

Pancreatic Precursors and Differentiated Islet Cell Types From Murine Embryonic Stem Cells

An In Vitro Model to Study Islet Differentiation

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Embryonic stem (ES) cells differentiating in vitro reproduce many facets of early embryonic development, including the expression of developmentally regulated transcription factors and the differentiation of multipotent precursor cells. ES cells were evaluated for their ability to differentiate into pancreatic and islet lineage-restricted stages including pancreatic duodenal homeobox 1 (PDX1)-positive pancreatic precursor cells, early endocrine cell progenitors, and islet hormone-producing cells. Following growth and differentiation in nonselective medium containing serum, murine ES cells spontaneously differentiated into cells individually expressing each of the four major islet hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide. PDX1 immunostaining cells appeared first, before hormone-positive cells had emerged. Hormone-positive cells appeared within focal clusters of cells coexpressing PDX1 and the nonclassical hormone markers peptide YY (YY) and islet amyloid polypeptide (IAPP) in combination with the definitive hormones, characteristic of endocrine cells appearing during early pancreaticogenesis. This system allows the investigation of many facets of islet development since it promotes the appearance of the complete range of islet phenotypes and reproduces important developmental stages of normal islet cytodifferentiation in differentiating ES cell cultures. *Diabetes* 52:2016–2024, 2003

Embryonic stem (ES) cells are pluripotent cell lines derived from the inner cell mass of blastocyst-stage embryos (1,2) that possess the ability to differentiate into a wide variety of specialized cell types in chimeric animals or in vitro (rev. in 3,4). ES cells differentiating in culture display a pattern of differ-

entiation that reproduces characteristic features of early embryonic development. Significantly, single ES cells in suspension aggregate and differentiate in a manner that resembles the formation of the mouse egg cylinder. After 7 days of differentiation, embryoid bodies (EBs) are two-layered structures comprised of an inner layer of columnar ectoderm surrounding a proamniotic-like cavity and an outer layer of primitive endoderm (5). During further differentiation promoted by allowing EBs to attach and spread on an adhesive substrate, expression of specific lineage-restricted genes is initiated in tightly regulated temporal sequences (6,7). Ultimately, lineage-committed multipotent precursor cells, which can develop into terminally differentiated mature cell types, appear (8,9). Consequently, ES cells are a useful model system to study the regulation of tissue specification in mammalian development (3,6,10).

Pancreatic islet cells in mammals develop through the stepwise commitment of foregut endoderm, characterized by the patterned expression of lineage-restricted transcription factors including pancreatic duodenal homeobox 1 (PDX1), and by early marker proteins such as peptide YY (YY), a pancreatic polypeptide (PP)-related neuropeptide, and islet amyloid polypeptide (IAPP) (rev. in 11–13). Morphogenesis of the pancreas is initiated when the posterior foregut endoderm evaginates to form first a dorsal and subsequently a ventral pancreatic bud. Here, PDX1 is expressed in the early pancreatic epithelium at embryonic day 9.5 (e9.5) in mice and is required for the specification of all three functional tissue types of the pancreas, including islet, acinar, and ductal tissues (14–16). Among PDX1-positive cells at e9.5, the first endocrine hormone-positive cells expressing glucagon (Glu) appear (17). Shortly thereafter, a subset of Glu+ cells begins to express insulin (Ins). Most early Glu+ and Glu+/Ins+ cells coexpress YY and IAPP (12,13,18,19). At approximately e13.5, a secondary developmental transition occurs marked by rapid and massive expansion of the β -cell mass and emergence of single hormone-producing cell types, including definitive α -, β -, δ -, and PP cells.

Many critical questions regarding the genetic regulation of lineage commitment and the proliferation and differentiation of progenitor cell phenotypes in islet development remain unanswered. Previous studies have demonstrated that ES cells can commit toward embryonic endoderm lineages, including gut, liver, and pancreatic islet endocrine cell types (20–32). Therefore, ES cells afford the

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EB, embryoid body; ES, embryonic stem; IAPP, islet amyloid polypeptide; PDX1, pancreatic duodenal homeobox 1; PP, pancreatic polypeptide; YY, peptide YY.

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opportunity to investigate questions in pancreatic islet development in novel ways, providing characteristic stages of development are faithfully reproduced. Recent reports have indicated that insulin-producing cells could be produced with very high efficiencies in differentiating ES cells, ranging from 30 to 95% of the total population (28,31,32), using a multistep protocol previously developed to select for the survival and subsequent growth and differentiation of nestin-positive neuronal stem cells in ES cell cultures (8). If correct, this would represent a significant achievement toward deriving insulin-producing cells in quantities necessary for in vivo transplantation, as well as for investigating the underlying mechanisms of β -cell differentiation. As part of the neuronal-enhancing culture medium developed in the original protocol (33), high levels of insulin were supplied to cells throughout selection, including during final stages of differentiation. It appears now that insulin-positive cells resulting from protocols that utilize high levels of exogenous insulin can be explained by cellular uptake from the exogenous source, rather than endogenous synthesis by differentiating ES cells (34; P. Serup, personal communication, data not shown).

We describe here a straightforward, nonsupplemented culture protocol that supports the stepwise differentiation of mouse ES cells through characteristic stages of early pancreatic development, including precursor phenotypes of pancreatic endocrine differentiation and formation of each of the four major islet endocrine cell types, thus demonstrating this ES cell in vitro differentiation system recapitulates characteristic features of normal pancreatic development and islet cytodifferentiation.

RESEARCH DESIGN AND METHODS

In vitro differentiation of ES cells. Mouse ES D3 cells, obtained from the American Tissue Culture Collection (ATCC, Rockville, MD) were maintained undifferentiated on irradiated (5,000 R) STO cell feeder layers in Dulbecco's modified Eagle's medium (DMEM)-high glucose (GIBCO/BRL, Grand Island, NY) supplemented with 15% defined FCS (Hyclone, Logan, UT), 1,500 units/ml leukocyte inhibitory factor (GIBCO/BRL) 1% nonessential amino acids, 2 mmol/l L-glutamine, 0.1 mmol/l β -mercaptoethanol, and 100 units penicillin/100 μ g streptomycin/ml (GIBCO/BRL) at 37°C in 10% CO₂. To initiate differentiation, ES cells were removed from feeder layers with 2 mmol/l EDTA containing 2% chicken serum, filtered through 20- μ m NyteX filters, and plated in nontissue culture plastic dishes (2 × 10⁶ cells/60-mm dish). After 7 days of suspension culture, intact EBs were plated onto gelatin-coated surfaces at a density of 30–50 EBs per 13-mm glass coverslip in 24-well culture plates. EBs were allowed to differentiate further for 1–5 weeks in high-glucose DMEM containing 10% FCS in 5% CO₂, with daily medium changes. To determine the total number of cells present, six wells at each time point were preincubated with 2 mmol/l EDTA for 10 min and then dissociated in 0.25% trypsin plus 0.05% EDTA (GIBCO/BRL) for 10 min. Duplicate hemocytometer counts were performed on each well.

RNA extraction, cDNA synthesis, and PCR. To extract RNA, Trizol (GIBCO/BRL) was added to cells according to the manufacturer's instructions. RNA was washed and treated with RNase-free DNase (1 unit/10 μ g RNA; Promega, Madison, WI) at 37°C for 10 min. First-strand cDNA synthesis from 9 μ g total RNA was performed using Moloney murine leukemia virus RNase H⁺ Reverse Transcriptase (Promega) and random hexamer primers (Perkin Elmer, Foster City, CA) in a 60- μ l reaction according to manufacturer's specifications. One microliter of cDNA was amplified by PCR with gene-specific oligonucleotide primers. Oligonucleotide primer pairs were generated against mouse genes using Genbank sequences except where noted. Primer pairs, amplicon sizes, and cycle numbers are as follows: Pdx1: 5'-TGTAG GCAGTACGGGTCCTC, 3'-CCACCCAGTTTACAAGCTC, 325 bp, 33 cycles; Pax 4: 5'-GTGTTGGCTCAGTTCCTCC, 3'-AACCAAACCTCACCGTGTC, 215 bp (35) 35 cycles; Nkx6.1: 5'-TACTTGGCAGGACCAGAGAG, 3'-CGCTGGATT TGTGCTTTTTC, 271 bp, 35 cycles; Nkx2.2: 5'-AAAGGTATGGAGGTGACG CCT, 3'-AGCTGTACTGGGCGTTGTACT, 190 bp, 35 cycles; neurogenin 3:

(Ngn 3) 5'-TGGCGCCTCATCCCTTGGATG, 3'-CAGTACCCCACTTCTGCT-TCG, 160 bp, 35 cycles (36); BETA2/Neuro D1 (Neuro D): 5'-GGAGTAGGG ATGCACCGGGAA, 3'-CTTGGCCAAGAATACATCTGG, 231 bp, 35 cycles; p48 subunit of PTF-1 (p48): 5'-TGCAGTCCATCAACGACGC, 3'-GGACAGAGT TCTTCCAGTTC, 700 bp, 35 cycles (37); α -amylase 2A (Amy): 5'-CATTGTTG CACTTGTACAC, 3'-TCTGCTGCTTTCCTCAT, 300 bp, 33 cycles; carboxypeptidase A (Carb A): 5'-GCAAATGTGTGTTTGTATGCC, 3'-ATGAC-CAAACCTTGGACCG, 521 bp, 33 cycles; and ribosomal protein S26 (S26, internal control): 5'-GCTCCTTACATGGGCTTTGGTGG, 3'-TCGTGCCAAAAA GGGCCG, 300 bp, 26 cycles. Primers were selected from two different exons and spanned at least one intronic sequence.

PCR conditions were as follows: initial denaturation at 94°C for 3 min, cycling at 94°C for 1 min, 1 min at annealing temperature, 1 min at 72°C, and a final 5 min at 72°C. Primers were annealed at 58°C except for Pdx1, which was annealed at 62°C. Amplification of the ubiquitous ribosomal protein S26 mRNA was used as an internal control for equal cDNA input. A control sample without reverse transcriptase (-RT) was amplified in parallel in all cases, and mouse fetal pancreas RNA was used as a positive control. All PCR amplifications from a given experimental condition were performed from the same cDNA sample. Gel analysis was performed on 1.6% agarose, and bands were imaged with ethidium bromide. PCR products were sequenced to confirm amplicon identity.

Immunofluorescence microscopy. Glass coverslips in wells were rinsed three times with PBS and fixed with 4% paraformaldehyde for 45 min at 4°C. Cells were permeabilized with 0.2% Triton X-100 in PBS for 2–4 h at room temperature and blocked for 2–4 h at room temperature in 5% BSA, 2% goat serum, and 0.2% Triton X-100 in PBS. Primary antisera were diluted in 1% BSA and 0.2% Triton X-100 in PBS, centrifuged at 16,000g for 15 min, and then incubated with coverslips overnight at room temperature. Wells were washed with PBS plus 0.2% Tween 20 four times for 20 min with shaking and then treated overnight with secondary antibody and washed as before. Coverslips were washed once more in PBS and mounted on slides with ProLong Antifade (Molecular Probes, Eugene, OR) mounting medium. Stained ES cell cultures were visualized and photographed in Z-series sections using a Biorad MRC-1024 laser confocal microscope.

Antisera. Primary antibodies were mouse anti-insulin monoclonal antibody (clone DB9G8), 10 μ g/ml (ATCC no. HB124); mouse anti-insulin monoclonal antibody (clone K36aC10), 1:2,000 (Sigma); rabbit anti-mouse C-peptide 1 and C-peptide 2 (no. 657,660, gift of Dr. P. Serup, Hagedorn Research Institute, Denmark), 1:2,000; rabbit anti-rat IAPP, 1:2,000 (Phoenix Pharmaceuticals, Belmont, CA); mouse anti-pancreas-specific glucagon monoclonal antibody (clone K79bB10), 1:2,000 (Sigma, St. Louis, MO); rabbit anti-human glucagon, 25 μ g/ml (Dako, Carpinteria, CA); mouse anti-somatostatin monoclonal antibody (SOM 018, batch 900702/PC21, gift of P. Jorgensen, Novo Nordisk), 1:2,000; rabbit anti-synthetic somatostatin (15-28), 1:2,000 (DiaSorin, Stillwater, MN); rabbit anti-bovine PP, 1:2,000 (Accurate Chemical, Westbury, NY); guinea pig anti-rat PP, 1:100 (Linco, St. Charles, MO); guinea pig anti-synthetic porcine peptide YY, 1:2,000 (Accurate); rabbit anti-PDX1, 1:4,000 (no. 1858.5, gift from O. Madsen, Hagedorn Research Institute, Denmark); and mouse anti-Ki67 monoclonal antibody (clone B56), 1:25 (BD Biosciences, San Diego, CA).

Secondary antibodies [Alexa Fluor(AF)488 goat anti-mouse IgG, 1:2,000, A-11029; AF568 goat anti-mouse IgG, 1:2,000, A-11031; AF568 goat anti-rabbit IgG, 1:4,000, A-11036; AF488 goat anti-guinea pig IgG, 1:2,000, A-11073; AF647 goat anti-rabbit IgG F(ab)₂, 1:4,000, A-21246; and AF647 goat anti-guinea pig IgG, 1:2,000, A-21450] were obtained from Molecular Probes (Eugene, OR).

Morphology. Transmission electron microscopy was performed as previously described (38).

RESULTS

ES cell derivatives activate islet transcription factor genes. Pancreatic development and gene expression are exquisitely regulated by a sequence of specialized transcription factors, including PDX1, Fox A2 (HNF3 β), Fox B2 (HNF6), HB9, Isl1, Ptf1a (p48), neurogenin 3, Beta2/NeuroD1, Nkx2.2, PAX4, PAX6, and Nkx6.1. To determine whether these genes were expressed in differentiating ES cells, RT-PCR analysis was performed on cultures harvested 3 weeks postplating. *Pdx1*, *Pax4*, and *Nkx6.1* mRNA transcripts were undetectable in undifferentiated ES cells but present in differentiated ES cell progeny (Fig. 1A). *Foxa2* (HNF3 β), *Pax6*, and *Isl1* transcripts were also detected early during EB formation and through subse-

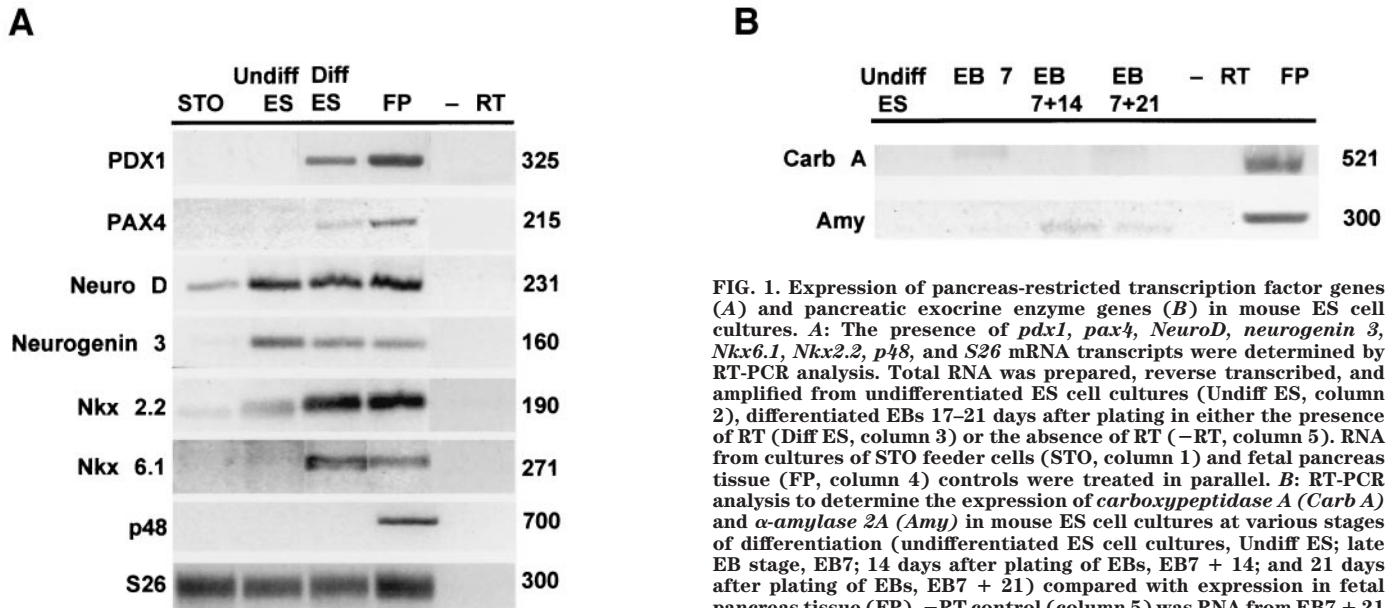


FIG. 1. Expression of pancreas-restricted transcription factor genes (A) and pancreatic exocrine enzyme genes (B) in mouse ES cell cultures. A: The presence of *pdx1*, *pax4*, *NeuroD*, *neurogenin 3*, *Nkx6.1*, *Nkx2.2*, *p48*, and *S26* mRNA transcripts were determined by RT-PCR analysis. Total RNA was prepared, reverse transcribed, and amplified from undifferentiated ES cell cultures (Undiff ES, column 2), differentiated EBs 17–21 days after plating in either the presence of RT (Diff ES, column 3) or the absence of RT (–RT, column 5). RNA from cultures of STO feeder cells (STO, column 1) and fetal pancreas tissue (FP, column 4) controls were treated in parallel. B: RT-PCR analysis to determine the expression of *carboxypeptidase A* (*Carb A*) and *α-amylase 2A* (*Amy*) in mouse ES cell cultures at various stages of differentiation (undifferentiated ES cell cultures, Undiff ES; late EB stage, EB7; 14 days after plating of EBs, EB7 + 14; and 21 days after plating of EBs, EB7 + 21) compared with expression in fetal pancreas tissue (FP). –RT control (column 5) was RNA from EB7 + 21 cultures not treated with RT during cDNA synthesis.

quent stages of development but not in undifferentiated cultures (data not shown). *Nkx2.2* mRNA transcripts, while present in undifferentiated ES cells, were notably more prevalent after differentiation. On the other hand, *Neurogenin3* and *NeuroD* genes were actively transcribed in both undifferentiated and differentiated ES cell cultures (Fig. 1A). RT-PCR analysis cannot readily determine whether a few differentiated cells, commonly present in undifferentiated cultures, or some undifferentiated ES cells express these transcripts. In fact, similar observations, in which undifferentiated ES cell cultures have been found to express tissue-specific gene transcripts with unknown functional significance, have been noted previously (28,39,40). In contrast, mRNA transcripts of *p48*, encoding the pancreas-specific subunit of the trimeric PTF1 transcription factor, were not detected during either EB or postplating stages of ES cell differentiation (Fig. 1A). Finally, pancreatic exocrine restricted *CarbA* and *Amy* genes were not actively transcribed (Fig. 1B).

ES cell derivatives express early islet endocrine marker proteins. To determine whether ES cells could differentiate into cells expressing endocrine phenotypes, cultures were costained with antibodies to each of the four major islet hormones and to the nonclassical hormones YY and IAPP. We used four criteria to judge the authenticity of cells stained with antibodies, including clear cytoplasmic staining and granular localization, colocalization with appropriate markers, and incorporation into specific nonrandom tissue areas. Stained cells usually existed in tightly knit groups or foci of cells, as opposed to singular cells, which were integrated within or above epithelial sheets or cysts and sometimes extended as three-dimensional structures through multiple cell layers.

Previous studies have demonstrated that YY and/or IAPP are expressed by all four islet cell types as they first emerge during development (12,13,18,19), suggesting that they may mark a common progenitor cell. We examined cultures for the presence of YY- and IAPP-immunostained cells to identify early as well as possibly infrequent events in ES cell differentiation. Surprisingly, we found YY and

IAPP expressed in many cells, clustered in multiple, discrete foci (Fig. 2A). Typically, a larger focus contained hundreds of cells of which the majority expressed YY and IAPP colocalized in cytoplasmic granules and in perinuclear regions, probably golgi (Fig. 2A insert). The number of stained cells continually increased over the culture period (Fig. 2B). Significant differentiation did not occur until the cultures were near their maximum density, which was relatively constant at day 10 postplating and thereafter at $\sim 2\text{--}3 \times 10^6$ cells per coverslip. Thus, the relative frequency of YY+ and IAPP+ cells increased over time and particularly between days 10 and 17 of culture; however, the overall frequency of YY+/IAPP+ cells under these conditions is very low at $\sim 10^{-4}$ or 0.01% of cells.

Applying the four criteria for authenticity, we also found cells expressing each of the four major islet hormones. Consistent with previous *in vivo* studies, many Glu+, Ins+, and Som+ cells coexpressed both YY and IAPP (Fig. 3A–N). Likewise, PP+ cells often coexpressed YY (Fig. 3O–Q). Compared with Fig. 6, however, many more YY+ or IAPP+ cells were detected at each stage of differentiation than other hormone-positive cells of all types. These results suggest the possibility that YY and IAPP mark an early progenitor cell type that appears in ES cell cultures before commitment to definitive islet lineages.

PDX1+ pancreatic progenitors differentiated from ES cells. During normal development, PDX1 expression is first initiated when foregut endoderm commits to a pancreatic fate and precedes specification of any definitive islet cell types. Given the importance of PDX1 in early pancreas development, we sought to determine the pattern of PDX1 protein expression by immunohistochemistry and identify early events in pancreatic lineage differentiation in ES cell cultures. PDX1+ cell clusters were first detected at 4 days after EB plating, before the appearance of Glu+ and other hormone-positive cells (Fig. 4A), and increased in frequency over the ensuing 2 weeks (data not shown). Interestingly, double staining with anti-YY revealed a subset of PDX1+ cells that also expressed YY (Fig. 4B, arrows) at early times before the

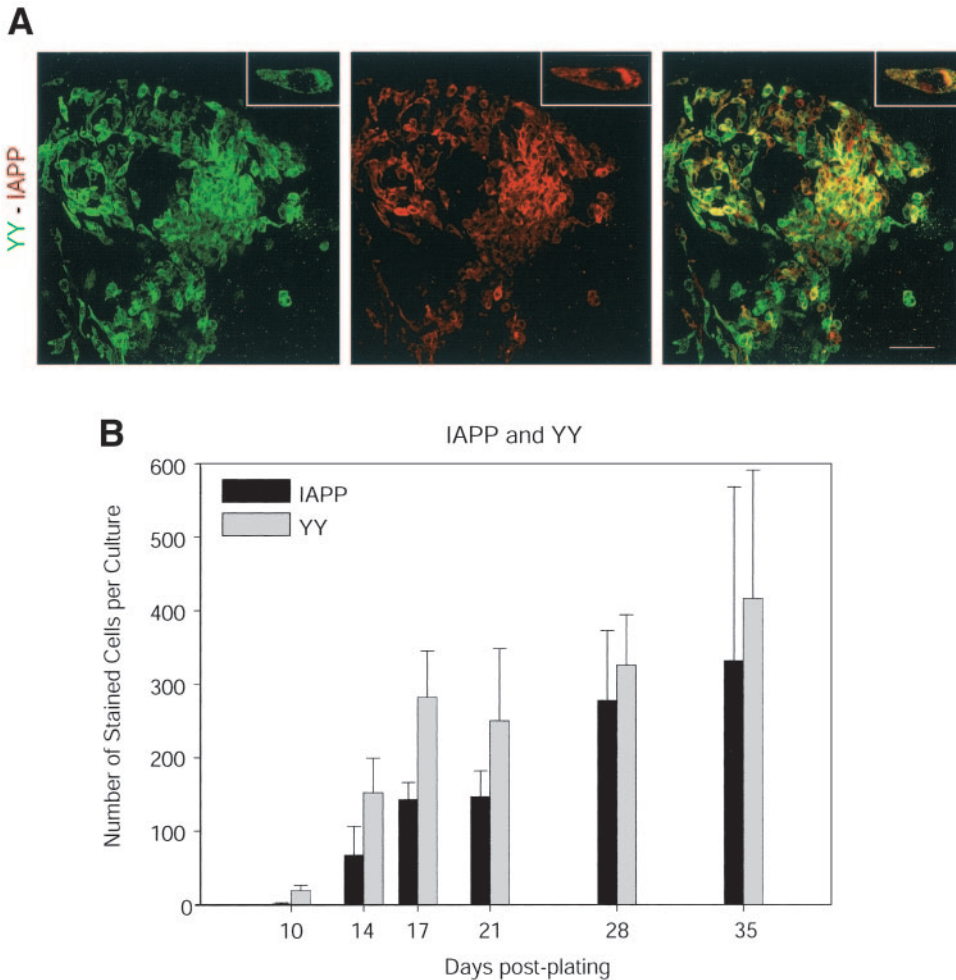


FIG. 2. Expression of early endocrine cell markers, peptide YY (YY), and IAPP in mouse ES cell-differentiated derivatives. **A:** Immunostaining of EBs 35 days after plating with anti-YY (green) and anti-IAPP (red) reveals numerous YY and IAPP coexpressing cells densely clustered, rather than randomly distributed throughout the culture. Scale bar 50 μ m. Insert: 3- μ m section of cell at higher magnification showing expression of YY and IAPP colocalized in cytoplasmic granules and in perinuclear region. **B:** Quantification of the number of YY- and IAPP-expressing cells per culture at several stages shows a continuous increase in the number of YY- and IAPP-positive cells in the 5 weeks post plating. Results are expressed as mean number of cells \pm SE of 3–10 independent cultures.

appearance of other, definitive hormones. Significantly, we found no expression of nestin associated with either PDX1+ or YY+/IAPP+ cells (not shown).

To determine if PDX1 was also expressed in hormone-producing cells, we costained day 17 and 21 cultures for PDX1 and Glu, Som, or Ins. PDX1 was coexpressed by nearly all Ins+ cells and many Som+ cells, as would be expected of mature β - and δ -cells (Fig. 4C and D). A few Glu+ cells also coexpressed PDX1 (data not shown), as has been reported previously in early mouse embryos (15,17)

To assess whether PDX1 or YY precursors were dividing, cultures were costained for PDX1 or YY and Ki-67, a nuclear proliferation marker. Although many proliferating cells were detected in close proximity to PDX1+ and YY+ cells in 4- to 14-day cultures, the absence of any observed costaining indicates that PDX1+ and YY+ cells were not mitotically active under these conditions (Fig. 4E and F).

Pattern of expression of major islet hormones produced by ES cell progeny. To determine the overall pattern of individual islet hormone expression in ES cell progeny, cultures were stained for the presence of Ins, Glu, Som, and PP at various stages of differentiation. Cells expressing each of the four major islet hormones appeared in a time-dependent manner (Figs. 5 and 6). Glu+ cells were the earliest hormone-positive cells, detected at day 7–10 postplating (Fig. 6), consistent with the early presence of Glu+ cells in pancreatic bud epithelium in vivo

(41). Double staining of cultures with all combinations of antibodies revealed only single hormone-staining cells (Fig. 5), a finding that mimics the expression profile of islet hormones in adult islets. The single exception was the early coexpression of Ins and Glu, as described below. Cells expressing different islet hormones appeared together in distinct foci, as described for YY and IAPP expression. The total number of cells expressing specific islet endocrine hormones increased substantially over the first 3 weeks but decreased in later stages (Fig. 6), suggesting that conditions leading to their derivation were not optimal to sustain either the differentiated state or their survival.

A consistent observation in studies of normal pancreas development is the appearance of Ins+/Glu+ double-positive cells within the early pancreatic bud epithelium (18). Subsequently, single hormone-positive cells expressing only Glu or only Ins appear during the secondary transition. To determine whether this pattern was reproduced in ES cultures undergoing islet differentiation in vitro, we stained cells for Ins and Glu at various times following EB plating. In early cultures (day 10–17), virtually all Ins+ cells were also Glu+, while only a subset of the more numerous Glu+ cells were Ins+ (Fig. 7A and C). This was in striking contrast to the pattern observed in later cultures (day 21 and thereafter) where essentially no costaining cells were seen (Fig. 7B and C). This abrupt transition to single hormone-positive cells observed in ES

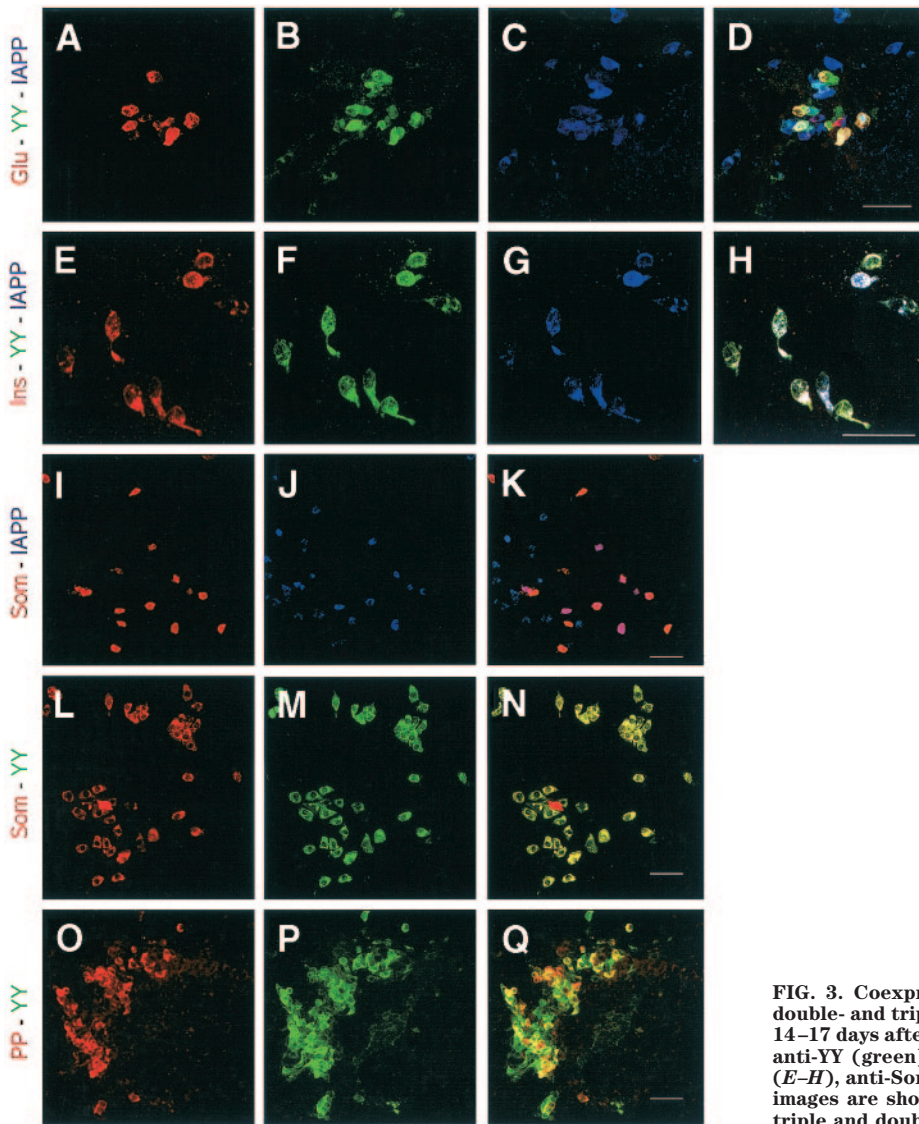


FIG. 3. Coexpression of YY and IAPP with islet hormones in double- and triple-immunostained differentiated ES cell cultures 14–17 days after plating EBs. Cultures were immunostained with anti-YY (green), anti-IAPP (blue) and anti-Glu (*A–D*), anti-Ins (*E–H*), anti-Som (*I–K* and *L–N*), or anti-PP (*O–Q*) (red). Merged images are shown in *D*, *H*, *K*, *N*, and *Q*, and demonstrate many triple and double hormone-expressing cells. Scale bars 50 μ m.

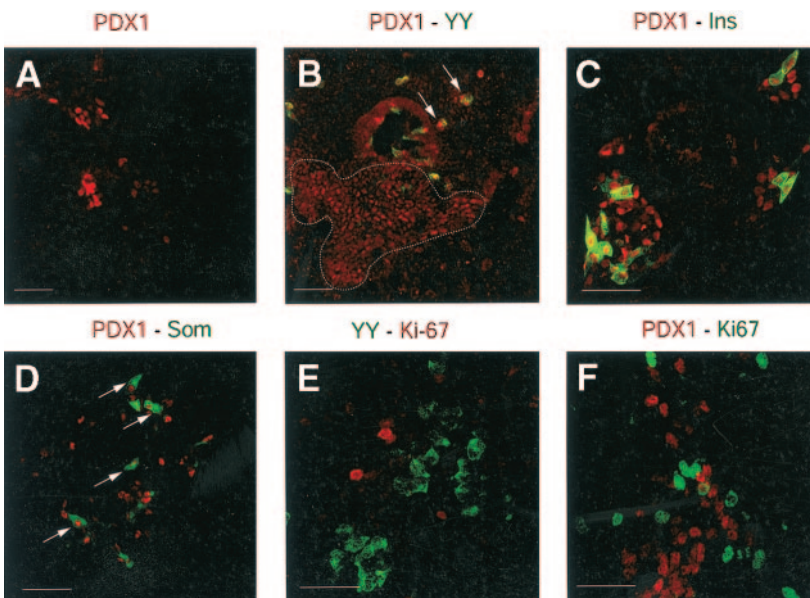


FIG. 4. PDX1 protein expression in mouse ES cell derivatives. *A*: Anti-PDX1-stained early stage culture (4 days postplating of EBs) shows PDX1-immunostained cells at a time when hormone-immunostained cells are not detected. *B*: Double-label immunofluorescence with anti-PDX1 (red) and anti-YY (green) shows a focus of PDX1+ cells in early stage cultures with a few cells coexpressing YY (arrows). Staining with anti-PDX1 (red) and anti-Ins (green) (*C*) or anti-Som (green) (*D*) shows all Ins+ cells, and the majority of Som+ cells in late-stage cultures (21 days' postplating of EBs) express PDX1. Cultures coimmunostained with anti-Ki67 (red) and anti-YY (green) (*E*) and with anti-Ki67 (green) and anti-PDX1 (red) (*F*) demonstrate no costaining. Scale bars 50 μ m.

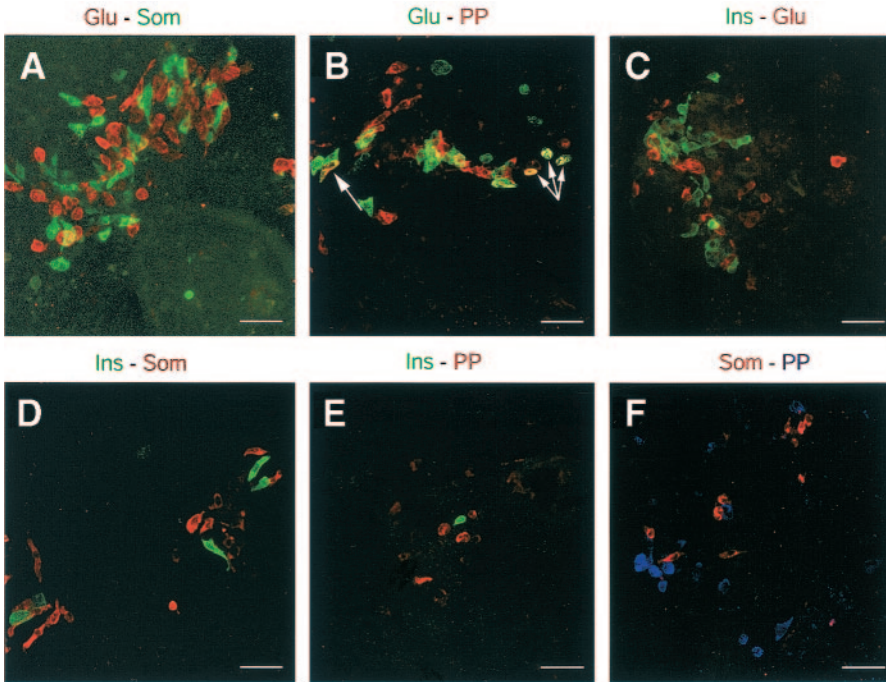


FIG. 5. Expression of islet hormone proteins in differentiated mouse ES cell cultures. Double labeling performed with all possible combinations of antibodies for Ins, Glu, Som, and PP. Cultures immunostained with anti-Glu (red) and anti-Som (green) (A), anti-Glu (green) and anti-PP (red) (B), anti-Ins (green) and anti-Glu (red) (C), anti-Ins (green) and anti-Som (red) (D), anti-Ins (green) and anti-PP (red) (E), and anti-Som (red) and anti-PP (blue) (F) demonstrate expression of each of the four major islet endocrine hormones in individual cells frequently found clustered together. There is essentially no coexpression of the islet hormones; Glu and PP coexpressing cells are rarely detected (B, arrows). Scale bars 50 μ m.

cell cultures closely parallels that occurring in vivo during pancreatic development, emphasizing that ES cells in this system are following the sequence expected for normal pancreatic development.

ES cell-derived insulin-producing cells resemble normal β -cells. The appearance of insulin alone is not sufficient to characterize an islet β -cell, as several other cell types also express the hormone (42,43). In addition, mice produce two nonallelic forms of insulin, Ins I and Ins II (44). Ins I is restricted to β -cells, whereas Ins II is more broadly expressed. Antibodies to insulin generally do not discriminate between the two forms, but antibodies exist that can specifically distinguish between the C-peptide cleaved from proinsulin I or II. Therefore, to determine whether ES cell-derived Ins⁺ cells are producing Ins I, we costained cultures with anti-Ins (HB124 or K36aC10) and anti-C-peptide 1. Numerous C-peptide 1 immunostain-

ing foci were detected and, importantly, within a focus all Ins⁺ cells were also C-peptide 1⁺ (Fig. 8A–D). C-peptide 1 and Ins colocalized in cytoplasmic granules and in perinuclear regions (Insert, Fig. 8A–C), and anti-C-peptide 1 detected significantly more positive cells within a region than either of the two monoclonal antibodies to Ins (Fig. 8D and data not shown), possibly because the polyclonal anti-C-peptide antibody is capable of detecting fewer insulin molecules. As expected, since normal β -cells produce both Ins I and II (45), ES cell-derived Ins⁺ cells were also positive for C-peptide 2, staining with patterns identical to those for C-peptide 1 (not shown). These results provide evidence that the insulin detected arose endogenously. As described earlier, Ins⁺ cells at 21–28 days postplating also expressed nuclear PDX1 (Fig. 4C) and all Ins⁺ cells also expressed IAPP (Fig. 2H and data not shown), which typically colocalizes with insulin in

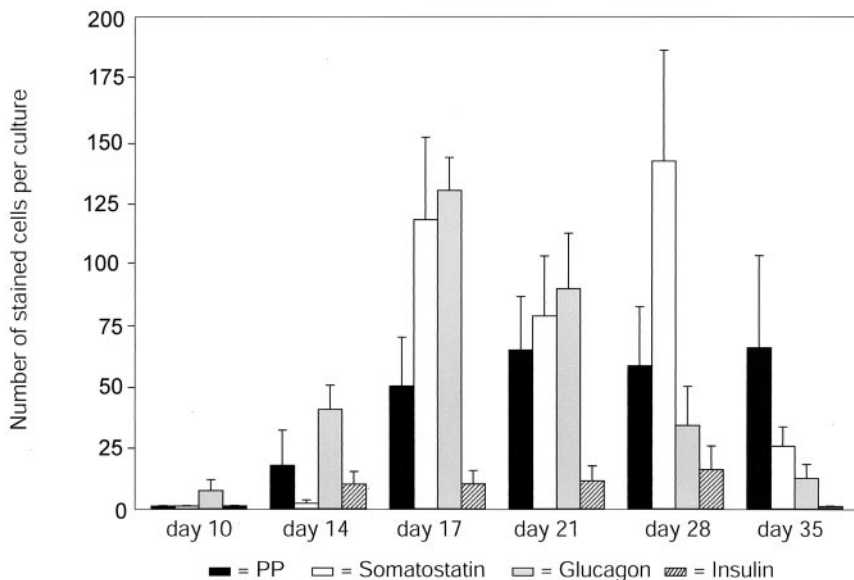


FIG. 6. Quantification of Glu⁺, Ins⁺ (stained with HB124), Som⁺, and PP⁺ immunostaining cells in differentiated ES cell cultures at various stages. Thirty to 50 EBs were plated onto gelatin-coated coverslips, allowed to differentiate for an additional 10–35 days, fixed, and immunostained. Three to 10 separate cultures were counted per time point. Results are expressed as a mean of the total number of positively staining cells per 13 mm coverslip \pm SE.

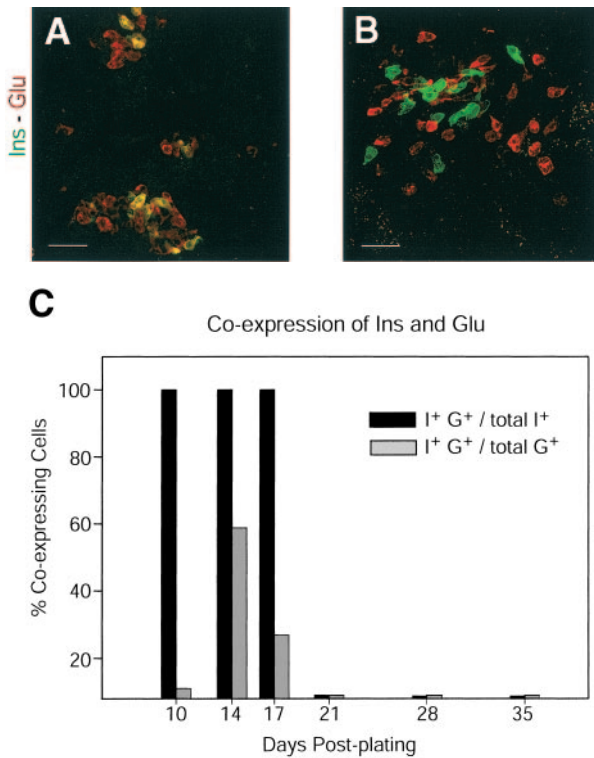


FIG. 7. Ins and Glu expression in ES cell progeny at various stages of differentiation (10–35 days after plating EBs). Cultures were immunostained with anti-Ins (green) and anti-Glu (red). Whereas all Ins⁺ cells also express Glu at early stages (shown in *A*) after differentiation, later stages (shown in *B*) are characterized by the absence of coexpression of these hormones. *C*: The ratio of double-positive (Ins⁺ Glu⁺) cells to Ins⁺ cells or to Glu⁺ cells at different stages is depicted. Note the abrupt transition in the percentage of Ins⁺ cells that are costained with Glu that occurs between 17 and 21 days postplating. Scale bars 50 μ m.

cytoplasmic granules. Assessment of ultrastructural morphology by transmission electron microscopy showed the presence of characteristic electron-dense secretory gran-

ules (Fig. 8*E*). These data indicate that ES cell-derived insulin-producing cells have several of the biosynthetic and morphological features expected of normal β -cells.

DISCUSSION

At present, there is insubstantial evidence to establish that Ins⁺ cells or islet structures can differentiate en masse from ES cells by pathways or through precursor cells distinct from those occurring in normal development. Recent studies have questioned whether previous observations of insulin staining in ES cell derivatives represent cellular uptake from media containing high concentrations of insulin (28–30,34). In this context, it is relevant to evaluate the ability of ES cells to differentiate toward islet lineages using a straightforward, non-insulin-supplemented, nonselective differentiation protocol. Our results indicate that under such conditions, murine ES cells are capable of differentiating into β -like cells, expressing nuclear PDX1 and C-peptide 1 in secretory granules and developing in concert with other islet cell types in rare foci, which nevertheless appear to include PDX1⁺ progenitor cells. This is the first study to systematically examine the spontaneous differentiation of all islet lineages from ES cells and uniquely shows that many features of normal islet cytodifferentiation are reproduced as islet-like cell types differentiate in vitro.

Important aspects of islet differentiation were observed to occur in ES cell cultures. Cells typical of islet precursor cells expressing PDX1 and YY or IAPP appeared first in discrete foci that occurred within a specific contextual organization of the culture. Glu⁺, Ins⁺, Som⁺, and PP⁺ cells subsequently emerged within similar foci, frequently coexpressing PDX1, YY, and/or IAPP markers. Following their initial appearance, an abrupt transition of Glu⁺/Ins⁺ cells to single hormone-positive cells occurred, as takes place during the secondary transition in normal pancreatic

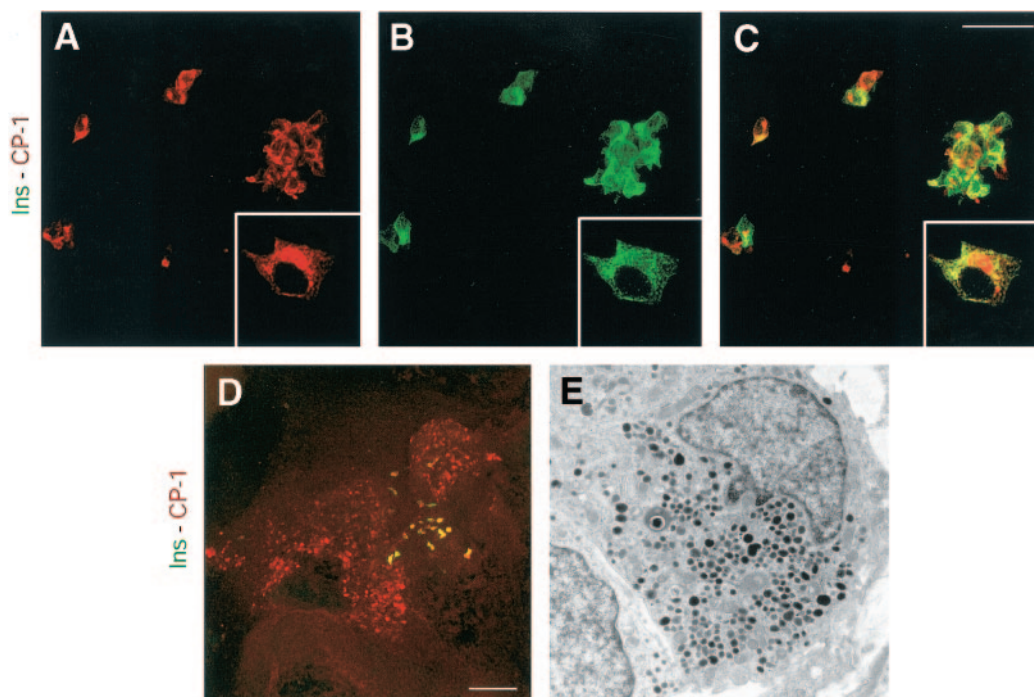


FIG. 8. Confocal immunofluorescence micrograph of differentiated ES cell progeny (day 17 postplating) costained with anti-C-peptide I (red) (*A*) and anti-Ins (HB124, green) (*B*). *C*: Merged image of *A* and *B*. Scale bar 50 μ m. Inserts: Single 1- μ m Z-section of cell at higher magnification showing localized expression in cytoplasmic granules and perinuclear region. *D*: Ins⁺ (K36aC10, green) and C-peptide I⁺ double-immunostaining cells in a region of many C-peptide I-positive cells (day 21 postplating). Scale bar 200 μ m. *E*: Transmission electron micrograph of EB cultures 17 days postplating demonstrating small seven-cell cluster of secretory endocrine cells possessing electron-dense secretory granules (one cell shown here). Final magnification 21,200 \times .

development. The expression of many islet-restricted transcription factor genes was also observed.

Given the importance of PDX1 and YY in pancreas development, it is notable that there is little data available on the coexpression of these markers during embryogenesis. A novel finding of this study is the demonstration of early YY expression in PDX1+ cells. Prior studies have shown that within the pancreatic bud epithelium, YY expression is generally limited to the endocrine cell pool (i.e., early Ins+ Glu+ YY+ cells) and does not appear to overlap the PDX1 expression domain (17,46). In this ES culture differentiation system, however, a population of PDX1+ and YY+ cells was identified before elaboration of hormone-positive cells. This coexpressing subset might provide clues to endocrine differentiation from committed PDX1+ epithelial progenitors.

The absence of *Ptf1-p48* and exocrine enzyme gene products in fully differentiated ES cultures suggests that islet differentiation programs are promoted in preference to acinar differentiation pathways under these conditions. However, a recent lineage-tracing study suggests a more global role of *Ptf1-p48*, with *Ptf1-p48* expression in the early pancreatic bud epithelium marking precursors of endocrine and duct as well as exocrine cells (47). Because homozygous null mice contain pancreatic Glu+ and Ins+ cells, the absence of a functional p48 gene does not prevent the development of all endocrine cells (37,47). Our data indicate that ES-derived islet cells were produced independently of *p48* gene products. Nonetheless, a more detailed study of *Ptf1-p48* expression profiles at earlier stages in differentiating ES cultures is necessary to determine the precise role of *Ptf1-p48* in endocrine lineage differentiation from ES cells.

The development of different islet endocrine cell types in close proximity to one another in small clusters raises the possibility that hormone-expressing cells may differentiate from a single, but not necessarily common, progenitor. The existence of PDX1+ cells within differentiating clusters and coexpression in hormone-positive cells within a focus suggest the latter could be derived from PDX1-positive progenitor cells in the population, consistent with pancreatic lineages described in normal development (15,17). The establishment of lineage relationships in this system, however, will require the use of genetically marked progenitor cells whose progress through the differentiation process can be closely monitored. We believe this study establishes the feasibility of performing lineage analyses of islet development using ES cells in vitro, with potential advantages over whole embryo usage.

We were unable to detect proliferating PDX1+ and YY+ cells at any stage, even though the numbers of these cells increased over the culture period. This observation, together with the nonrandom distribution of differentiating clusters, suggests that local inductive microenvironments are very important in the differentiation of precursor and hormone-expressing cells in this system. Significant differentiation did not occur until the cultures became heavily confluent, multilayered tissues containing a variety of cell types. Thus, either cell contact and/or locally secreted growth factors could have influenced spatially restricted lineage differentiation. The functional importance of α and δ endocrine cell types within the islet in providing hor-

monal counterregulation and interaction with β -cells is well known (48–50), and it is generally believed that optimal islet function is in part determined by islet structure and the presence of other non- β islet cell types. A more complete islet lineage differentiation program created using a nonselective strategy provides an appropriate context to investigate not only the embryonic mechanisms of islet development, but also the steps of maturation to fully functional cell types. In this regard, an analysis of functionality of the β -like cells described here is clearly of great importance and is currently being undertaken.

In summary, we describe a culture system in which mouse ES cells are able to initiate a developmental program that results in the differentiation of cells resembling all islet cell types, developing together in isolated foci in which PDX1 or YY and IAPP are coexpressed in cells producing definitive hormones. As such, mouse ES cells offer an important new tool to study how pancreatic islets and, in particular, β -cells develop from embryonic endoderm and pancreatic progenitors.

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