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*Original Article*

# Expression of regulatory genes for pancreas development during murine embryonic stem cell differentiation

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**ABSTRACT Insulin-producing cells derived from embryonic stem cells could be surrogates for beta cells in diabetes therapy. However, their derivation remains hard to achieve with current protocols which rely on initial embryoid body formation. We assume that factors known to inhibit pancreas development contribute to this limitation *in vitro*. To evaluate this hypothesis, embryoid bodies were examined after different culture periods by real time RT-PCR to profile the expression of genes known to regulate embryonic pancreas development. Our data indicate that transcripts for pancreas markers (*insulin, glucagon* and *amylase* ) were expressed during differentiation, but the highest levels achieved were at least $10^5$ times lower than in the adult mouse pancreas. *Notch* signalling was activated as suggested by *Delta, Jagged, Ngn3* and *NeuroD1* profiles. However, *Sonic hedgehog,* a known inhibitor of pancreas induction *in vivo* drastically increased in day 6 embryoid bodies, while *Inhibin* $\beta$*A* and $\beta$*B* were down-regulated and *follistatin* up-regulated. Members of the Fibroblast- and Transforming Growth Factor families which pattern the endoderm were expressed at low levels, while those that inhibit pancreas development were highly transcribed. The profile of pancreas regulators expressed in embryoid bodies is therefore not compatible with differentiation of pancreatic and insulin-producing cells. These findings provide an explanation for the limited derivation of such cells to date, in addition to basic information for establishing novel differentiation protocols.**

KEY WORDS: *embryonic stem cell, pancreas development, differentiation factors, Beta cells, insulin.*

## Introduction

Since the establishment of the first embryonic stem cell line in 1981, the potential of their derivatives as a cell replacement in degenerative diseases is still under investigation (Evans and Kaufman, 1981; Martin, 1981). ES cell-derived insulin-producing cells are regarded as a possible means of treating diabetes and overcoming limitations imposed by scarcity of islet donors. Soria *et al.* (2000) investigated the potential of ES cells to develop into insulin-producing cells and other studies claimed similar findings in mouse and human ES cells (Assady *et al.*, 2001; Lumelsky *et al.*, 2001; Shiroi *et al.*, 2002; Kahan *et al.*, 2003). However, there is no generally accepted and efficient paradigm for derivation of beta cells from ES cells and current protocols yield a limited fraction of immature and/or apoptotic insulin-positive cells within a heterogeneous population (Kahan *et al.*, 2003; Rajagopal *et al.*, 2003; Hansson *et al.*, 2004; Sipione *et al.*, 2004). Actually, such cells have been proposed to represent immature neuron-like cells rather than beta cells. Therefore, strategies to differentiate ES cells towards a beta cell-like phenotype, preferably without the need of genetic modifications, are yet to be developed. These will rely mainly on the knowledge acquired from embryo development studies.

Pancreas develops from the foregut endoderm following three major steps: endoderm formation regulated by forkhead box factors, pancreatic morphogenesis regulated by homeobox factors and differentiation of endocrine versus exocrine cells regulated by basic helix-loop-helix factors (reviewed in Chakrabarti and Mirmira, 2003). Molecules influencing endoderm layer establishment are progressively deciphered, highlighting to some extent the well-organised and complex process of pancreas morphogenesis. For instance, expression of Hedgehog factors is not compatible with pancreas initiation. Their exclusion from the presumptive pancreatic region by notochord-derived Activin βB and Fibroblast Growth Factor 2 (Fgf2) allows initiation of a pancreatic program (Apelqvist *et al.*, 1997; Hebrok *et al.*, 1998). By regulating neurogenin 3 (*Ngn3* ), Notch signals as well as epithelial-mesenchymal interactions

*Abbreviations used in this paper:* bHLH, basic helix loop helix; EBs, embryoid bodies; ES, embryonic stem; TF, transcription factor.

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modulate a TFs cascade that control differentiation of exocrine and endocrine subsets (Gradwohl *et al*., 2000; Schwitzgebel *et al*., 2000; reviewed in Jensen, 2004).

Expression of several pancreas/endocrine-related TFs and markers has been extensively studied during ES cell differentiation (Assady *et al*., 2001; Lumelsky *et al*., 2001; Shiroi *et al*., 2002; Kahan *et al*., 2003; Rajagopal *et al*., 2003; Hansson *et al*., 2004; Sipione *et al*., 2004). Forced expression of key endocrine TFs (*Ngn3*, *Pax4* ) in teratocarcinoma or ES cell lines didn't entirely reinforce endocrine differentiation (Blyszczuk *et al*., 2003; Vetere *et al*., 2003). However, very little is known of the expression of extracellular molecules that also regulate pancreas development. Nonetheless, a recent examination of the rare population of pancreatic lineages spontaneously differentiated from ES cells showed reproduction of many features of normal islet differentiation (Kahan *et al*., 2003).

It is undoubtedly that the gene expression program activated early after EBs initiation plays a critical role in the selection of particular developmental pathways by ES cells. Therefore, soluble molecules known to regulate pancreas development *in vivo* might be able to induce or to suppress islet cell differentiation when expressed *in vitro* by differentiating ES cells. To our knowledge, no previous study using the *in vitro* system of ES cell differentiation thoroughly examined the components of major pathways that (negatively) influence early pancreas formation. To test our hypothesis that pancreas inhibitors limit acquisition of pancreatic fate in early EBs, we cultured EBs in non insulin-supplemented conditions and screened for mRNA profiles of genes regulating early pancreas development.

## Results

On morphological examination, early stage EBs showed an outer endoderm and an underlying primitive ectoderm cell layer (Martin, 1981) surrounding a cavity with some apoptotic cells (Fig. 1A: image of a whole EB on upper panel, section of paraffin embedded EB on lower panel). Late EBs displayed cells with various phenotypes representing – as expected – the three germ layers of mammalian development (not shown).

### *Expression of endoderm and pancreas specific markers*

We sought to confirm expression of marker genes for endoderm and more specifically pancreatic tissues. Transcripts of *alpha-fetoprotein* were absent in ES cells and early EBs, but were identified later on (peak around day 22) and the protein was localized in the endoderm layer (Figs. 1B, 2A). Similarly, *albumin* mRNA was detected in EBs and the protein was located in a subset of endoderm cells (Figs. 1B, 2B). Contrary to EBs, undifferentiated ES cells were negative or showed low mRNA levels of *alpha-1 antitrypsin, intestinal fatty acid binding protein* and *caudal homeobox gene 2*. The epithelial and gut endoderm marker *indian hedgehog* (*Ihh* ) was present in ES cells at a moderate level and increased thereafter. In contrast, *sonic hedgehog* (*Shh*) was detected in EBs, but not in undifferentiated ES cells. Similarly, the endoderm marker *Sox17* was not expressed by ES cells, but only low levels could be detected in EBs (Fig. 1B and data not shown). Furthermore, transcripts for *MyoR*, a bHLH TF repressor of definitive endoderm differentiation were detected at high levels (Fig. 1B), suggesting that only a limited definitive endoderm population arose from these cultures.

The beta cell specific marker *insulin1* was detected only at 3 weeks culture, whereas *insulin2* was expressed from the second week and increased towards day 29. *Amylase, glucagon* and *islet amyloid polypeptide* (*Iapp* ) were detected in ES cells and showed a substantial increase afterwards (Fig. 1C). Maximal expression levels of these markers were very low compared to adult mouse pancreas levels, namely $10^{-8}$ to $10^{-5}$ fold by real time PCR. By immunocytochemistry, few insulin-positive cells (<0.1%) were detected by the end of week 3, but glucagon-immunoreactive cells were more frequently identified, consistent with the higher signal in RT-PCR (Figs. 1C, 2C-D). Since we were not interested in showing

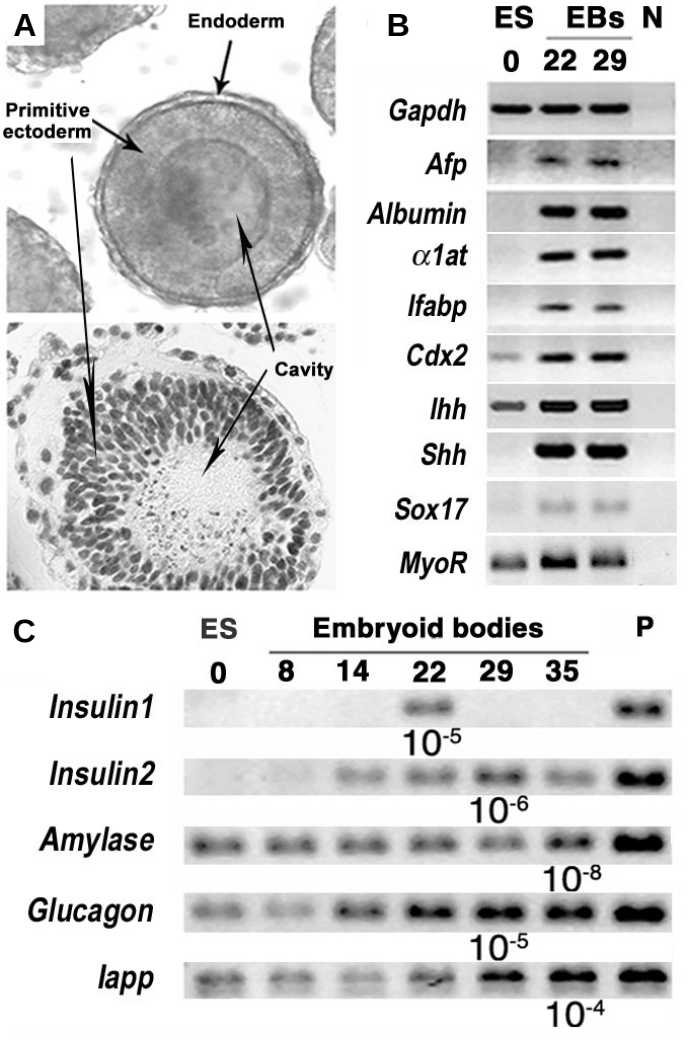


**Fig. 1. EB morphology and expression of endoderm and pancreas markers.** *Four to 6 days old EBs show well organised endoderm and primitive ectoderm layers surrounding a cavity both on whole EB (upper panel) and on paraffin section (lower panel). Original magnifications x200* **(A)**. *Several markers expressed by primitive and definitive endoderm are absent in ES cells and abundant in EBs, except for the definitive endoderm specific marker Sox17* **(B)**. *Pancreas markers are expressed at various levels and stages of differentiation, but remain very low compared to adult mouse pancreas ($10^{-8}$ to $10^{-5}$ fold by real time RT-PCR;* **(C)**). *N, no template control; P, adult mouse pancreas.*

that true beta cells differentiate from ES cells but that insulin-producing cells are very rare, we didn't further characterise these cells by C-peptide detection for instance.

### *Expression profiling of regulatory genes for pancreas development*

The restricted differentiation of pancreatic cells in this system is concordant with the literature and prompted us to critically analyze the profile of major regulatory pathways for pancreas development known from mouse embryo.

### *Hedgehog pathway*

We detected no *Shh* transcripts in ES cells and early EBs. As from day 6, *Shh* was expressed with gradual increase to a pinnacle at day 18 (128 fold increase). In contrast, *Ihh* was expressed in ES cells and increased further up to day 18 (2.8 fold). The hedgehog receptors *Ptc1* and *Ptc2*, as well as the membrane protein *Smo* were all expressed in ES cells and EBs at high levels. Intracellular effectors were present in ES cells and remained so (*Gli2* ) or increased (*Gli1* ; 3.1 fold at day 4) after EBs initiation. The fluctuations of these components paralleled those of the hedgehog molecules (Figs. 3A, 4A).

Considering the very early *in vivo* requirement for *Shh* repression during embryonic pancreas initiation, we sought to confirm the expression of this pancreas inhibitor at the protein level. Indeed, we found a widespread Shh expression in embryoid bodies, encompassing both the outer and the inner cells in many of them (Fig. 2 E-F).

### *Notch pathway*

Several components of the Notch pathway were detected in ES cells, of which some further increased in EBs. *Notch1, -2 and -3* transcripts showed minor increase from day 2 (2.9 fold for Notch1) and did not significantly change thereafter. *Notch4*, expressed at lower levels than other receptors displayed the major variation with down regulation through week 3 (Fig. 3B). Notch ligands *Delta1*, *Jagged1* and *Jagged2,* which are known regulators of pancreas development were all induced in EBs (2.6 to 5 folds). The relative level of *Delta3* remained low compared to other ligands and the initial increase was followed by down regulation through weeks 3 to 5 (Figs. 3B, 4B). We also found a sustained expression of Notch antagonists *Hes6* and *Sel-1l* (Fig. 3B).

### *Wnt pathway*

We measured the expression level of a set of *Wnt* members recently described in the developing pancreas. Our data showed the expression of the *Wnt* receptor *Fz2* at all stages of differentiation with a peak at day 14 (3.1 fold), while *Fz1* was absent from ES or early EBs up to day 6. The three members of the Wnt family (Fz ligands) described in mouse embryonic pancreas were low in early phases of culture and increased substantially only after day 14 (*Wnt2b*, 2.9 fold; *Wnt5a*, 21 fold; *Wnt7b*, 2.1 fold). In contrast, most of the secreted antagonists (*Sfrp1*, *Sfrp2*, *Frzb*, *Sfrp4* ) showed higher levels in the earlier phases of cultures. These patterns suggest a reduced activity of Wnt signals described in mouse embyonic pancreas during the initiation of EBs cultures. (Fig. 4C).

### *Growth factors of the FGF and TGFβ families*

During embryonic development, notochord-derived growth factors (Activin βB and FGF2) repress *Shh* in the dorsal pancreatic anlagen; *in vitro* they activate *Pdx1* expression from the isolated dorsal pancreatic epithelium. Since Activins are homo- or heterodimers of inhibin α, βA and βB (*Inha*, *InhbA*, *InhbB* ), we examined the expression of the inhibins to deduce the Activin profile. ES cells showed minute amounts of *Inha* and *InhbA* subunits, but high levels of *InhbB*, suggesting a higher amount of Activin βB compared to Activin βA, Inhibin A or B. Upon EBs formation, *Inha* followed a biphasic pattern with peaks at days 4 and 22, corresponding to a 3.8 and 11.4 fold respectively. *InhbA*, which was already low in ES cells was further down regulated (40 to 50 times) and showed a slight increase during the last 3 weeks. *InhbB* gradually decreased to a barely detectable level at days 8 to 10 and was then up-regulated to its initial levels in late EBs (Figs. 3C, 4D). *Follistatin* is a potent inhibitor of Activin/TGFβ family members. ES cells expressed its transcripts, which increased

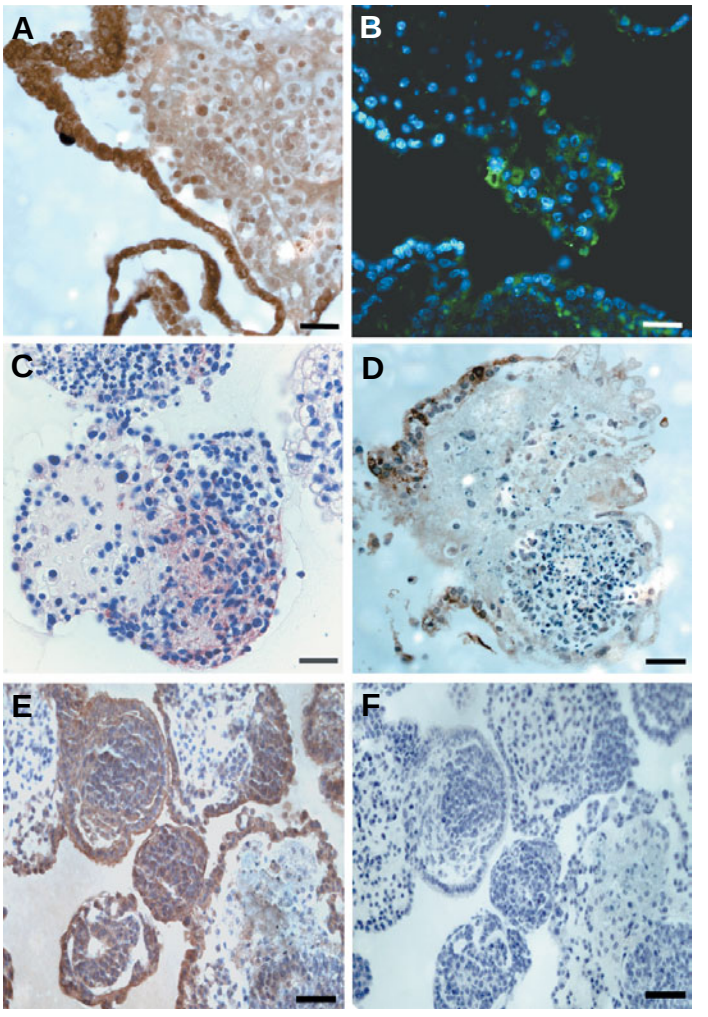


**Fig. 2. Immunodetection of endoderm proteins, pancreatic hormones and Shh**. *Alpha-fetoprotein was confined to the endoderm* **(A)** *as well as Albumin detected in subsets of endodermal cells* **(B)**. *Though very rare, Insulin-positive cells were mainly detected after 3 weeks of culture* **(C)** *as well as Glucagon-positive cells* **(D)**. *The signaling protein Shh was identified in the endoderm and ectoderm layers of many EBs* **(E)**. **(F)** *Negative control. Scale bar, 100 μm (A-D) and 200 μm (E-F).*

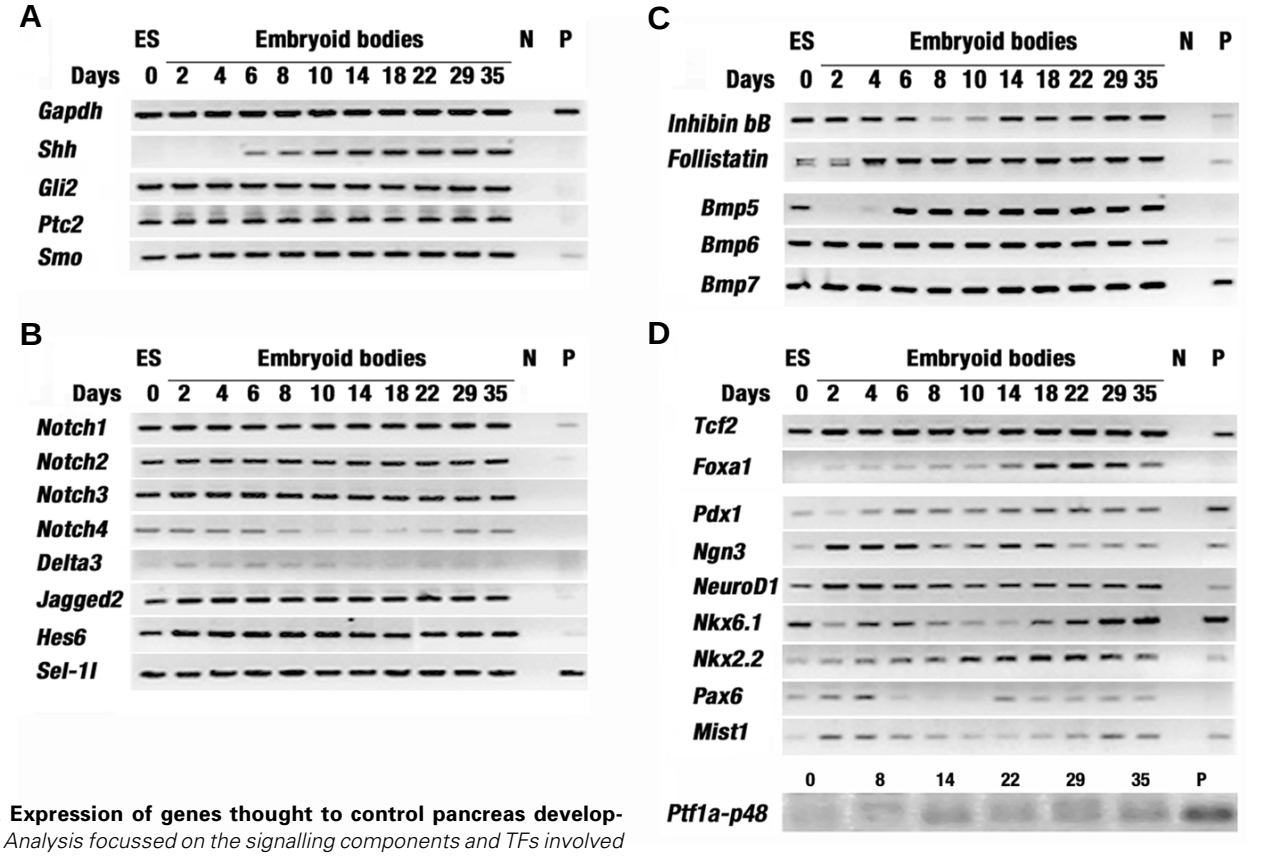


**Fig. 3**. **Expression of genes thought to control pancreas development.** *Analysis focussed on the signalling components and TFs involved in pancreas development including the Hedgehog pathway* **(A)**, *the Notch signalling pathway* **(B)**, *growth factors of the TGFβ family and their inhibitor follistatin* **(C)** *and TFs expressed in pancreas progenitor cells* **(D)**. *N, no template control; P, adult mouse pancreas.*

upon differentiation up to 31 fold by day 18.

Among the bone morphogenetic proteins evaluated in our system, *Bmp6* and *-7* were highly expressed in ES cells and did not considerably change thereafter. *Bmp5* showed a transient shut-off at days 2-4 (30 fold decrease; Figs. 3C, 4D).

*Fgf2* mRNA was detected in ES cells and substantially decreased upon differentiation with a nadir at day 8 (50 fold lower), whereas intermediate levels of *Fgf4* transcripts were identified with a profile similar to *InhbB*. *Fgf10* increased from its high level in ES cells, showing two peaks at days 4 (11.7 fold) and 22 (13.1 fold; Fig. 4D).

### *TFs expressed by pancreatic progenitor cells*

To complete the analysis of the aforementioned extrinsic regulators, we further dissected the profile of TFs expressed by pancreatic progenitors. Of the 4 hepatocyte nuclear factors tested, the expression of *Tcf2*, *Foxa1* and *Foxa2* gradually increased to a pinnacle at day 18-22 representing 28, 134 and 34 fold respectively (Figs. 3D, 4E). *Onecut1* (α and β) profile was biphasic with peaks at days 4 and 22 (9 and 9.6 fold). The pro-endocrine gene *Ngn3* was expressed in ES cells at hardly detectable levels. An increase in its expression was noticed early after EBs initiation (day 2 to 6), but was rapidly followed by a gradual decrease. Likewise, its target *NeuroD1* showed the same pattern with a slower kinetic (Fig. 3D). ES cells expressed high levels of *Nkx6.1*, which was gradually repressed during the initial two weeks. In contrast, *Nkx2.2* increased gradually, showing an overall profile akin to that of *Pdx1*. The pattern of *Pax6* and *Mist1* mimicked that of *Nkx6.1,* whereas *Ptf1a-p48* was only barely detected from day 14 (Fig. 3D).

## Discussion

The limited derivation of insulin-producing cells from ES cells in the framework of published protocols is progressively documented (Kahan *et al.*, 2003; Rajagopal *et al.*, 2003; Hansson *et al.*, 2004; Sipione *et al.*, 2004). Although in EBs definitive endoderm lineages are overwhelmed by preferential ecto- and mesodermal differentiation, most studies relied on the initial formation of these embryo-like structures. Only recently, enhanced derivation of definitive endoderm from Activin-treated embryoid bodies was reported (Kubo *et al.*, 2004). Expression of the analysed endoderm markers followed trends extensively described in literature, but they are not exclusively expressed by definitive endoderm that still lacks a specific marker not expressed elsewhere (Abe *et al.*, 1996; Hamazaki *et al.*, 2001; Kubo *et al.*, 2004). Nevertheless, the patterns of *Sox17* and *MyoR* point to a limited population of definitive endoderm cells contributing to EBs (Kubo *et al.*, 2004; Yu *et al.*, 2004). In the first part of this study, we confirmed EBs expression of pancreatic markers by quantitative RT-PCR and immunocytochemistry. As expected, very few cells express *insulin* or *glucagon* and the maximum transcripts levels achieved were $10^5$ times lower compared to normal mouse pancreas, suggesting that a meaningful differentiation strategy

should increase these markers by at least 1000 fold to represent 1% of pancreas levels. Selection of EBs harbouring dithizon-positive clusters (Shiroi *et al*., 2002) did not significantly enrich for *insulin* mRNA (unpublished observations).

Following the hypothesis that inhibitory pancreas regulators are expressed during EBs cultures, we aimed at establishing a comprehensive and descriptive profile of those pathways so as to build the ground for further work towards pancreatic cell differentiation from ES cells. We therefore screened the expression profile of components of major pathways known to influence embryonic pancreas development over a 5 week-period. In this screening, we focused on extracellular molecules, most of which are soluble and known to have effects at long distance from their site of expression. Transcripts of *Shh* were absent from ES cells, but were highly induced in the first week of culture. As *Shh* is a diffusible potent inhibitor of pancreas induction, it is very likely that its high expression by EBs limits the acquisition of pancreatic fate in ES cell differentiation (Apelqvist *et al*., 1997, Hebrok *et al*., 1998). On the other hand, hedgehog signals favour the development of neurons, dermomyotome, sclerotome and liver at the expense of pancreas and the pattern of its components explains the relative abundance of neuroectodermal cell types as mentioned before (Wichterle *et al*., 2002; Loebel *et al*., 2003; Maye *et al*., 2004). This suggests that the mandatory *Shh* repression in embryonic endoderm should be reproduced in EBs to allow patterning to pancreatic lineages.

Induction of Notch ligands in the presence of their receptors points to a functional Notch pathway in EBs. Indeed, the Notch ligands known to participate in pancreas development (*Delta1, Jagged1, Jagged2*) where found up-regulated between 2 to 5 fold and the *Notch* target *Hes1* was initially repressed 8-fold at day 2, then modulated from that point between 50 – 225%, giving a

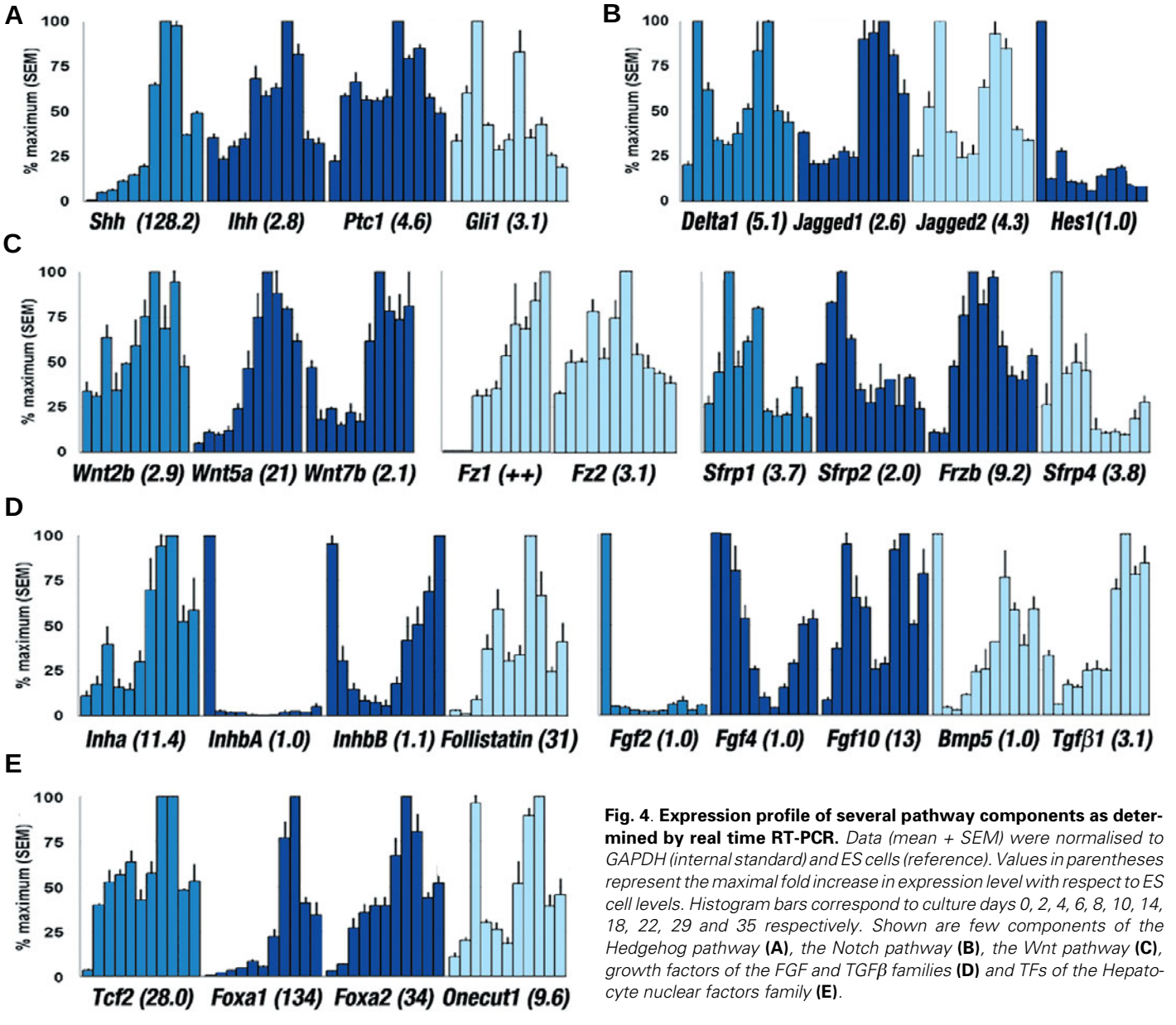


**Fig. 4**. **Expression profile of several pathway components as determined by real time RT-PCR.** *Data (mean + SEM) were normalised to GAPDH (internal standard) and ES cells (reference). Values in parentheses represent the maximal fold increase in expression level with respect to ES cell levels. Histogram bars correspond to culture days 0, 2, 4, 6, 8, 10, 14, 18, 22, 29 and 35 respectively. Shown are few components of the Hedgehog pathway* **(A)**, *the Notch pathway* **(B)**, *the Wnt pathway* **(C)**, *growth factors of the FGF and TGFβ families* **(D)** *and TFs of the Hepatocyte nuclear factors family* **(E)**.

biphasic profile that somewhat followed *Delta1*, *Delta3* and *Jagged2* patterns. Although *Hes1* shows a small response to Notch signals in some tissues, the initial down-regulation suggests a tight control of the pathway by Notch antagonists *(Notch3*, *Hes6* and *Sel-1l)* (Donoviel *et al.*, 1998; Bae *et al.*, 2000). The secondary down-regulation of *Ngn3* and *NeuroD1* was likely coupled to *Hes1* activity, revealing a prolonged Notch signalling in EBs. *In vivo*, Notch signalling defects accelerate endocrine differentiation while constitutively active *Notch1* represses both endocrine and exocrine development (Apelqvist *et al.*, 1999; Hald *et al.*, 2003; Hart *et al.*, 2003; Norgaard *et al.*, 2003). As demonstrated *in vivo*, a sustained Notch activity in EBs would impede the differentiation of tissues such as pancreas that rely on the attenuation of this pathway.

*Wnt* signalling was recently suggested to participate in pancreas development, based on the expression of several *Wnt* genes, receptors and antagonists in the developing and adult pancreas (Heller *et al.*, 2002, Heller *et al.*, 2003). Although convincing data are still lacking on the requirement of *Wnt* signals during initiation of pancreatic buds *in vivo* and on its exact role in pancreas development, our expression profiling suggests that if those Wnt genes are required for the pancreatic cells to develop, this role is not achieved in EBs system as they are not consistently expressed during the major patterning events.

Activin βB and Fgf2 repress endodermal *Shh*, although this exclusion is not sufficient to induce pancreatic genes outside the presumptive anlagen. Patterning of the early endoderm by Fgf4 is necessary to define a window of competence to pancreatic fate (Hebrok *et al.*, 1998; Wells and Melton, 2000; Kumar *et al.*, 2003). Expression patterns of *Inhibin* in our EBs compare to that found by Albano *et al.* (1993) and illustrate a decrease of Activins that would otherwise repress *Shh*. Indeed, *Shh* expression in EBs suggests that its ordinary repression by Fgf2/Activin βB signals was not effective and this is consistent with the observed profiles of both molecules in our study. Furthermore, the *follistatin* profile was compatible with the complete inhibition of the low levels of Activins achieved in these cultures (de Winter *et al.*, 1996). Activin was recently reported to induce definitive endoderm from ES cells; therefore the observed down regulation of *InhbA* and *InhbB and* up-regulation of *follistatin* upon EBs formation also explains the limited differentiation into endoderm tissues (Kubo *et al.*, 2004). Considering the profiles and effects of FGF and TGFβ family members in endoderm/pancreas development, we presume that their combined supplementation would be one option for improving the pancreatic fate in differentiating ES cells; especially because EBs treatment with Fgf2 alone induced meso- and ectodermal derivatives, while exposure to cyclopamine or Activin A increased insulin by only 2-4 fold (Schuldiner *et al.*, 2000; Skoudy *et al.*, 2004). Similarly, the blocking of *Bmp6* could be associated in this strategy as its expression under *Pdx1* promoter leads to complete pancreas agenesis (Dichmann *et al.*, 2003). The call for this concomitant modulation of several pathways is even made imperative by data from Léon-Quinto *et al.* (2004) showing significant increase in insulin-producing cells (19%) only after EBs co-culture with pancreatic rudiments, but not upon application of anti-Shh antibody.

The majority of TFs expressed by pancreas progenitors were detected, however the expression of these factors is not a *bona fide* illustration of pancreatic differentiation within heterogeneous EBs since many of them are shared with neuroectoderm. The selection of the mesendoderm progenitor from early EBs might help to resolve this issue (Kubo *et al.*, 2004). During development, islet progenitors arise from *Ptf1a-p48*+/*Pdx1*+ cells through removal of repressive and stimulation of inductive pathways, a phenomenon unlikely to occur in EBs considering the low *Ptf1a-p48* expression profile (at least $10^5$ times lower compared to the mouse pancreas). As ES cell lines and the differentiation protocols used by several groups may differ considerably, it remains unclear whether observed insulin-positive cells arise from such progenitors, because of the contradictory data on *p48* expression (barely detected in this study; not detected by Kahan *et al.* (2003); easily detected by Moritoh *et al.* (2003) and by Skoudy *et al.* (2004). Nevertheless, early pancreatic epithelium bears insulin and glucagon cells in the absence of *Pdx1* (Ahlgren *et al.*, 1996), a phenomenon that might characterize immature pancreatic cells and that cannot be ruled out in the case of *Ptf1a-p48* on the basis of present knowledge.

In conclusion, we examined the mRNA profile of extracellular pancreas regulatory factors in embryoid bodies in order to assess the actual limited ES cell differentiation into insulin-producing cells. Our data indicate that pancreas fate acquisition is blocked at several stages within EBs starting from definitive endoderm. Although we used a crude but highly sensitive approach that provides no information on cell types expressing the mRNA, or their actual translation into proteins, it first of all allows screening for useful targets that can be further confirmed and modulated in cultures. The generation of beta cells from ES cells might benefit from these observations through implementation of strategies that integrate, as we are currently testing, the inhibition of suppressive pathways (Shh, Notch, Bmp6, Fgf10) and supplementation of defective inductive factors (Activin, Fgf2, Fgf4, Wnt) with the ultimate goal of reproducing the features of normal development.

## Materials and Methods

### *ES cells and EB cultures*

The E14 ES cell line derived from 129P2/Ola mice was used in this study. Mitomycin C (Sigma-Aldrich, St Louis - USA) inactivated STO cells were plated on gelatine-coated tissue culture dishes and used as feeder layers. ES cells were expanded on feeders and passaged every 2 to 3 days (following trypsin dissociation and separation from feeder cells by a short passage on gelatine-coated dishes). Expansion medium consisted of knockout DMEM (Gibco, California - USA) supplemented with 15% knockout serum replacement (Gibco, California - USA), L-glutamine (Sigma-Aldrich, St Louis - USA), non-essential amino acids (Sigma-Aldrich), beta-mercaptoethanol (Sigma-Aldrich), penicillin-streptomycin (Sigma-Aldrich) and 1000U/ml leukaemia inhibitory factor (LIF, Sigma-Aldrich).

EBs were initiated by dissociating ES cells and removing feeders, then plating in suspension culture dishes in the absence of LIF. The differentiation medium consisted of DMEM supplemented with 15% FCS (PAA, Pasching - Austria), L-glutamine, non-essential amino acids, beta-mercaptoethanol and penicillin-streptomycin. At day 4 of culture, EBs were transferred to the medium of same composition, but lower glucose concentration (6.25mM). As from day 8, cultures were supplemented with 10mM nicotinamide (Merck, Darmstadt - Germany) and maintained for 4 more weeks, the medium being renewed every other day.

### *Immunocytochemistry and immunofluorescence*

Embryoid bodies were harvested at several time points (day 8, 14, 22, 29 and 35) and fixed in 4% formol for 90 min, then rinsed in PBS and processed for paraffin embedding. Staining was carried out on 5 μm sections that were dewaxed, blocked with 10% pre-immune serum and

incubated overnight at 4°C with a primary antibody. The primary antibodies used were monoclonal mouse anti-insulin (1/2000, Sigma-Aldrich, St Louis, USA), rabbit anti-glucagon (1/3000, Dr. C. Van Schravendijk, Vrije Universiteit Brussel, Belgium), rabbit anti-alpha fetoprotein (1/200, Dako, Glostrup, Denmark), sheep anti-albumin (1/100, Biogenesis, Poole, England) and goat anti-sonic hedgehog (1/200, Santa Cruz, California, USA). After rinsing in PBS, slides were incubated at room temperature for 1h with the appropriate secondary antibody and further developed when applicable. For negative control slides, the primary antibody was omitted. Slides were examined on a Leica microscope and pictures taken with an Axiocam cold camera using AxioVision software.

### *Classical RT-PCR analysis*

Total RNA was extracted from EBs using TRIzol (Gibco, California - USA) and following the manufacturer's recommendations. cDNA was synthesised from 1 μg DNase-treated RNA with random hexamers and an 80ng equivalent used in each PCR set-up. All cDNA synthesis and PCR reaction components were from Invitrogen (California - USA). Whenever possible, intron-spanning primers were designed from published mouse sequences (EMBL) and were BLASTed against the nucleotide databank to check for their specificity. The complete list and sequences of primers are available upon request. All reactions underwent a standard amplification program for 28-35 cycles. PCR products were analysed on 1.2% agarose gel and images acquired with a UV sensitive camera. Three independent experiments were carried out from three different ES cell aliquots and the expression profile of target genes found to be reproducible.

### *Real time PCR analysis*

Selected target genes from each pathway that already indicated sharp and interesting differences upon classical PCR were further analyzed by real time PCR in order to quantify their expression level. For this purpose, cDNA was prepared from 500 ng of total RNA following DNase treatment and 10 ng RNA equivalent used for PCR with specific primers (previously tested and confirmed on gel) in the presence of SYBR Green I. A melt curve analysis was performed at the end of each reaction. Data (mean + SEM) are from triplicate runs of one samples set representative of the three independent experiments. Expression levels were normalized to individual GAPDH (internal control) and to undifferentiated ES cells (reference). The profile was obtained by plotting relative expression levels as a percentage of the maximum value. For pancreatic gene expression analysis, 150 ng RNA equivalent was used (Fig.1C, *Ptf1a-p48* in Fig. 3D) and adult mouse pancreas taken as reference.

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