Neurogenin3 triggers β -cell differentiation of retinoic acid-derived endoderm cells

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Neurogenin3 is a member of the basic helix-loop-helix ('bHLH') family of transcription factors. It plays a crucial role in the commitment of embryonic endoderm into the pancreatic differentiation programme. This factor is considered to act upstream of a cascade of other transcription factors, leading to the fully differentiated endocrine phenotype. Direct observation of the sequential activation of these factors starting from Neurogenin3 had never been demonstrated. By using retinoic acid-derived-endoderm F9 cells as a model, the present study indicates that the ectopic expression of Neurogenin3 is able to start the differentiation pathway of endocrine pancreas. Neuro-

genin3 triggers the expression of several pancreatic transcription factors following a well defined temporal activation sequence. By reverse transcriptase PCR, immunohistochemistry and RIA, it is shown that stable transfected cells are able to form embryod bodies that produce insulin in response to glucose stimulation. This is the first report of a differentiation event induced by the ectopic expression of a transcription factor in embryonic pluripotent stem cells.

Key words: diabetes, insulin, pancreatic differentiation, transcription factor.

INTRODUCTION

During embryogenesis the pancreas develops from the region of the duodenum posterior to the stomach. After the formation of the gut tube, dorsal and ventral pancreatic buds, which will later fuse, grow from the endodermal epithelium that is surrounded by mesenchyme [1]. Some cells in the bud will differentiate into exocrine cells retaining epithelial characteristics and eventually forming ducts and acini. A more consistent part of these cells, fated to become endocrine cells, will migrate from epithelium and aggregate into islets within the mesenchyme. The pancreatic islets of Langerhans are composed of four different cell types, namely α -, β -, δ - and pancreatic polypeptide (PP) cells. They produce the four principal pancreatic hormones, i.e. glucagon, insulin, somatostatin and PP, respectively. A specific hierarchy in the appearance of these hormones has been demonstrated. The glucagon-producing α -cells appear very early during development [at embryonic day 9.5 (E9.5)] [2,3]. On the following day (E10.5), insulin-producing cells can be detected. Cells producing somatostatin and PP appear only at E15.5 and at birth, respectively [2,3]. Indeed, the development of pancreas appears to be a very complicated event. During development, the formation and functional differentiation of the pancreas result from the activation and extinction of a large number of genes. Specific sets of transcription factors expressed in the developing and the mature pancreas control these gene-expression events. Some of them are expressed very early, like Hlxb-9, required for dorsal bud initiation [4], and the pancreas-duodenum homeodomain protein (Pdx1) is required for bud expansion [5]. Several transcription factors have been identified for their involvement in the differentiation of specific pancreas cell types, after bud formation. Paired homeodomain proteins Pax4 [6–8] and Pax6 [7–10], LIM homeodomain protein Isl1 [11], Nkx6.1 and Nkx2.2 [12,13], as well as the basic helix-loop-helix ('bHLH') proteins NeuroD [14] and Neurogenin3 (Ngn3) [15] are all necessary for differentiation

of endocrine cell types. By immunohistochemical analysis these transcription factors have been demonstrated to be (co)expressed in different regions of the embryonic pancreas and their importance has been determined by genetic approaches. However, it is not yet fully clear either how they are switched on or what their specific relationships are. To this end, no cell-model approach has been reported so far.

Among the various transcription factors involved during pancreas development, Ngn3 is likely to be the earliest expressed in the pathway of endocrine differentiation. The aim of this work was to show unambiguously that Ngn3 is indeed the triggering factor for the expression of transcription factors involved in pancreas differentiation. In this study it is shown that ectopic expression of Ngn3 is sufficient to commit retinoic acid (RA)-derived-endoderm cells to the endocrine differentiative pathway. Furthermore, the specific dependence of the expression of transcription factors NeuroD, Pax6 and Isl1 on Ngn3 is also shown. Embryonic carcinoma F9 stem cells were used as a model. Upon treatment with RA, these cells can differentiate into primitive endoderm cells [16], mimicking mouse early embryogenesis.

The present investigation reports on the genomic approach to studying early pancreatic development as an alternative to the use of embryonic pancreas at different stages of differentiation.

EXPERIMENTAL

Materials

Mouse teratocarcinoma F9 cells were purchased from the ATCC (Manassas, VA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, L-glutamine, penicillin/streptomycin solution, LipofectAMINE, OPTIMEM and Moloney murine leukaemia virus reverse transcriptase (M-MLV-RT) were from Gibco-BRL (Grand Island, NY, U.S.A.). RA, *Taq*

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; E9.5 (etc.), embryonic day 9.5 (etc.); EB, embryod body; EBSS, Earle's buffered salt solution; IBMX, 3-isobutyl-1-methylxanthine; M-MLV-RT, Moloney murine leukaemia virus reverse transcriptase; Ngn3, Neurogenin3; PP, pancreatic polypeptide; RA, all-*trans*-retinoic acid; RT, reverse transcriptase; TRITC, tetramethylrhodamine isothiocyanate.

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Table 1 Primers used for PCR analyses

In Ngn3 primers, the underlining indicates *SacI* and *BamHI* cloning sites.

	Forward primer	Reverse primer
β -Actin	5'-CATGTTTGAGACCTTCAA-3'	5'-AAGAGTGCCTCAGGGCA-3'
Ngn3	5'- <u>GAGCTCATGGCGCCTCATCC</u> TTG-3'	5'-GGATCCTTATTACAAGAAGTCTGAGA-3'
NeuroD/BETA2	5'-CTTGCCAGAACTACATCTGG-3'	5'-GGAGTAGGGATGCACCGGAA-3'
Pax6	5'-TGAAGCGGAAGCTGCAAGAAA-3'	5'-TTTGGCCCTTCGATTAGAAAACC-3'
Pax4	5'-GAGATCCAACACAGCTTTGCACTG-3'	5'-GGAGAAGATAGTCCGATTCTGTG-3'
Isl1	5'-ATGGGAGACATGGCGAT-3'	5'-TCATGCCTCAATAGGACTGGC-3'
Pdx1	5'-CTCGCTGGGAACGCTGGAACA-3'	5'-GCTTTGGTGGATTTCATCCACGG-3'
Nkx2.2	5'-ATGTCGCTGACCAACACAAAG-3'	5'-GGGAGATTGGAGGCCCTC-3'
Insulin	5'-AAGTCCCGCCGTGAAGT-3'	5'-TTAGTTGCAGTAGTTCTCCAGCTGG-3'
Glucagon	5'-ATGAAGACCATTACTTTGTGGCT-3'	5'-GGTGTTCATCAACCACTGCAC-3'
Somatostatin	5'-ATGCTGTCTGCCGTCTCC-3'	5'-CTAACAGGATGTAATGTCTTCCAGA-3'
α -Amylase	5'-CATTGTTGACCTTGTACAC-3'	5'-TTCTGCTGCTTCCCTCAAT-3'
GATA4	5'-CGCCGCCTGTCCGCTCC-3'	5'-TTCGGCTTCCGTTTCTGTTTGA-3'

DNA polymerase, anti-(mouse laminin) antibody, Geneticin (G418), 3-isobutyl-1-methylxanthine (IBMX), FITC-conjugated anti-mouse IgG, and monoclonal anti-(mouse insulin) (clone K36AC10) and monoclonal anti-(mouse glucagon) (clone K79bB10) antibodies were from Sigma (Milwaukee, WI, U.S.A.). Colloidal secondary anti-mouse 30 nm-gold-conjugated IgG antibody was from British Biocell Int. (Cardiff, Wales, U.K.). Polyclonal rabbit anti-(mouse insulin) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit antibody was from Jackson ImmunoResearch (West Grove, PA, U.S.A.). Nunclon™ Delta MicroWell™ plates were from Nunc (Rochester, NY, U.S.A.). OMNIzoP reagent was from Euroclone (Wetherby, W. Yorks., U.K.). Expression vector pIRES-EGFP was from Clontech (Palo Alto, CA, U.S.A.). Vector pCMV SPORT- β -Gal was from Promega (Madison, WI, U.S.A.). Nylon membrane GeneScreen Plus was from Dupont (Wilmington, DE, U.S.A.). Rat insulin radioimmunoassay kit was from Linco Research (St. Charles, MO, U.S.A.).

Cell culture

Mouse teratocarcinoma F9 cells were grown in DMEM supplemented with 10% fetal bovine serum containing 2 mM L-glutamine, 100 units/ml penicillin and 100 μ l/ml streptomycin at 37 °C in an atmosphere of 5% CO₂. Cells were induced to differentiate by adding RA to a final concentration of 0.1 μ M. Cells were allowed to differentiate for 4 days before being used for transfection. These cells were designated F9/RA cells. Differentiation ratio was assessed by measuring laminin liberated into the conditioned medium by means of an ELISA [17]. Cell culture medium was changed every 24 h after RA treatment and the amount of secreted laminin was measured. Signal was normalized with respect to the number of cells.

Cloning of Ngn3 and cell transfection

Mouse cDNA of complete coding sequence of Ngn3 was provided kindly by Dr David J. Anderson (Division of Biology, Howard Hughes Medical Institute, Pasadena, CA, U.S.A.) and it was subcloned into the green fluorescent protein (GFP) expression vector pIRES-EGFP.

Transient transfections were carried out using 7 μ l of LipofectAMINE reagent and 2 μ g of both recombinant construct pIRES/Ngn3 and control vector pCMV SPORT- β -Gal, in 35 mm Petri dishes containing 50000 F9 cells and 100000 F9/RA

cells, according to the standard conditions for mammalian cells described by the manufacturer. The transfected cells were grown for 72 h prior to RNA extraction. Efficiency of transfection was determined by testing β -galactosidase activity in fixed cells and in cell extracts [18], and by detecting fluorescent cells, fixed with 4% paraformaldehyde for 20 min and mounted with coverslips, using a fluorescence microscope (Leica). The transfection efficiency for both F9/RA and F9 cells varied from 25 to 35% in different experiments.

For stable transfectants, 48 h post-transfection cells were seeded in 96-well plates at 1:500 dilution with DMEM containing 1 mg/ml G418. After 14 days of selection, individual resistant clones were transferred to 24-well plates and maintained in medium containing 0.5 μ g/ml G418 for further propagation. In the subsequent 2 weeks, cells were transferred to flasks and cultured in non-selective complete medium.

Reverse transcriptase PCR (RT-PCR)

Total RNA from F9 cells, F9/RA cells and embryonic bodies was isolated by using OMNIzol according to the manufacturer's instructions. Genomic DNA was removed by treatment with DNase I. For cDNA synthesis, 1 μ g of total RNA was denatured at 70 °C for 10 min and quickly chilled on ice, added to the RT mixture containing 1 $\times$ M-MLV-RT buffer, 40 units of RNase out, 10 mM dithiothreitol, 25 pmol of random hexanucleotides, 0.2 mM dNTPs and 200 units of M-MLV-RT to a total volume of 50 μ l, and incubated for 1 h at 37 °C. Reaction was blocked by heating the mixture at 95 °C for 5 min.

PCR was carried out in a 100 μ l reaction volume using 5 μ l of the cDNA reaction product (corresponding to 100 ng of RNA equivalent) as a template mixed with 95 μ l of PCR mix (1 $\times$ Taq buffer, 50 pmol of each primer, 0.1 mM dNTPs and 0.5 units of Taq polymerase). Specific primers pairs used in this study are shown in Table 1.

The reaction was conducted on a Progene DNA Thermo Cycler under the following conditions: for β -actin, Ngn3 and NeuroD, 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; for Pax6, Pax4, Isl1, Nkx2.2, insulin, glucagon and somatostatin, 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. The products of the investigated genes were all within the linear phase of the reaction using 35 PCR cycles for β -actin, Ngn3 and Pax6, 29 cycles for NeuroD, Pax4, Isl1 and insulin primers and 25 cycles for glucagon, somatostatin and Nkx2.2 primers.

To confirm that no contamination of genomic DNA occurred, samples without RT treatment were prepared.

Dot hybridization analysis

Dot hybridization of amplified materials were performed with 20 μ l of RT-PCR mixture blotted by vacuum on to a nylon membrane and then cross-linked at $1.2 \times 10^5 \mu\text{W-s/cm}^2$. Membrane hybridization was carried out with 500 ng of an internal ^{32}P -end-labelled probe, at a temperature 10 °C lower than the probe melting temperature in $6 \times \text{SSC}$ (where $1 \times \text{SSC}$ is 0.15 M NaCl/0.015 M sodium citrate) containing 0.25 % powdered milk. The membranes were washed for 10 min, twice with $6 \times \text{SSC}$ containing 0.1 % SDS and twice with $3 \times \text{SSC}$ containing 0.1 % SDS. Membranes were then washed for 15 min once in $1 \times \text{SSC}$ containing 0.1 % SDS at a temperature 10 °C higher than the hybridization temperature. After washing, membranes were exposed and single spots were counted from the membranes using a computerized PhosphorImaging system (Beckman). Relative quantification of the specific cDNA was standardized to the β -actin mRNA expression level to normalize for the degree of RNA degradation, which can be quite different between samples.

The nucleotide sequences of the internal probes used for hybridization are as follows. β -Actin, 5'-TCATGAAGAT-CCTCACCGAGCG-3'; Ngn3, 5'-AAGAGCGAGTTGGCA-CTCAGCAAACAGC-3'; Pax6, 5'-TTTACCCAAGAGCAA-ATTGAGGCCCTGGAG-3'; Isl1, 5'-ACCACATCGAGTGT-TTCCGCTGTGTAGCCT-3'; NeuroD, 5'-CAAAGCCCTG-ATCTGGTCTCCTTCGTACAGA-3'.

Induction of formation of embryoid bodies (EBs)

About 10^6 cells were seeded into 100 mm bacterial-grade Petri dishes and cultured in RPMI 1640 supplemented with 10 % fetal bovine serum containing 2 mM L-glutamine, 100 units/ml penicillin and 100 μ l/ml streptomycin at 37 °C in an atmosphere of 5 % CO_2 .

Structural studies of EBs

EBs of 1, 5 and 10 days of age were fixed with 4 % paraformaldehyde and 0.1 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 40 min. The materials were washed several times in a cacodylate buffer, dehydrated in a graded series of ethanol up to absolute (99.9 %) and embedded in LR White M acrylic resin polymerized at 54 °C for 24 h. Semithin sections (1 μ m) were counterstained with 1 % Toluidine Blue in 1 % borax.

Immunocytochemistry on whole EBs

EBs were collected after 5 days of culture, washed twice with 500 μ l of Earle's buffered salt solution (EBSS) and then fixed with 500 μ l of 2 % formaldehyde in PBS for 20 min. During the fixation time, EBs were vortexed to avoid aggregation. EBs were washed twice with 200 μ l of EBSS containing 0.1 % saponin (EBSS-saponin) by centrifugation at 400 g for 5 min and then incubated overnight at 4 °C with 100 μ l of a 1:100 solution of rabbit anti-insulin or anti-glucagon monoclonal antibody, both in EBSS-saponin. After that EBs were washed twice with 200 μ l of EBSS-saponin as before and then incubated overnight at 4 °C with 100 μ l of a 1:100 solution of anti-rabbit TRITC-IgG or of anti-mouse FITC-IgG. After incubation EBs were washed twice with 200 μ l of EBSS-saponin followed by a final washing with 200 μ l of EBSS. EBs were resuspended in 20 μ l of EBSS, dropped on glass slides and air-dried.

Immunolabelling on thin section of EBs

Immunoelectron microscopy of ultrastructural details was carried out on ultrathin sections (120 nm) of 1-, 5- and 10-day-old EBs.

The ultrathin sections were mounted on 300-mesh nickel grids, floated on a 25 μ l drop of specific blocking buffer containing 1 % BSA and 20 % normal goat serum in 0.1 M Tris/HCl, pH 7.4, for 1 h at room temperature, and then incubated on a drop of Tris-buffered saline containing 1 % BSA, 1 % normal goat serum, 4 % fetal calf serum, 0.1 % Tween-20 and monoclonal anti-insulin and anti-glucagon primary antibodies (final dilution 1:10) for 2 h at room temperature. After several washes in Tris-buffered saline to remove the antibody excess, the sections were incubated in colloidal secondary anti-mouse IgG 30 nm-gold-conjugated antibody (diluted 1:100) in the incubation medium, pH 8.4, for 2 h at room temperature. The sections were finally counterstained with 4 % uranyl acetate (2 min) and 0.25 % lead citrate (2 min). The primary reagent anti-insulin antibody was omitted in the controls. The specimens were examined with Philips EM 208 electron microscope (Philips Electronoptics BV).

Insulin secretion assay

EBs were harvested from 100 mm bacterial-grade Petri dishes at 1, 5 and 10 day intervals and washed twice with 1 ml of glucose-free DMEM. Three aliquots of EBs were incubated in 1 ml of glucose-free DMEM for 1 h at 37 °C in an atmosphere of 5 % CO_2 . After that, each aliquot was treated with glucose-free DMEM, DMEM containing 5 or 16.7 mM glucose or DMEM containing 5 or 16.7 mM glucose/1 mM IBMX, and incubated for 2 h at 37 °C in an atmosphere of 5 % CO_2 . Subsequently, conditioned media were collected and insulin levels were measured using a rat insulin RIA kit.

RESULTS

The teratocarcinoma F9 murine cell line has been used as a cellular model in our study. F9 is an embryonic cell line that, being almost completely unable to differentiate spontaneously, propagates primarily as stem cells. Upon exposure to RA, F9 cells differentiate into primitive and visceral endoderm-like cells, resembling in many biochemical properties the extra-embryonic endoderm of the early mouse embryo [16,19–21]; for this reason they are often used in studies of early embryonic development. The differentiated F9 cells express endoderm markers such as GATA4 [22], GATA6 [23], Dab2 [24], and basement-membrane components including collagen IV [25] and laminin [26].

F9 cells were allowed to differentiate into endoderm cells using RA at a final concentration of 0.1 μM . To assess the efficiency of differentiation of F9 cells, laminin levels in the culture media were measured by an immunosorbent assay. As shown in Figure 1(A), laminin levels increased during the first 72 h, reaching a plateau after 96 h. In contrast, there was only a modest amount of immunoreactive material in the media from F9 cells not treated with RA.

RA-induced differentiation of F9 cells is accompanied by induction of transcription of GATA4 mRNA, a marker of definitive (embryonic) and visceral (extra-embryonic) endoderm [22]. The expression of this gene was verified by RT-PCR in RA-treated cells. As shown in Figure 1(B), only F9/RA cells expressed GATA4 on the fourth day of treatment whereas no such expression was detectable in F9 cells cultured in absence of RA.

After 4 days, both F9 non-differentiated cells and F9/RA endoderm cells were transfected with a recombinant vector harbouring the complete coding sequence of the mouse *Ngn3* gene. After 72 h the cells were harvested, total RNA was extracted and subjected to RT-PCR using primers for the pancreas development transcription factors NeuroD, Pax6, Pax4, Nkx2.2,

Figure 1 Differentiation of F9 cells into endoderm-type cells

(A) Detection of laminin by ELISA assay in cell culture media of RA-treated and untreated F9 cells. F9 cells were grown for 4 days in the absence of RA (●) or in media containing 0.1 μ M RA (○). Cell-culture medium was changed every 24 h and the amount of secreted laminin was measured in 100 μ l of medium when indicated. An arbitrary unit was established by dividing the absorbance value (410 nm) by the number of cells in each plate. Results are means $\pm$ S.E.M. from triplicate analyses. (B) Expression of GATA4 in F9/RA and F9 cells on the fourth day of RA treatment. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Pdx1 and Isl1 and for the pancreatic hormones insulin, glucagon and somatostatin. Primers for β -actin mRNA were used as positive controls for RT-PCR analysis. The results are reported in Figure 2.

As a negative control, the expression of Ngn3 and of all the analysed transcription factors was also investigated in F9 and F9/RA non-transfected cells. Ngn3 was found to be expressed in both F9 and F9/RA transfected cells at rather high levels, but expression of the pancreas cell markers NeuroD, Pax6 and Isl1 was detected only in the endoderm-derived F9/RA cells (see Figure 2A). No evidence for the transcription of mRNAs of factors Pdx1, Nkx2.2 and Pax4 was detected in either transfected or non-transfected F9 and F9/RA cells (results not shown). The RT-PCR for insulin, glucagon and somatostatin was negative, indicating that the transfected cells were unable to express pancreatic hormones that are produced only by differentiated and mature endocrine cells (results not shown).

The results of RT-PCR experiments showed that 72 h after transfection there was a substantial difference between the levels of expressed transcription factors analysed. In fact, NeuroD seems to be expressed at a higher level with respect to Pax6 and Isl1. Particularly so, the latter factor showed only a very low signal (Figure 2B).

Figure 2 Expression of pancreatic transcription factors in Ngn3-transfected and non-transfected cells

(A) RT-PCR analysis of the expression of β -actin, Ngn3, NeuroD, Pax6 and Isl1 genes in F9 and F9/RA cells, non-transfected (—) and transfected (+) with pIRES/Ngn3 recombinant vector. F9/RA cells were induced to differentiate into endoderm cells by adding RA to a final concentration of 0.1 μ M for 4 days before transfection. At 72 h after transfection total RNA was extracted and processed for the RT-PCR analysis of the gene transcription. As a control, the same quantity of RNA was subjected to PCR directly without RT treatment. Each row shows ethidium bromide-stained agarose gel of the PCR amplification of the indicated transcripts. (B) Expression of β -actin, Ngn3, NeuroD, Pax6 and Isl1 genes in transfected F9/RA cells at the indicated times. Total RNA was extracted from the cells 24, 48 and 72 h after transfection, and RT-PCR was performed with the same amount of RNA and primers pairs specific for Ngn3, NeuroD, Pax6 and Isl1 and the control gene β -actin.

It was therefore important to assess the temporal expression of transcription factors, their hierarchy of appearance in F9/RA cells transiently expressing Ngn3 and the relative quantification of their mRNAs. A time-course experiment was then performed analysing the expression of the pancreatic markers 24, 48 and 72 h after transfection. As reported in Figure 3, the mRNA expression of Ngn3 was detected just after 24 h and its signal persisted during the following 48 h. Its expression preceded by 24 h that of NeuroD and Pax6, whereas the Isl1 expression took place only 72 h after transfection. Moreover, the low intensity of the signal denotes late activation of the Isl1 gene.

To analyse gene expression of the analysed transcription factors, levels of mRNA were also estimated by a dot hybridization analysis of their RT-PCR products [27].

Figure 3 Dot-blot analysis of the expression of transcription factors Ngn3, NeuroD, Pax6 and Isl1 in transfected F9/RA cells 24, 48 and 72 h after transfection

The magnitude of gene expression was determined by densitometry and the relative signal was normalized to the level of actin expression. The ratio between the analysed genes and the internal standard is shown. Panel (A) shows the β -galactosidase activity detected in protein extracts, at the indicated times after transfection from non-transfected (striped bars) and transfected (black bars) F9/RA cells.

As shown in Figure 3, Ngn3 was expressed within the first 24 h of transfection and increased slightly during the next 48 h (Figure 3A). Only after 72 h was a significant decrease in Ngn3 expression observed, due to recombinant plasmid degradation and dilution by cell division, as assessed by β -galactosidase expression assays (results not shown).

Gene expression of transcription factors NeuroD and Pax6 was detected 48 h after transfection and their expression increased on the third day (Figure 3). It is worth noting that although on the second day after transfection both NeuroD and Pax6 showed a similar level of expression, after 72 h the signal intensity of NeuroD had almost doubled, reaching an expression level higher than that of Ngn3, whereas the signal of Pax6

increased only slightly. In a quite different way, the expression of Isl1 started only on the third day after transfection (Figure 3).

The evidence obtained by transient transfection regarding a cascade activation of transcription factors starting from Ngn3 prompted us to analyse transcription-factor activation over a longer period of time. To do this a selection of stable transfectants of F9/RA by treatment with G418 was carried out. Resistant clones were analysed for their ability to express Ngn3 by RT-PCR. We obtained nine resistant clones, five of which were positive for Ngn3 expression. We used the G6 stable transfected clone as a representative clone for the results reported here, but similar results were obtained with the other stable clones. G6, F9 and F9/RA cells were normally maintained in DMEM in cell

Figure 4 Expression of β -cell-related genes in 10-day-old G6-derived EBs, G6 high-density adherent cells, F9/RA cells, F9-derived EBs and F9/RA-derived EBs

cDNA was synthesized from 1 μ g of total RNA and random hexanucleotides. INS, insulin; GLUC, glucagon.

Figure 5 RT-PCR analysis of the expression of OCT4, GATA4, insulin (INS) and β -actin in F9, F9/RA and G6 adherent cells, and in 1-, 5- and 10-day-old G6-derived EBs

Figure 6 Morphology of whole 1-day-old EBs (A) and of histological sections of 1- (B) and 10- (C) day-old EBs

Cells were cultured in RPMI 1640 medium in 100 mm bacterial-grade Petri dishes. Semi-thin sections (1 μ m) were prepared from whole EBs embedded in LR White M acrylic resin and counterstained with 1% Toluidine Blue. Magnification, $\times$ 400.

culture flasks and in RPMI 1640 when cultured in bacterial Petri dishes.

After cells were transferred from suspension culture to bacterial-grade Petri dishes, they failed to adhere and formed small aggregates. Acquisition of the configuration of discrete EBs started as early as 2 days after transfer to suspension culture.

According to the results reported above, the expression of β -cell-related genes using RT-PCR analysis on total RNA extracted from high-density stable-transfected adherent cells and from EBs

Figure 7 Insulin- and glucagon-expressing cells in EBs

(A and B) Immunofluorescence microscopy on whole 5 day-old EBs was performed as described in the Experimental section. Insulin- (A) and glucagon- (B) producing cells are organized in three-dimensional clusters; magnification, $\times 320$. (C–E) Transmission-electron-microscope immunocytochemistry on ultrathin sections (120 nm) of 10 day-old EBs. Insulin (C and D) and glucagon (E) are indicated by black spots. They appear localized in the cytoplasm, near the plasma membrane and in secretory granules (arrows).

was examined. RT-PCR conditions were the same used for transiently transfected cells. As shown in Figure 4, lanes 1 and 2, all the tested transcription factors, Ngn3, NeuroD, Pax6, Pax4,

Nkx2.2, Pdx1 and Isl1, were found in 10-day-old G6-derived EBs and in G6 cells. Interestingly, insulin was expressed only in G6-derived EBs but not in G6 cells. The presence of all analysed

transcription factors and of insulin was investigated in non-transfected F9/RA cells and in F9- and F9/RA-derived EBs, but no expression of these factors was detected (Figure 4, lanes 3–5).

To examine whether Ngn3 overexpression restricts cell fate to the endocrine lineage, the presence of other endocrine markers such as glucagon and somatostatin and the exocrine marker α -amylase was analysed by RT-PCR in 10-day-old G6-derived EBs. Glucagon was detected whereas somatostatin and α -amylase were both absent (Figure 4, lane 1).

In another set of experiments, the expression of OCT4 [28], a marker of the pluripotent state, of GATA4 and of insulin in F9 cells, F9/RA cells, G6 high-density adherent cells and in 1, 5 and 10-day-old G6-derived EBs was examined (Figure 5). Expression of OCT4 was detected in F9 cells and at lower levels in F9/RA cells, whereas it became almost undetectable in high-density G6 cells and their derived EBs. The activation of *GATA4* gene expression took place after RA treatment of F9 cells and its expression persisted at lower levels in G6 adherent cells even after activation of markers of pancreatic cells occurred. Its expression decreased drastically in 5 day-old G6-derived EBs. As judged by expression of the marker OCT4, F9 cells seemed to be composed predominantly of undifferentiated cells, whereas the down-regulation of OCT4 expression in F9/RA cells is consistent with the observations that the differentiation process induced by GATA factors may include active repression of *OCT3/4* genes [28]. The decrease of OCT4 in G6 cells and in corresponding EBs can be ascribed to an efficient conversion of undifferentiated cells into differentiated progenitor cells and to the achievement of a fully differentiated status for EBs. This hypothesis is confirmed by the observation that insulin expression was consistently revealed only from G6-derived EBs of 5 days and its level was increased in EBs of 10 days, but it seemed to be not relevantly expressed in 1-day-old EBs and, as expected, it was completely absent in F9, F9/RA and G6 cells (Figure 5).

A preliminary study was performed to analyse the gross structure of G6-derived EBs. The results are reported in Figure 6. The EBs appear as spherical structures with primitive endodermal layers, surrounding inner cells (Figure 6A) that grow in size, developing a pronounced cyst structure. The morphology of the thin sections shows the presence of active and synchronous proliferating cells (Figure 6B) with the mean diameter of EBs increasing from 250 μ m (1 day old; Figure 6B) to $\approx$ 400–500 μ m (10 days old; Figure 6C).

To gain more-detailed information on the distribution of insulin-/glucagon-producing cells, immunocytochemistry analyses were performed on whole EBs as well as on sections of them. Results are reported in Figure 7. As shown in Figure 7(A), positive signals were detected in whole EBs. Occasionally cells expressing insulin were present either as scattered cells throughout the EBs or organized in small clusters (Figure 7A). Similar results were observed for glucagon (Figure 7B). No staining was evident using non-immune serum. Based on these results immunocytochemical analysis was performed on ultrathin resin-embedded sections of EBs. Insulin and glucagon distribution was analysed on sections of 10 day-old EBs. The results are reported in Figures 7(C)–7(E). Insulin (Figures 7C and 7D) and glucagon (Figure 7E) signals were present in cytoplasm, in secretory granules and near to the plasma membrane. From a quantitative point of view there was a more intensive distribution of positive signals in 10 day-old EBs than in 1 day-old ones, thus indicating a progressive maturation and/or proliferation of EBs.

To characterize these insulin-containing cells, insulin liberated into the medium upon glucose stimulation was measured by RIA. EBs were harvested at 1, 5 and 10 days of culture and incubated in 35 mm bacterial-grade Petri dishes with 1 ml of

Table 2 Insulin release from EBs in response to glucose and IBMX stimulation

	Insulin release (ng/mg of protein)		
	1 day-old EBs	5 day-old EBs	10 day-old EBs
0 mM Glucose	$(0.06 \pm 7.1) \times 10^{-3}$	$(0.04 \pm 3.5) \times 10^{-3}$	$(0.05 \pm 2.1) \times 10^{-3}$
5 mM Glucose	$(0.05 \pm 2.1) \times 10^{-3}$	3.9 ± 0.28	11.0 ± 1.4
5 mM Glucose + IBMX	$(0.05 \pm 2.1) \times 10^{-3}$	4.5 ± 0.21	8.4 ± 1.5
16.7 mM Glucose	$(0.05 \pm 7.1) \times 10^{-3}$	9.1 ± 0.51	25.0 ± 4.2
16.7 mM Glucose + IBMX	$(0.06 \pm 2.8) \times 10^{-3}$	11.2 ± 0.28	25.0 ± 4.6

glucose-free DMEM. After 1 h of incubation at 37 °C, 5 and 16.7 mM glucose was added in the presence or absence of 1 mM IBMX. Immunoreactive insulin concentration was measured at the end of the second hour of incubation (Table 2). In cells not stimulated with glucose and in 1 day old EBs, insignificant immunoreactive insulin could be detected. To the contrary, in response to glucose both 5- and 10-day-old EBs released insulin following rapid dose-dependent kinetics. Moreover, the data in Table 2 show that a progressive maturation of EBs cells occurred, going from 5 to 10 day-old EBs, as indicated by the almost 3-fold increase of the glucose-stimulated insulin release. To establish if the cells use physiological signalling pathways to regulate insulin release, we examined the effect of agonist IBMX [29] on insulin secretion. Release of insulin from cells is coupled to multiple phosphorylation events modulated, among others by protein kinase A, which is activated by cAMP. IBMX is an agonist since it acts as an inhibitor of cAMP phosphodiesterase. As shown in Table 2, IBMX seemed to weakly stimulate insulin secretion in the presence of a low concentration (5 mM) of glucose, whereas at a higher glucose concentration (16.7 mM) no effect of IBMX on insulin release was observed.

DISCUSSION

The analysis of cellular differentiation is often hampered by various factors, among which the heterogeneity of the cell population is one of the most important. Pancreatic differentiation and development is a good example of this heterogeneity [1,30,31]. Despite the large amount of data regarding the molecular events accompanying pancreatic development and differentiation [15,30,32,33], understanding of these events suffers from a lack of suitable cellular models. In fact, most of studies of pancreas development are performed on dissected embryonic tissues, thus making the search for a suitable cellular model a prominent goal [34,35]. Indeed, cell models have often been used in pancreas studies, but mainly limited to those on fully differentiated β -cells. Moreover, there is a growing research interest in the possibility of pancreatic stem cells being used for the treatment of type 1 diabetes [36–42]. In this respect, however, it should be stressed that a full understanding of the hierarchy of the molecular events involved in pancreatic differentiation is a necessary prerequisite to their utilization for therapeutic scopes. So far, most of the available information results from the analysis of the distribution of known transcription factors in the pancreas at different times of development [31,33,43,44]. This approach, albeit rich in qualitative information, is unfortunately unable to highlight the time correlation of the different transcription factors, not providing a picture of their sequential role and hierarchy. Among these factors, Ngn3 plays a central role in initiating the differentiation of islet cells [15]. Mice carrying a targeted disruption of the *Ngn3* gene fail to express other

pancreas-specific transcription factors, including NeuroD, Pax6 and Isl1 [32,45]. This finding suggests that Ngn3 acts upstream of these factors as a genetic switch that specifies the endocrine cell fate in pluripotent pancreatic progenitors. All immunohistochemical observations support this hypothesis, but until now a direct demonstration of the activation of pancreas-specific transcription factors by Ngn3 did not exist.

The data here reported demonstrate the existence of a precise temporal hierarchy of activation of transcription factors triggering the pancreatic differentiation. According to the data here reported, the first transcriptional activation sequence is *Ngn3* $\rightarrow$ *NeuroD* = *Pax6* $\rightarrow$ *Isl1*. Moreover, our results clearly demonstrate that Ngn3 is sufficient to initiate and complete the β -cell differentiation pathway.

Among the analysed transcription factors, NeuroD was detected 48 h after transfection with Ngn3. This finding is consistent with a direct effect of Ngn3 on the activation of NeuroD, as postulated by Huang et al. [46]. It was observed that overexpression of Ngn3 could cause activation of the 1.0 kb NeuroD promoter in islet-derived cell lines. NeuroD is a basic helix-loop-helix transcription factor, expressed in all endocrine cells of the pancreatic epithelium and is likely to be implicated in their proliferation rather than in their differentiation [14]. However, recent studies of the development of pancreatic α - and β -cells [45] suggested that NeuroD is the earliest marker of endocrine-differentiating cells.

Our experiments also show that Ngn3 leads to the expression of Pax6 48 h after transfection. The important role of Pax6 was only recently revealed in the differentiation of α -cells during pancreas development using knockout mice [9]. Expression of Pax6 can be detected around E9.0. It is not only required for the final differentiation of α -cells, but it is also involved in the proliferation of all endocrine precursor cells and in the regulation of islet morphology [8]. Although the *Pax6* gene is very closely related to the *Pax4* gene, Pax4 expression was not detected in F9/RA cells. In contrast with Pax6, which can be detected throughout early pancreas development in all endocrine cells, Pax4 is required exclusively in cells restricted to the β - and δ -cell lineages. In fact, mice lacking the *Pax4* gene generate neither insulin-producing β -cells nor somatostatin-producing cells [6].

The third transcription factor detected in the endoderm transiently transfected F9/RA cells was Isl1, but its signal appeared only after 72 h. Isl1 is a LIM homeodomain protein expressed in all types of islet cells in the adult; its expression in the embryo is initiated soon after the islet cells have left the cell cycle [47,48]. Isl1 appears in glucagon-positive cells in E9.5 embryos, before the differentiation of the first cells expressing insulin and somatostatin [15]. It seems to act downstream of Pdx1 and NeuroD in islet differentiation, being described as one of the factors involved in the formation of endocrine progenitor cells [11]. Although some immunohistochemical evidence suggests that Isl1 expression precedes *Pax6* gene activation [43], in our study the very small signal obtained for Isl1 24 h after *Pax6* gene detection indicates a later activation and a very low level of expression of this gene. On the other hand, in stable transfectants *Isl1* expression is present in low levels and increases after EB formation and insulin production.

Interestingly, Pdx1 was not revealed in the transient transfection, but it was instead present in the stable clones. This result is not surprising since in early pancreatic differentiation Pdx1 is mainly involved in processes surrounding bud formation, allowing cells to invade the mesenchyme [49] and disappearing afterwards. Only subsequently, in a fully differentiated and functional pancreas, does Pdx1 appear again, albeit restricted to insulin-producing cells. On the contrary, the differentiation events

controlled by Ngn3 act only later with respect to those involved in bud formation [45]. Our results are consistent with this hypothesis since we detected Pdx1 only in stable transfectants and in EBs that were able to express insulin.

Among the various transcription factors involved and activated during the development of mouse pancreas there is also the NK-homeodomain factor Nkx2.2. It is initially expressed very early throughout the pancreatic epithelium, it immediately precedes the expression of Nkx6.1, and it is only later restricted in the endocrine lineage during the final differentiation of α -, β - and PP cells. Nkx2.2 seems to be required for terminal differentiation of β -cells and maintenance of Nkx6.1 expression [12,13]. Again the appearance of these transcription factors has been detected at a significant level only in stably transfected G6 cells and in insulin-producing EBs.

The data here reported demonstrate that Ngn3 is the first event in the temporal sequence of activation of some transcription factors specifically involved in the differentiation of β -cells. Interestingly, gene activation triggered by Ngn3 was restricted to the cells that underwent endodermal differentiation after RA treatment (F9/RA). In contrast, ectopic Ngn3 expression in cells not treated with RA (i.e. F9 cells) was not sufficient for inducing expression of genes involved in the signalling pathways that promote endocrine pancreas differentiation. These results should be consistent with experimental observations in zebrafish endoderm [50] in which specification of both pancreas and liver requires RA signalling. That RA-dependent step in pancreatic specification occurs significantly earlier than the expression of markers of pancreatic progenitors. Besides, we could not detect endocrine differentiation in non-transfected F9/RA cells, even after culture as EBs, indicating that retinoic acid signalling is critical but not sufficient to induce the proper differentiation programme towards pancreatic endocrine cells.

Very recently, employing an inducible Cre-ERTM-LoxP system it has been shown that Ngn3-expressing cells are islet progenitors but not exocrine progenitors during embryogenesis and adulthood in mice [51]. Our data are consistent with these results, demonstrating that ectopic Ngn3 is enough to trigger differentiation in endocrine pancreas cells but not in exocrine cells.

The sequence of events, growth factors, cell-cell interactions, extracellular matrix factors and signalling pathways involved in the endoderm lineage pathway represent only the first steps towards deciphering how to induce differentiation in endoderm-derived cells. The approach normally followed to enrich a specific cell lineage is the selection of particular culture conditions that can allow for enrichment of spontaneously differentiated cells [40]. Another approach was followed in a previous study [49], in which it was reported that ectopic expression of Ngn3 in chick embryos turned endodermal cells of any region into α -type but not β -type endocrine islet cells, not requiring the co-operation of all other pancreas-specific patterning genes.

Here, using a cellular model, we demonstrated that it is possible to drive both β - and α -cell differentiation of RA-derived-endoderm cells, controlling the cascade of molecular events by ectopic activation of the earliest endocrine pancreatic transcription factor Ngn3.

It is known that the formation of EBs reproduces some of the conditions required for cell differentiation: they are polarized, there are cell-cell interactions, there exist asymmetric contacts and growth factors are being produced. We followed this approach to obtain structures able to secrete insulin in response to physiologically appropriate glucose concentrations. The low number of cells able to produce and secrete insulin that we obtained cannot be considered a drawback of the approach since other factors can contribute to the enhancement of the starting

number of β -cells in the population within the EBs. Among them are vascular endothelial growth factor [52], hepatocyte growth factor [53], transforming growth factor [54], insulin-like growth factor [55], epidermal growth factor [56] activin A and betacellulin [57]. None of these factors were analysed in the present study. The results of the present study are an important achievement since they provide evidence that the start of the differentiation programme of the β -cells in embryonic stem cells can be triggered *in vitro* by Ngn3, which activates a cascade of specific transcription factors that can drive full differentiation into insulin-producing cells. These results provide new insight in the understanding of the complex mechanism of control of the differentiation of stem cells towards the production of β -cells.

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