Mist-1 (55 °C) 5′-CTAAAGCTACGTGTCCTTGT-3′ sense and 5′-AGAAGAGCAGGCAGGC-3′ antisense (760 pb). Other primers were as described previously: glucagon and insulin [27]; amylase, elastase, chymotrypsinogen, Pdx-1, and p48 [28]; HNF3β (specific for the endodermal isoform) and β-actin [29]; and ngn3 [30]. All experiments were carried out in duplicate and the reproducibility of the observations was confirmed in two or three independent experiments. RNA expression was estimated based on the intensity of the bands of the PCR products as compared with the intensity of the band corresponding to β-actin. Semi-quantitative analysis of PCR

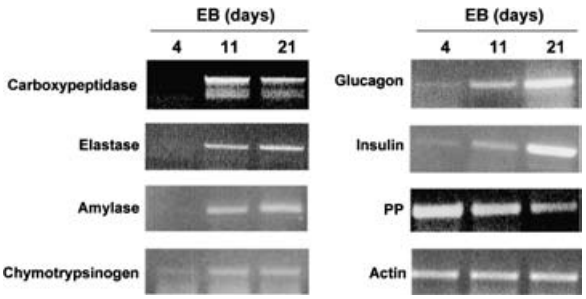


Figure 1 Kinetics of expression of exocrine and endocrine markers in EB

RNA isolated from EB at the indicated times was analysed by RT-PCR using gene-specific primers. Transcripts encoding digestive enzymes (carboxypeptidase, elastase, amylase, chymotrypsinogen) are expressed later than transcripts encoding endocrine hormones (insulin, glucagon, PP). Actin transcript amplification is shown as a control of RNA amount and integrity.

products was performed using an Alpha Imager™ 2000 instrument (Alpha Innotech, San Leandro, CA, U.S.A.) and the alpha ease™ analysis program (Alpha Innotech). Products obtained from mRNA were analysed by densitometry and the ratios of pancreatic markers to β-actin were calculated.

RESULTS

Expression of an exocrine pancreatic phenotype in ES cells differentiated *in vitro*

EB were cultured in suspension for 4 to 21 days, and the expression of endocrine and exocrine differentiation markers was examined by semi-quantitative RT-PCR. Results from a representative experiment are shown in Figure 1. Low levels of glucagon and insulin mRNAs are detected as early as day 4. Higher levels are detected at day 11 and they increase markedly at day 21. PP transcripts are consistently detected in undifferentiated ES cells and in EB. In contrast, none of the exocrine differentiation markers studied (carboxypeptidase A, elastase and amylase) are detected at day 4. These mRNAs are first detected at day 11 and their levels remain constant at day 21. An exception was observed for transcripts encoding chymotrypsinogen that were occasionally barely detected at day 4. These results show that, both endocrine and exocrine markers are detected during ES cell differentiation. In addition, the expression of the majority of the exocrine differentiation markers appears to be activated later than that of endocrine markers.

Expression of mRNA encoding transcription factors involved in pancreatic differentiation

Expression kinetics of a panel of exocrine and endocrine transcription factors were analysed. Several transcription factors expressed during pancreatic differentiation are known to play a dual role, first during early development and later during the specification of pancreatic lineages and terminal differentiation. For instance, Pdx-1 is expressed in endocrine and exocrine progenitors [31]. Subsequently, Pdx-1 is involved in the activation of endocrine and exocrine genes [32]. Similarly, p48 is required at early stages of development, as $p48^{-/-}$ mice lack a pancreas, and it is also required for the activation of the expression of acinar genes [28,32,33]. During EB formation, both Pdx-1 and p48 mRNAs are detected as early as day 4 (Figures 2A and 2B). As shown in Figure 1, transcripts coding for acinar enzymes are not detected at this early stage, suggesting that the p48 and Pdx-1 transcripts detected at day 4 reflect mostly, if not exclusively, progenitor cells. Both p48 and Pdx-1 mRNA levels increase at day 21 in parallel with acinar gene mRNAs. A similar kinetics of expression was observed for HNF3β, a transcription factor involved both in acinar and endocrine gene expression. In contrast, mRNA for Mist-1, a protein involved in the maintenance of acinar cell organization and identity [34], is first expressed at day 11, in parallel with transcripts coding for acinar enzymes, possibly reflecting the emergence of terminally differentiated acinar cells.

Ngn3, a basic helix-loop-helix transcription factor, is critical for the development of all endocrine cells. It is expressed in endocrine progenitors and it is turned off during terminal differentiation [31,35]. Pax4 is likely to be involved in the specification of the β-cell lineage. Its expression is also down-regulated during terminal differentiation [36]. Interestingly, in the developing EB, both ngn3 and Pax4 are strongly expressed at 4 days of differentiation and their levels decrease at the later time points, in accordance with their *in vivo* expression kinetics. In contrast, Nkx6.1 mRNA, whose expression is dependent on Pdx-1 expression in early differentiating β-cells [37] and Pax6, which is expressed in all endocrine cells throughout early pancreatic development [15, 37], are also detected at day 4 but their levels are up-regulated at later time points.

We examined next the expression of p48 and Pdx-1, two factors involved in the expression of acinar genes, and amylase, in day 21 EB by immunocytochemistry (Figure 2C). A very low proportion of cells express either protein. p48 and Pdx-1 are mainly found in the nucleus. Cells immunoreactive for exocrine pancreatic markers are present as singlets or as doublets, though some clusters of reactive cells are also observed. No labelling is detected in undifferentiated ES cells (results not shown).

A comparison of the expression kinetics of pancreas-specific genes during *in vitro* differentiation of ES cells and during *in vivo* foetal development is given in Table 1. It shows that pancreatic markers that are expressed at early stages of pancreatic development *in vivo* (E8.5–9.5; where 'E' indicates days after fertilization) are already expressed at day 4 in early differentiating EB, whereas pancreatic markers whose expression is switched on later (E10.5) are first detected at day 11 in EB, indicating a close temporal pattern of pancreatic gene expression between the two developmental models.

Activation of exocrine differentiation by co-culture with conditioned medium from pancreatic rudiments

The low proportion of ES cells that spontaneously adopt an endodermal phenotype during EB formation prompted us to assess the ability of ES cells, or their early differentiated derivatives, to respond to signals involved in pancreatic development. A schematic representation of the general experimental protocol used for ES cell differentiation is shown in Scheme 1. Conditioned media were prepared by culturing pancreatic rudiments from E13.5, E15.5 and E16.5 mouse foetuses, in medium containing 3% FBS. These conditioned media were then applied to differentiating EB for 7 days and EB were further grown for 1 week in normal medium. A significant increase in the levels of Pdx-1 (2.5-fold) and amylase (4-fold) transcripts is consistently observed (Figure 3A). Conditioned media prepared from E13.5, E15.5 and E16.5 foetuses appear equally efficient at promoting expression of the two markers. The effect of conditioned medium prepared from E15.5 rudiments on the expression of pancreatic markers is confirmed by an immunohistochemical analysis, which shows a marked increase in the number of cell foci immunoreactive for Pdx-1 and amylase (Figure 3B).

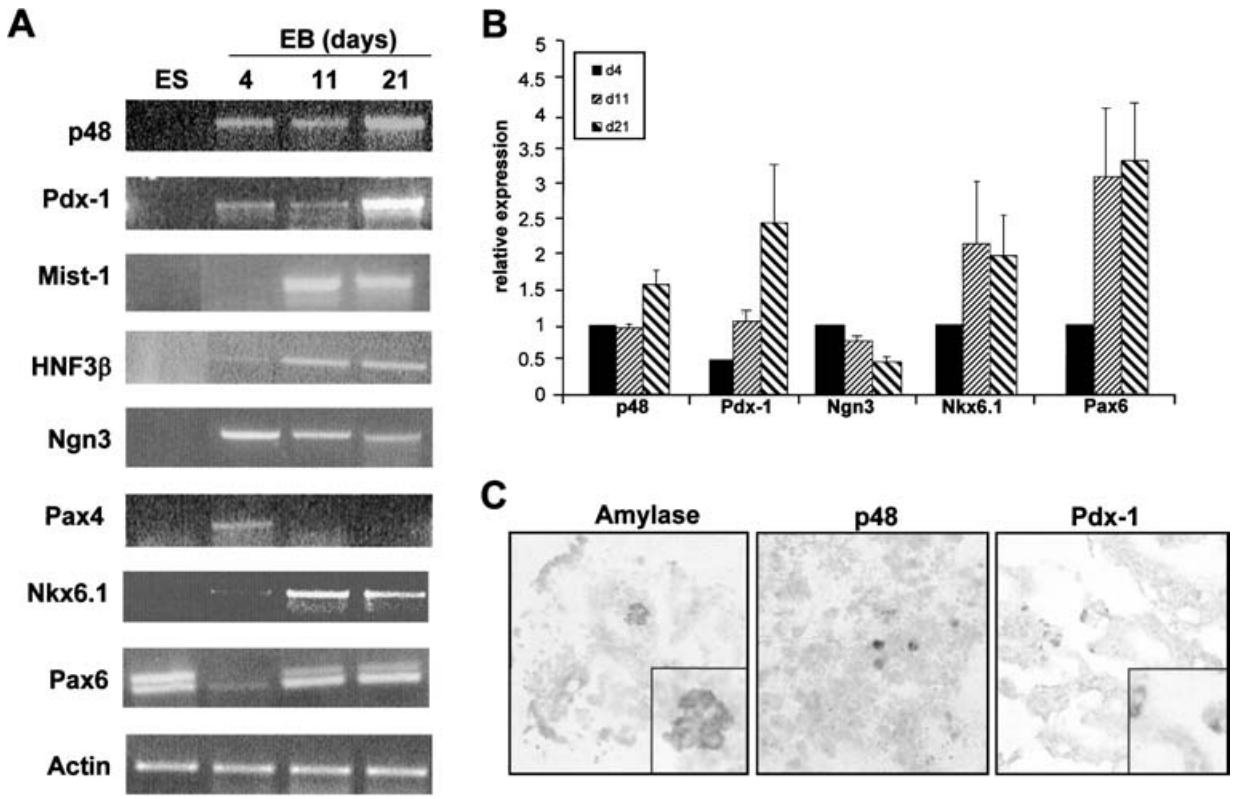


Figure 2 Time-course analysis of expression of genes involved in pancreatic development

(**A**) RNA isolated from undifferentiated ES cells and from EB at the indicated times was analysed by RT-PCR using gene-specific primers (as shown on the left-hand side). Actin transcript amplification is shown as a control for RNA loading and integrity. (**B**) Histograms represent the relative level of expression of selected pancreatic markers quantified by densitometry. mRNA levels present at day 4 (d4) were taken as reference and values were normalized to β-actin mRNA levels. Bars indicate the standard deviations of three independent experiments. (**C**) Immunocytochemical analysis of acinar gene expression in EB. Cryosections of pools of EB grown for 21 days were fixed and subjected to immunocytochemistry using antibodies specific for amylase, p48, and Pdx-1 and an enhanced tyramide technique. Insets show that Pdx-1 immunoreactivity is nuclear whereas amylase is found in the cytoplasm. Original magnification $\times$ 200.

Activation of exocrine differentiation by alteration of specific signalling pathways

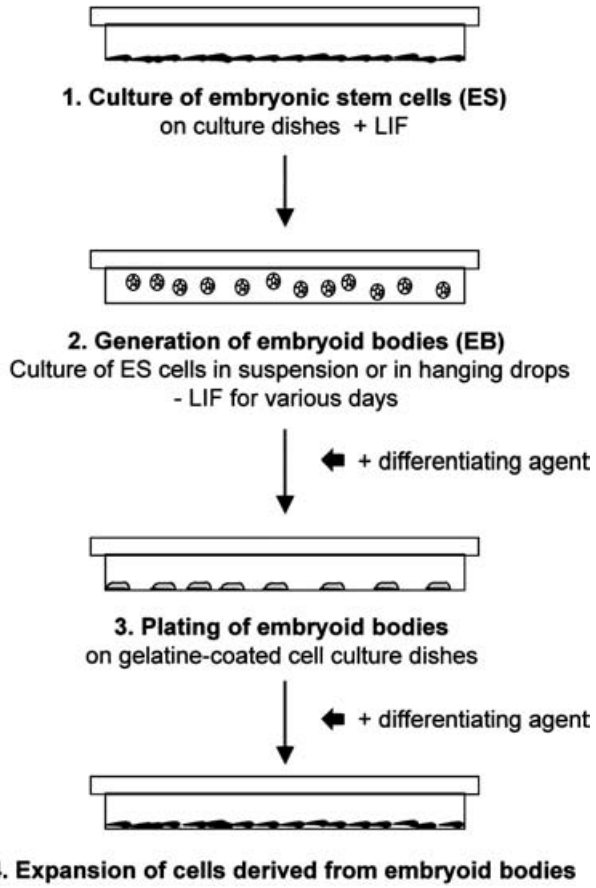
The next step was to investigate which signals could up-regulate exocrine pancreatic differentiation in this system.

Inhibition of Shh-dependent signalling in gut endoderm cells has been shown to be critical for the specification of the pancreatic epithelium [12,21]. Therefore, we examined whether inhibition of Shh signalling during EB differentiation could promote pancreatic differentiation. EB were grown in suspension for 4 days, then allowed to attach to the culture dish in the presence of cyclopamine, an inhibitor of HH signalling, for 24 h. In the absence of cyclopamine, transcripts encoding Pdx-1, insulin, and

Table 1 Pancreatic marker expression in EB as compared with expression during mouse development

(+) Indicates positive signals for RT-PCR reactions after 40 cycles, whereas (−) indicates the absence of signal. Scoring was estimated based on blind assessment of the results of 3 independent experiments performed in duplicate.

Markers	EB (days) 4	11	21	Onset of expression in mouse embryos (days after fertilization)	Expression in adult pancreas	References
Pdx-1	+	+	+	8.5	acinar cells, β-cells	[14,15,22]
Ngn3	+	+	+	9	not detected	[14,15,22]
Nkx6.1	+	+	+	9	β-cells	[15,22]
Pax-6	+	+	+	9	β, α, δ, PP-cells	[15,22]
Insulin	+	+	+	9	β-cells	[47]
Glucagon	+	+	+	9	α-cells	[47]
PP	+	+	+	10	PP cells	[47]
Pax-4	+	−	−	10	not detected	[15,22]
p48	+	+	+	9.5	acinar cells	[33]
Mist-1	−	+	+	10.5	acinar cells	[34]
Carboxypeptidase A	−	+	+	10.5	acinar cells	[47]
Elastase	−	+	+	12	acinar cells	[47]
Amylase	−	+	+	12	acinar cells	[47]
Chymotrypsinogen	−	+	+	?	acinar cells	



Scheme 1 Schematic representation of the general experimental protocol

Undifferentiated ES cells (1) were grown in suspension after LIF removal and induced to form EBs (2) in suspension. In some experiments, EB formation was performed in hanging drops. After culture in non-adhering conditions, EB were pooled and plated in gelatine-coated cell culture dishes (3), to allow EB-derived cells to grow (4). The symbol (⬅) indicates at which stage the differentiating agents have been added, depending on the experiment.

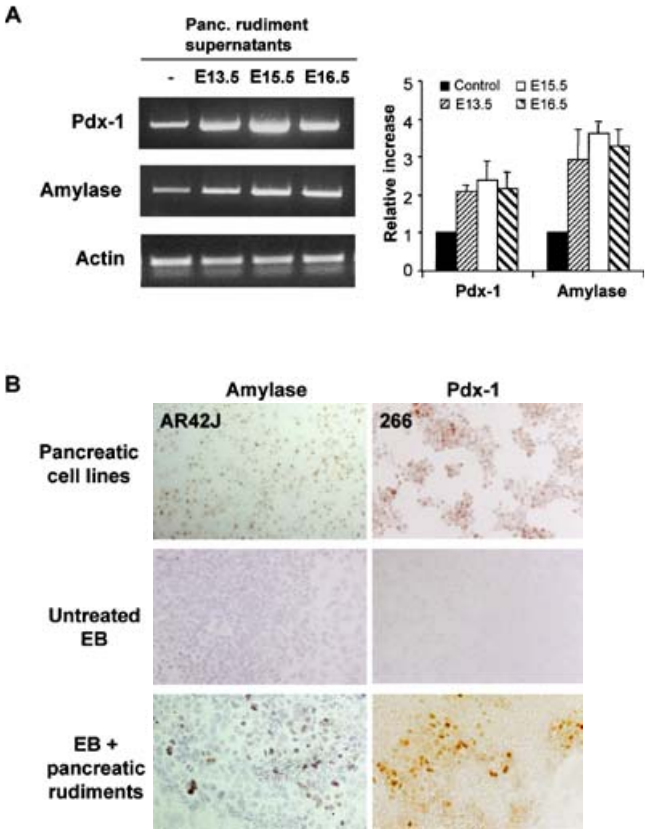


Figure 3 Expression of amylase and Pdx-1 in EB incubated with supernatants generated from embryonic pancreatic rudiments

ES cells were cultured in suspension in 3 % FBS until day 7 with supernatants generated from pancreatic rudiments. Cells were transferred to culture dishes for 7 additional days in the absence of conditioned media. (**A**) RNA isolated from control or supernatant-treated EB was analysed by RT-PCR using gene-specific primers. The developmental stage of the embryonic pancreas used for generating the supernatants is indicated. Actin transcript amplification is shown as a control of RNA amount and integrity. Histograms represent the relative level of expression of pancreatic markers quantified by densitometry. mRNA levels present in control cells were taken as reference and values were normalized to β-actin mRNA levels. Bars indicate the standard deviations of two independent experiments performed in duplicate. (**B**) Immunocytochemical analysis using antibodies specific for amylase and Pdx-1 and an EnVision amplification technique on control cells derived from untreated EB and cells derived from EB incubated with E15.5 pancreatic supernatants. The AR42J and 266-5 pancreatic cells were used as positive controls.

glucagon are barely detectable (Figure 4A). Cyclopamine induces a moderate increase in glucagon mRNA level whereas it induces a strong increase both in Pdx-1 and in insulin mRNA levels. However, no effect on the level of exocrine markers (p48 and amylase) is observed (results not shown).

Several members of the activin TGFβ superfamily have been shown to be expressed in developing pancreatic rudiments, where they regulate the early stages of pancreas development [21]. An increasing amount of data also supports a role of activin signalling in both endocrine and exocrine differentiation [38–40]. The effect of activin A on pancreatic gene expression in developing EB was tested. EB were grown in suspension for 4 days, then allowed to attach to the culture dish for 24 h, and further grown in the presence of activin A for 48 h. Activin A up-regulates the expression both of exocrine (p48 and amylase) and of endocrine genes (Pdx-1, insulin, and glucagon). Importantly, maximal effect is observed at physiological concentrations (1 nM) of activin A (Figure 4B).

Recently, it has been proposed that retinoids play a role in the specification of exocrine phenotypes during pancreatic development [41,42]. To assess the effect of RA on exocrine differentiation of ES cells, EB were grown in suspension for 7 days in the presence of 1 μM RA and then allowed to re-attach and grow in RA-free medium for 7 days. RA up-regulates expression of exocrine-specific (p48 and elastase) genes. In contrast, the expression level of insulin and Pdx-1 mRNA is not affected (Figure 5A).

FGF7 is also emerging as another growth factor that participates in pancreatic development [15]. Incubation of EB with 3 nM FGF7 results in increased levels of transcript pancreatic (Pdx-1) and exocrine differentiation markers (p48, amylase) (Figure 5B).

DISCUSSION

Unlike the endocrine lineage, less is known about the genes and signalling pathways which control acinar pancreatic development and differentiation. This results largely from the availability of a very limited number of immortalized cell lines and the lack of relevant primary cell culture models. Hence, acinar cells undergo a ductal differentiation after 2 days in culture [43] which precludes propagation and genetic analyses of the original cell types. ES cells could provide an alternative system to develop cell culture models representing different stages of acinar differentiation. In this study, we show that (i) mouse ES cells can differentiate *in vitro* into cells expressing markers of the exocrine lineage, (ii) the expression kinetics of pancreatic regulatory genes follows a hierarchy that is reminiscent of pancreas development, and

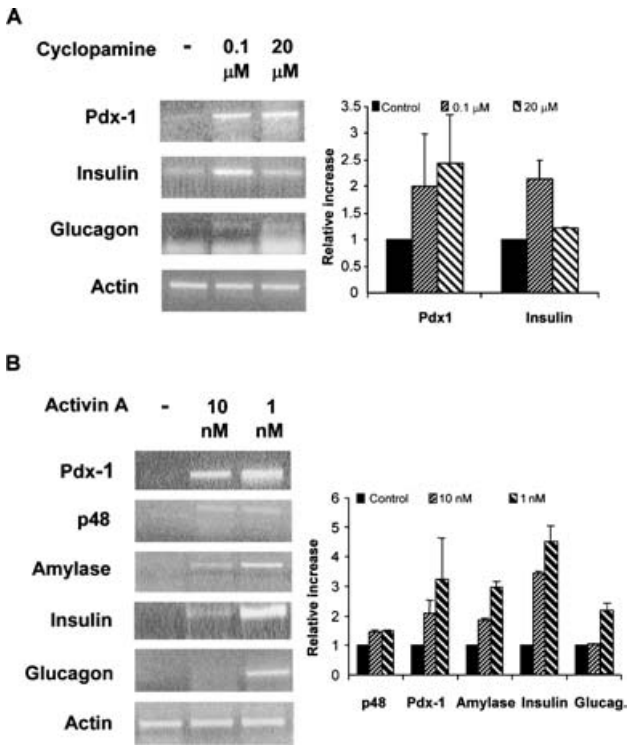


Figure 4 Expression of pancreatic genes in EB incubated with cyclopamine or activin A

ES cells were grown in hanging drops in the absence of LIF, then cultured in suspension until day 4, and transferred to culture dishes. After adhesion, EB were incubated with cyclopamine (**A**) or activin A (**B**) for 24 h or 48 h, respectively. RNA from control, cyclopamine- or activin A-treated EB was analysed by RT-PCR using gene-specific primers. Actin transcript amplification is shown as a control of RNA amount and integrity. Histograms represent the relative level of expression of pancreatic markers quantified by densitometry. mRNA levels present in control cells were taken as reference and values were normalized to β-actin mRNA levels. Bars indicate the standard deviations of two independent experiments performed in duplicate.

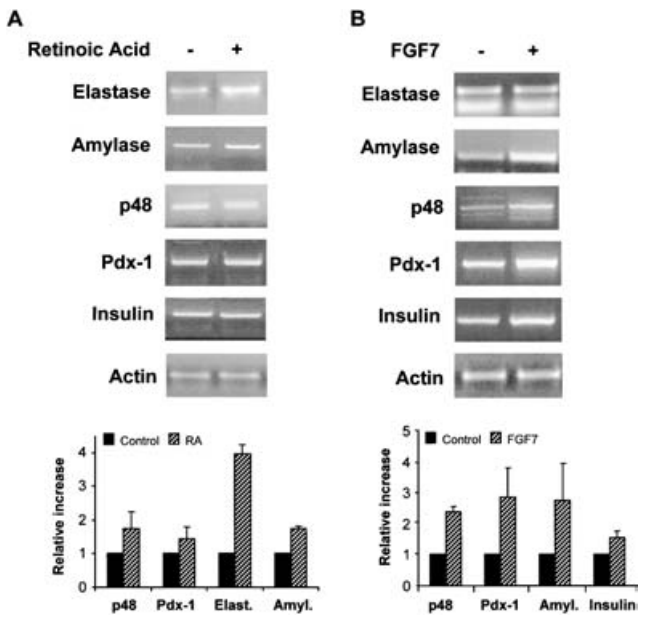


Figure 5 Expression of pancreatic genes in EB incubated with retinoic acid or FGF7

ES cells were cultured in suspension until day 7 with 1 μM RA (**A**) or 3 nM FGF7 (**B**) in 3 % FBS, and transferred to culture dishes. Cells were then cultured for another 7 days without treatment. RNA isolated from control, RA-, or FGF7-treated EB was analysed by RT-PCR using gene-specific primers. Actin transcript amplification is shown as a control of RNA amount and integrity. Histograms represent the relative level of expression of pancreatic markers quantified by densitometry. mRNA levels present in control cells were taken as reference and values were normalized to β-actin mRNA levels. Bars indicate the standard deviations of two independent experiments performed in duplicate.

(iii) signalling pathways that have been shown to be important for pancreatic development can be exploited to bias ES cell differentiation towards the exocrine lineage *in vitro*.

Mouse ES cells differentiate into cells expressing markers of the exocrine lineage

Our results indicate that ES cells can adopt a pancreatic acinar phenotype when spontaneously induced to differentiate, although they do so with a very low efficiency, as reported previously for cells expressing markers of the β-cell lineage [9]. Interestingly, the differentiated cells expressing exocrine markers often appear as small clusters within developing EB, as has been described for insulin producing cells [44], suggesting that local signals produced within the EB are able to promote the exocrine differentiation programme. It also suggests that cell density during EB aggregation may be crucial to elicit this process, as other groups have barely detected an exocrine phenotype when cells were induced to differentiate at higher densities [45]. Alternatively, it could reflect the variability in the ability of ES cell lines to differentiate into the exocrine lineage under similar culture conditions.

The expression kinetics of pancreatic regulatory genes follows a hierarchy that is reminiscent of pancreas development

Pdx-1, Nkx6.1, and Pax4 are expressed concomitantly with the appearance of insulin and glucagon-positive cells during early development of the pancreas, whereas ngn3 is transiently expressed before endocrine differentiation [15,22,37]. It is tempting, therefore, to speculate that transient expression of ngn3 in the developing EB reflects the emergence of endocrine progenitors that later differentiate into more mature endocrine cell types. Taken together, these data suggest that the ES-derived pancreatic cells differentiate beyond the primary transition state as defined by Pictet and Rutter [46], which corresponds to a phase during which a wave of proto-differentiated insulin cells develop. This stage is molecularly characterized by the expression of Pdx-1 and ngn3, whereas progression to the second transition is accompanied by the expression of Pax6, Pax4, Nkx6.1 and Nkx2.2 [14], most of which are found to be spontaneously expressed in our cultures.

Another important result that emerges from our kinetic analysis is that exocrine differentiation is detected after the activation of endocrine markers in the developing EB. This, again, reflects the situation observed during development in which the emergence of endocrine markers precedes that of exocrine markers [47]. In addition, p48 *in vitro* is detected prior to acinar genes in developing EB, a finding that goes along with the fact that p48 is required during early steps of pancreatic endoderm differentiation [33]. In contrast, Mist-1 expression is only detected in late differentiating EB, concomitantly with amylase and carboxypeptidase, reflecting the involvement of Mist-1 in the terminal differentiation of acinar cells [34].

Taken together, these data support the notion that the major molecular pathways which are known to control differentiation of the pancreatic exocrine lineage in the developing mouse foetus may also be operative during the *in vitro* differentiation of mouse ES cells.

Therefore, ES cells provide a good model to analyse the biological role of pancreatic regulatory genes at very early stages. This may help in the understanding and identification of new genes involved in the pancreatic acinar lineage commitment.

Signalling pathways that have been shown to be critical for pancreatic development *in vivo* can be used to bias ES cell differentiation towards the exocrine lineage *in vitro*

We hypothesized that the signals governing pancreatic development, which spontaneously are induced within EB, could be modulated by extrinsic factors to bias ES cell differentiation towards the exocrine lineage. Our data show that pancreatic rudiments release growth factors which promote the expression of Pdx-1 and amylase in differentiating EB. This increase in transcript levels is accompanied by an increase in the proportion of Pdx-1- and amylase-expressing cells, suggesting that conditioned media act by increasing the fraction of gut endoderm cells which commit to pancreatic fate, by promoting the growth of committed cells, or through both mechanisms.

Concerning the possible signalling routes involved in this effect, we have postulated that HH molecules in the developing EB [48] could repress pancreatic differentiation. We therefore used cyclopamine to inhibit HH signalling in differentiating cells. Cyclopamine, known to promote β-cell development and to induce pancreatic heterotopia in chicks [49], induces an increase in the levels of Pdx-1, insulin, and glucagon transcripts in EB. However, cyclopamine does not seem to have any effect on the level of exocrine transcripts. This is unexpected since inhibition of HH signalling in the pancreatic endoderm takes place prior to the endocrine/exocrine specification. However, as pancreatic development proceeds, HH signalling becomes necessary to regulate an appropiate pancreas size [50]. Moreover, the study of mice that have lost Hhip and patched 1 (Ptc), two HH inhibitors, has revealed the requirement of a tight regulation of this pathway to elicit a correct pancreas morphogenesis, islet formation and endocrine proliferation [51]. In contrast, acinar differentiation remains unperturbed in these mutant embryos [51]. Therefore, it is possible that in our system, the inhibition of HH signalling with cyclopamine reflects this scenario.

Repression of Shh expression in the prospective pancreatic epithelium is mediated by FGF2 and activin B [21]. Our data show that physiological concentrations of activin A, which exerts effects redundant to those of activin B, stimulate Pdx-1, insulin, and glucagon expression in a similar manner to cyclopamine. In addition, it activates exocrine gene expression in the developing EB. This activation could result from an indirect effect of the activin A promoting the differentiation of mesenchymal cells [52]. Activin A is well known as a potent inducer of mesoderm development and the mesenchyme is known to promote exocrine differentiation. This hypothesis is further supported by the observation that activin A induces a dramatic increase in transcripts encoding follistatin in EB [52]. In turn, follistatin is a potent inducer of exocrine differentiation of pancreatic precursors [53].

We have also investigated the role of retinoid signalling in pancreatic differentiation of ES cells. RA has been proposed to act as a posteriorising agent in embryonic endoderm in zebrafish (reviewed in [13]). In the chick, RA is sufficient to induce Pdx-1 expression in endoderm anterior to the pancreas [54]. A later role for RA has also been suggested based on *in vitro* experiments. Early embryonic pancreas cultured for 7 days with exogenous RA results in a predominant induction of acini formation with no obvious effect on the endocrine compartment [41]. In our system, incubation of the developing EB cells with all-*trans* RA leads to a specific increase in the level of exocrine gene expression, whereas expression of endocrine markers remains unchanged, suggesting that in these conditions, RA is acting on EB cells already committed to the pancreatic fate. Unlike RA, FGF7 up-regulates expression of both acinar and endocrine genes in the developing EB, which may indicate that pancreatic progenitors are expanded. FGF7 has been shown to increase proliferation of isolated pancreas epithelium and to mimic the effects of the mesenchyme on acinar differentiation [55]. In addition, it has recently been demonstrated that after removal of FGF7 *in vitro*, most of the expanded progenitor cells differentiate into endocrine cells [56]. A combination of these processes could explain the increase of the expression of both amylase and insulin in our system.

A combination of strategies is needed to generate acinar cells from ES cells

We show that signalling pathways that regulate pancreatic development *in vivo* can be used to drive pancreatic differentiation in ES cells. In these experiments, however, where single differentiating agents were used, the variations observed in pancreatic gene expression by RT- PCR were not accompanied by a significant increase in the proportion of pancreatic cells detected by immunostaining. This probably reflects the need to operate in more than one signalling pathway to achieve a significant increase in the efficiency of the cell culture methods, as occurs when foetal pancreatic conditioned media are used (Figure 3). But even when the hypothetical best combinations of pancreatic soluble factors are used, the number of pancreatic acinar cells is far from good enough, which indicates that for detecting a very significant statistical number of cells, the efficiency should be increased by a factor of 10^3–10^4. It seems unlikely that with a single strategy of differentiation this could be satisfactorily achieved. Indeed, the ability of ES cells to differentiate into the endocrine lineage has been shown to be very limited, independently of the biotechnology used [8–9,11,45]. It is reasonable to believe that the integration of different strategies in a single protocol may result in a better optimization of the culture methodologies in future. For instance, the knowledge obtained from our data may be useful to optimize the strategies based on pancreatic acinar cell-specific genetic selection. Identification of molecules, and their optimal combination, to selectively engage an acinar cell specification programme is underway.

We thank the investigators mentioned in the text for providing reagents and Ms Yolanda Tor, for technical assistance. A. S. was supported by CIRIT, Generalitat de Catalunya, and Instituto de Salud Carlos IIII. M. R. was recipient of a graduate fellowship from Ministerio de Ciencia y Tecnología. This work was supported by grants from Ministerio de Educación (PM97-0077; PM99-0142), Ministerio de Ciencia y Tecnología (SAF2001-0432), CIRIT (2001SGR 00410), La Marató de TV-3, European Foundation for the Study of Diabetes, Cardion AG, AVENIR and Association pour la Recherche contre le Cancer (ARC).

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Received 20 November 2003/19 January 2004; accepted 20 January 2004
Published as BJ Immediate Publication 20 January 2004, DOI 10.1042/BJ20031784