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Mouse ES cells over-expressing the transcription factor NeuroD1 show increased differentiation towards endocrine lineages and insulin-expressing cells

MÉLANIE MARCHAND[1,2,3,4,5], INSA S. SCHROEDER[6], SUZY MARKOSSIAN[1,2,3,4], ANOUCHKA SKOUDY[7], DIDIER NÈGRE[3,4,10,11], FRANÇOIS-LOÏC COSSET[3,4,10,11], PACO REAL[7,8,9], CHRISTIAN KAISER[6], ANNA M. WOBUS[6] and PIERRE SAVATIER[1,2,3,*]

[1]Inserm, U846 and [2]Stem Cell and Brain Research Institute, Bron, France, [3]Université de Lyon and [4]Université Lyon I, Lyon, France, [5]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, CA, USA, [6]In vitro Differentiation Group, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Germany, [7]Cell and Molecular Biology Unit, Institut Municipal d'Investigació Mèdica (IMIM), Barcelona, Spain, [8]Departament de Ciències Experimentals I de la Salut, Universitat Pompeu Fabra, Barcelona, Spain, [9]Centro Nacional de Investigaciones Oncologicas (CNIO), Madrid, Spain, [10]Inserm, U758, Human Virology Department and [11]Ecole Normale Supérieure de Lyon, Lyon, France

ABSTRACT Embryonic stem (ES) cells which constitutively express the Pdx-1, Ngn-3, NeuroD1, Nkx2.2, and Nkx6.1 transcription factors were engineered by means of lentiviral vectors, following a multi-step infection procedure to successively generate ES cell lines expressing one, two, and three factors, respectively. Each ES cell line was allowed to differentiate into nestin[+]/Isl-1^{+} endocrine precursors, then into more mature pancreatic cells, and subsequently analysed for expression of *Glc, Ins,* and *Sst*, markers of α, β and δ cells, respectively. Each ES cell line generated displayed a unique pattern of gene expression. The ES cell line expressing NeuroD1 displayed vastly elevated levels of *Glc, Ins-1, Ins-2* and *Sst*, and showed an increase in *Pdx-1, Pax-4, Nkx6.1, Isl-1, Glut-2* and *GK* transcript levels. Furthermore, immunofluorescence analysis revealed that differentiation of NeuroD1-expressing ES cells in nestin[+]/Isl-1^{+} multilineage progenitors, followed by the formation of C-peptide[+]/insulin[+] clusters, was accelerated. Together, these results indicate that stable expression of NeuroD1 in ES cells facilitates differentiation into endocrine and insulin-producing cells.

KEY WORDS: *embryonic stem, endocrine differentiation, insulin, NeuroD1, lentiviral vector*

Introduction

Embryonic stem (ES) cells are derived from the pluripotent cells of the blastocyst stage embryo. They are able to self-renew indefinitely *in vitro* while retaining pluripotency and to differentiate into diverse cell types of ectodermal, mesodermal and endodermal lineages (Smith, 2001; Wobus and Boheler, 2005). The differentiation of ES cells into insulin-producing β-like cells constitutes a field of intensive investigations as it represents a possible alternative both to the use of immortalized endocrine cell lines for drug screening and functional genomics and to the limited availability of human islets needed for transplantation in type I diabetes therapy (Blyszczuk *et al.*, 2004; Bonner-Weir and Weir, 2005; Nir

and Dor, 2005).

The pancreas is a heterogeneous organ composed by exocrine, ductal and endocrine cells. The exocrine compartment is responsible for the production of digestive enzymes, which are transported through pancreatic ducts formed by ductal cells. The endocrine compartment, organized in islets of Langerhans, is

***Address correspondence to:** Dr. Pierre Savatier. INSERM U846, 18 avenue Doyen Lépine, 69500 Bron, France. Tel: +33-47291-3442. Fax: +33-47291-3461. e-mail: pierre.savatier@inserm.fr - Web: http://wwwsbri.fr

responsible for hormone production. The endocrine α, β, δ and PP cells are secreting glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. During embryonic development, the pancreas arises as dorsal and ventral buds from the definitive endoderm layer. This complex and regulated process involves the integration of signals from adjacent mesodermal tissues (reviewed in Kumar *et al.*, 2003). Information provided by these signals activates a cascade of transcription factor expression (reviewed in Jensen, 2004; Bonal and Herrera, 2008) leading to differentiation and proliferation of pancreatic cells within the competent endoderm. Among known transcription factors, pancreatic duodenal homeobox 1 (Pdx-1 or Ipf1), a homeodomain protein, is involved in the development of pancreatic progenitor cells (Offield *et al.*, 1996; Gu *et al.*, 2002), and in insulin gene transcription and glucose response in mature β cells (Holland *et al.*, 2002). Gain-of-function studies indicate that neurogenin 3 (Ngn-3) and neurogenic differentiation 1 (NeuroD1), two bHLH factors, share the common ability to drive endocrine differentiation in the appropriate cell context (Apelqvist *et al.*, 1999; Schwitzgebel *et al.*, 2000) (Heremans *et al.*, 2002) (Gasa *et al.*, 2004). However, loss-of-function studies in mice revealed important differences in the roles of these two factors during endocrine differentiation. While *Ngn-3 null* animals lack all pancreatic endocrine cells (Gradwohl *et al.*, 2000), *NeuroD1 null* mice die postnatally from severe diabetes due to a dramatic loss of insulin-producing β cells, whereas the numbers of glucagon-producing α cells and somatostatin-producing δ cells are only modestly reduced (Naya *et al.*, 1997). Lineage tracing experiments indicate that Ngn-3 is a cell-autonomous determinant and a true marker of endocrine progenitor cells (Gu *et al.*, 2002). Taken together, these data suggest that the transient expression of Ngn-3 in the developing pancreas epithelium specifies which cells among the Pdx-1^{+} pancreatic progenitor pool will follow an endocrine fate, while its downstream target NeuroD1 carries on the endocrine differentiation program initiated by Ngn-3 and participates in the maintenance of the differentiated phenotype of the mature islet cells. The differentiation of Ngn3^{+} endocrine progenitor cells through the β cell pathway also requires the expression of other factors, such as Nkx2.2 (Sussel *et al.*, 1998), Pax-4 (Sosa-Pineda *et al.*, 1997) and Nkx6.1 (Sander *et al.*, 2000). NeuroD1 and Nkx2.2 are also involved in the regulation of insulin gene transcription in mature β cells (Naya *et al.*, 1995) (Cissell *et al.*, 2003).

Over the past few years, insulin-producing cells were derived from both mouse and human ES cells. Insulin-producing cells were obtained from ES cells by spontaneous differentiation (Assady *et al.*, 2001; Kahan *et al.*, 2003), lineage selection using pancreas-specific promoters (Soria *et al.*, 2000; Leon-Quinto *et al.*, 2004), directed differentiation using growth factor-based stepwise protocols (reviewed in Blyszczuk *et al.*, 2004) or co-culture with embryonic pancreatic buds (Vaca *et al.*, 2006) (Brolen *et al.*, 2005). More recently, pancreatic-hormone-expressing endocrine cells (D'Amour *et al.*, 2006; Kroon *et al.*, 2008) and insulin-producing islet-like clusters (Jiang *et al.*, 2007) were generated by using growth factors and signalling molecules stimulating the *in vitro* formation of definitive endoderm and the *in vivo* development of functional β-like cells from human ES cells. The overexpression of transcription factors, such as Pdx-1 (Lavon *et al.*, 2006), Ngn-3 (Treff *et al.*, 2006), Nkx2.2 (Shiroi *et al.*, 2005) or Pax-4 (Blyszczuk *et al.*, 2004), in ES cells has been shown to facilitate their differentiation into insulin-producing cells.

In the present work, we report the generation of mouse ES cell lines stably expressing Pdx-1, Ngn-3, NeuroD1, Nkx2.2 and Nkx6.1. We studied the capacity of these ES cell lines to differentiate into endocrine cells by using the three-step protocol via multilineage progenitors established by Blyszczuk *et al.* (2004), shown to result in the formation of functional glucose-responsive insulin-producing cells (Boyd *et al.*, 2008). The data presented show that NeuroD1 over-expression increases the expression of endocrine markers and facilitates differentiation of pancreatic precursors into insulin-producing cells.

Results

Generation of genetically modified ES cell lines stably expressing Pdx1, Ngn-3, NeuroD1, Nkx2.2, and Nkx6.1 transcription factors

ES cell lines that constitutively express the *Pdx-1, Ngn-3, NeuroD1, Nkx2.2* and *Nkx6.1* genes were engineered from CGR8 cells (here-
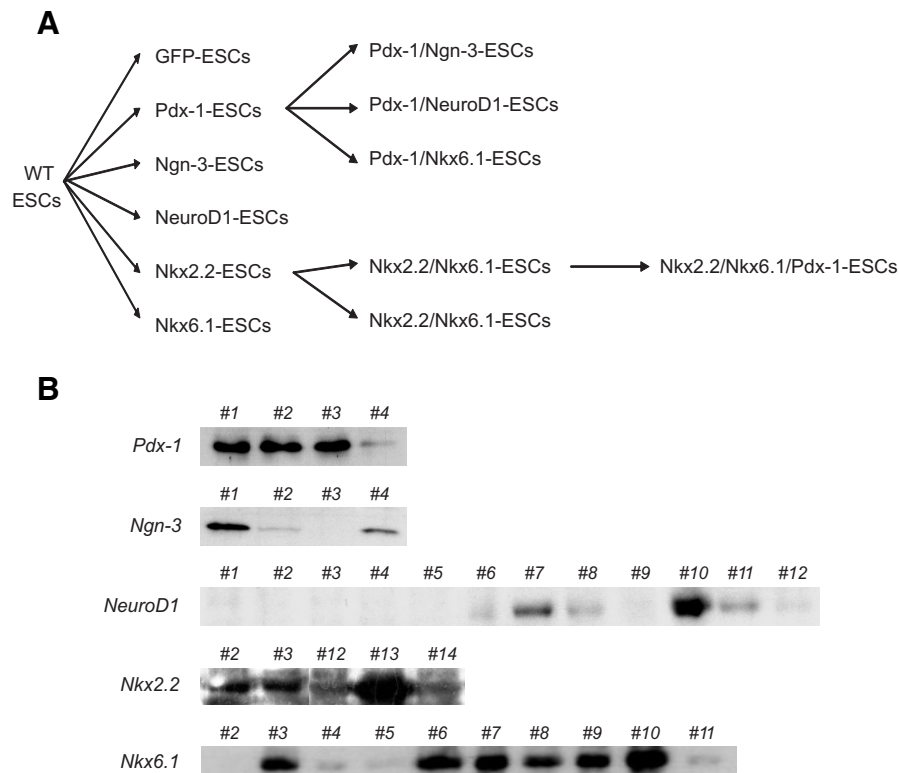
Fig. 1. Derivation of engineered ES cell lines stably expressing transcription factors Pdx-1, Nkx2.2, Nkx6.1, NeuroD1, and Neurogenin-3 (Ngn-3), by means of lentiviral vector infection. **(A)** *Schematic representation of the derivation of 12 ES cell lines expressing one, two and three transcription factors, from wild-type CGR8 ES cells (WT-ESC).* **(B)** *Immunoblotting analysis of ES cell lines expressing Pdx-1 (Pdx-1-ESC), Nkx2.2 (Nkx2.2-ESC), Nkx6.1 (Nkx6.1-ESC), NeuroD1 (NeuroD1-ESC) and Ngn-3 (Ngn-3-ESC) transcription factors.*

after called WT-ESC). This was achieved by means of lentiviral vector infection and sub-cloning following a multi-step infection procedure to successively generate ES cell lines expressing one, two, and three transcription factors (Fig. 1A). An ES cell line expressing the Green Fluorescent Protein (GFP) was used as control (hereafter called GFP-ESC). In a first step, ES cell lines expressing one single transcription factor (Pdx-1, Ngn-3, NeuroD1, Nkx6.1, and Nkx2.2) were generated. For differentiation studies, clones expressing transduced genes at the highest level were selected for subsequent infection steps: clone #1 expressing Pdx-1 (hereafter called Pdx1-ESC), clone #1 expressing Ngn-3 (hereafter called Ngn3-ESC), clone #10 expressing NeuroD1 (hereafter called NeuroD1-ESC), clone #7 expressing Nkx6.1 (hereafter called Nkx6.1-ESC), clone #13 expressing Nkx2.2 (hereafter called Nkx2.2-ESC) (Fig. 1B). In a second step, Pdx1-ESC was re-infected with the lentiviral vectors expressing Ngn-3, NeuroD1, and Nkx6.1, respectively. Clones (hereafter called Pdx1/Ngn3-ESC, Pdx1/NeuroD1-ESC and Pdx1/Nkx6.1-ESC) were selected for differentiation analysis. Similarly, Nkx2.2-ESC was re-infected with the lentiviral vector expressing Nkx6.1 to generate Nkx2.2/Nkx6.1-ESC, and with the lentiviral vector expressing Pdx-1 to generate Nkx2.2/Pdx1-ESC lines. The Nkx2.2/Nkx6.1-ESC line was subsequently re-infected with the lentiviral vector expressing Pdx-1 to generate Nkx2.2/Nkx6.1/Pdx-1-ESC.

Transgene expression was analysed by immunofluorescence in undifferentiated ES cells using specific antibodies (Fig. 2). Immunostaining was specific and localized in the nucleus in all clones analysed. To determine if transgene expression compromised self-renewal, all clones were analysed for expression of the pluripotency markers Oct-4 and Nanog by flow cytometry. This analysis revealed differences in the percentage of cells expressing Oct-4 and Nanog, ranging from 40% to 96% in transgenic ES cell lines, compared to 80% in control ES cells (Fig. 3 and Supplementary Fig. 1). Therefore, constitutive expression of most transgenes obviously affects expression levels of the pluripotency-associated genes *Oct4* and *Nanog*, which may lead to an increased rate of spontaneous differentiation. However, all transgenic ES cell lines could be propagated for several months in culture and formed embryoid bodies (EBs) upon growth in suspension (see next sections),
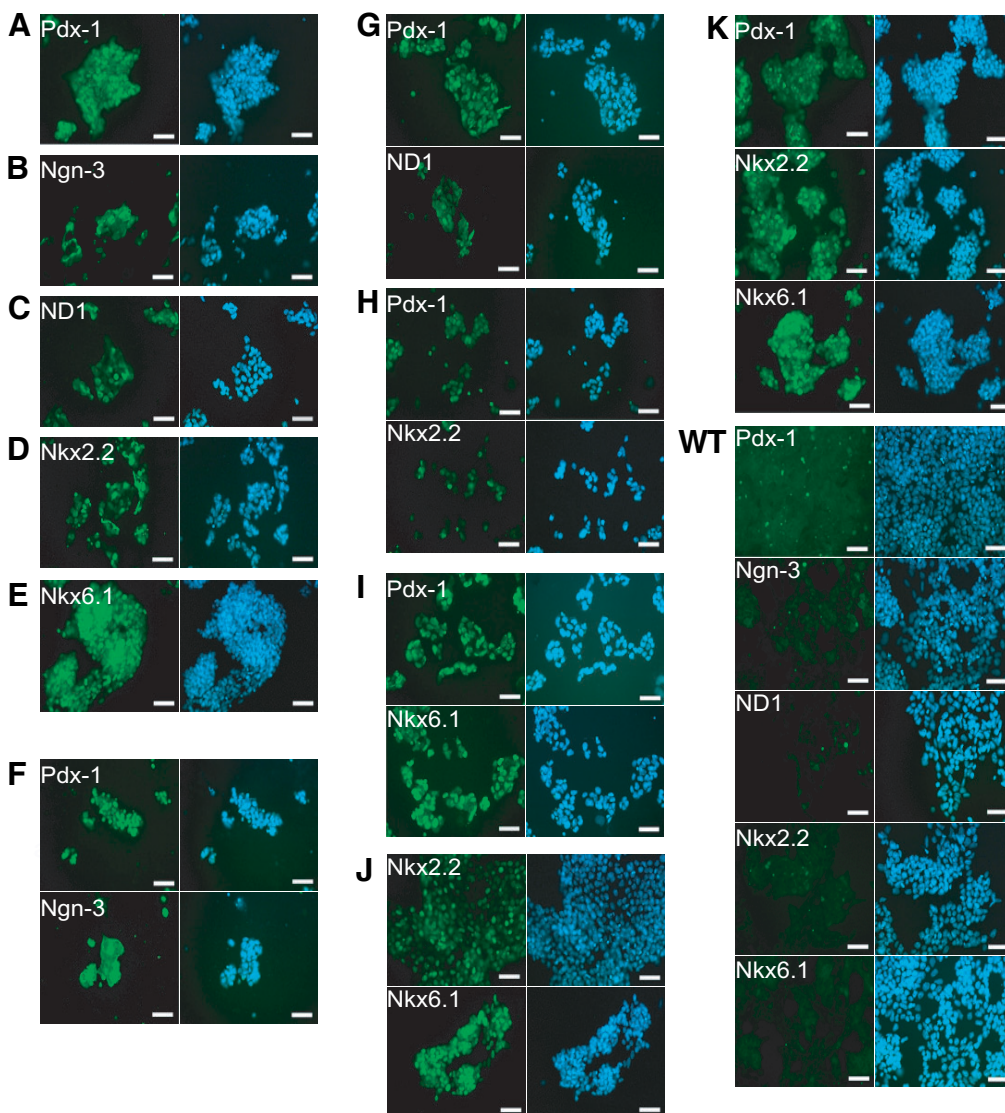

Fig. 2. Expression of transcription factors in engineered ES cell lines. *Immunofluorescence labelling of ES cell lines stably expressing Pdx-1 (A), Ngn-3 (B), NeuroD1 (C), Nkx2.2 (D), Nkx6.1 (E), Pdx-1+Ngn-3 (F), Pdx-1+NeuroD1 (G), Pdx-1+Nkx2.2 (H), Pdx-1+Nkx6.1 (I), Nkx2.2+Nkx6.1 (J), and Pdx-1+Nkx2.2+Nkx6.1 (K). Cells are labelled with FITC-conjugated antibodies (green) and counterstained with Hoechst to label nuclei. WT-ESC (WT) are shown as negative controls. Scale bar, 200 mm.*

indicating that they retained strong self-renewal capabilities.

NeuroD1-ES cells exhibit strongly increased expression of endocrine markers after differentiation

ES cell lines were induced to differentiate *via* EBs, as described previously (Blyszczuk *et al.*, 2004; Schroeder *et al.*, 2006; Boyd *et al.*, 2008). Expression of the endocrine markers *insulin-1 (ins-1), insulin-2 (ins-2), glucagon (Glc)* and *somatostin (Sst)* was analysed at differentiation stage 5+28d. During mouse embryonic development, *Ins-1* is only expressed in β-cells, while *ins-2* is expressed both in β-cells and in the developing brain (Deltour *et al.*, 1993). *Glc* and *Sst* expression characterizes α and δ cells of the Langerhans islets, respectively. Nkx2.2-ESC, Pdx-1/Nkx6.1-ESC, Pdx-1/Nkx2.2/Nkx6.1/NeuroD1-ESC showed a strong increase in *Glc* expression after differentiation, when compared to
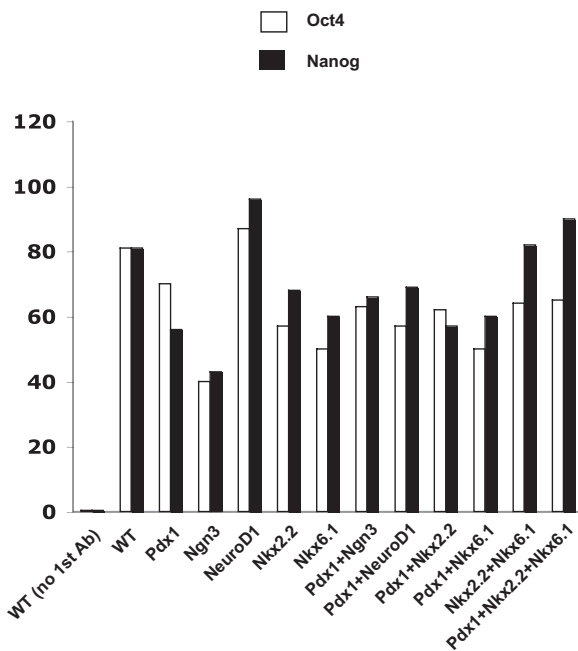
Fig. 3. Expression of pluripotency factors Oct-4 and Nanog in engineered ES cell lines. *Histograms showing the percentage of Oct-4+ and Nanog+ cells after immunolabelling with specific antibodies and analysis by flow cytometry.*

the GFP control line, whereas expression of the other endocrine markers analysed were not different to the control (Fig. 4A). Pdx-1/Nkx2.2-ESC showed a strong increase in *ins-2* expression at 5+28d of differentiation. Pdx-1/Ngn-3-ESC showed an increase in both *ins-1* and *ins-2* expression, but *Glc* expression remained below detection level. In contrast, Ngn3-ESC and NeuroD1-ESC showed a strong increase in the expression of all four endocrine markers. NeuroD1-ESC showed the highest increase in *Glc* level.

Strong induction of *Glc*, *Sst*, *Ins-1* and *Ins-2* expression at differentiation stage 5+28d in NeuroD1-ESC was confirmed in an independent experiment (Fig. 4B). When compared to WT-ESC, NeuroD1-ESC showed a significant increase in *Glc* expression (24 +/- 2.4 fold, p < 0.01, n = 3), in *ins-1* expression (13 +/- 2.7 fold, p < 0.05, n = 3), and in *Sst* expression (10 +/- 0.2 fold, p < 0.001, n = 3). *Ins-2* expression was moderately increased (6 +/- 1.2 fold, p < 0.2, n= 3).

NeuroD1-ESC also showed a strong increase in the expression of the endocrine transcription factors Pdx-1, Nkx6.1, Pax-6 and Isl-1 (Fig. 4C). Nkx2.2 is the only factor analysed which did not show elevated expression. Expression of the β-cell-specific glucose transporter-2 (*Glut-2*) and glucokinase (*GK*) genes was also strongly up-regulated in NeuroD1-ESC compared to WT-ESC. Of note, only low transcript levels of the neuronal genes *Nfm* (neurofilament heavy chain) and *Syn* (synaptophysin) were seen in NeuroD1-ESC. *Nfm* and *Syp* expression disappeared after differentiation under reduced glucose conditions (data not shown).

Together, these results show that stable expression of NeuroD1 in ES cells dramatically increases the expression of endocrine and β-cell markers upon differentiation.

NeuroD1 over-expression increases proliferation of nestin+ precursors and accelerates formation of pancreatic-committed progenitors

NeuroD1-ESC showed increased cell proliferation during *in vitro* differentiation relative to control, evidenced by a rapid exhaustion of medium and a dramatic overgrowth at stage 5+28d. We asked if the population of precursor cells exhibited an increased proliferation rate. To this aim, a proliferation assay based on BrdU incorporation was performed during WT-ESC and NeuroD1-ESC differentiation. Nestin+ progenitors in the S-phase of the cell cycle (BrdU+) were detected by double-immunofluorescence (Fig. 5A). No difference in the frequency of nestin+/BrdU+ cells was observed between WT-ESC and NeuroD1-ESC until differentiation stage 5+7d. By contrast, from differentiation stage 5+9d onwards, when multilineage progenitors (5+9d) and committed precursors (5+12, 5+16d) form (see Blyszczuk *et al.*, 2004), a larger fraction of nestin+ precursors derived from NeuroD1-ESC were BrdU+ (5+9d: 1.6 +/- 0.2 fold, p < 0.05, n = 4; 5+12d: 1.5 +/- 0.3 fold, p < 0.05, n = 4; 5+16d:1.5 +/- 0.2 fold, p <0.05, n = 4). These results indicate that NeuroD1-ESC-derived nestin+ progenitors exhibit a higher proliferation rate.

At early stage 5+9d, pancreatic progenitors can be distinguished by the co-expression of neurofilament nestin and cytokeratin 19 (CK19). By contrast, at stage 5+16d, nestin is co-expressed with Isl-1, a LIM homeodomain protein that controls cell-fate decision required for the differentiation of islet cells (Ahlgren *et al.*, 1997). Double labelling of nestin and Isl-1 has been used to characterize endocrine-committed pancreatic progenitors from stage 5+16d onwards (Blyszczuk *et al.*, 2004). Immunostaining of nestin+/Isl-1+ cells was performed during differentiation of WT-ESC and NeuroD1-ESC to identify endocrine-committed progenitors. In WT-ESC, nestin+/Isl-1+ clusters were first detected at stage 5+16d as previously described (data not shown, see (Blyszczuk *et al.*, 2004)). Some occasional nestin+/Isl-1+ cells were detectable as early as stage 5+9d, but they were dispersed and did not form clusters (Fig. 5B). This contrasted with NeuroD1-ESC that showed numerous nestin+/Isl-1+ positive cells organized in clusters as early as stage 5+9d. These results suggest that in NeuroD1-ESC the formation of nestin+/Isl-1+ endocrine-committed progenitors is accelerated.

### NeuroD1 overexpression accelerates the formation of insulin-producing clusters

NeuroD1-ESC showed an elevated expression of β-cell markers in differentiated cells at 5+28d (Fig. 4). We then asked if the yield of C-peptide+/insulin+ cells was increased. Double immunostaining for insulin and C-peptide was performed at the differentiation stage of 5+16d (Fig. 5). NeuroD1-ESC formed 3.4-fold more C-peptide+/insulin+ clusters than control WT-ESC (Fig. 6A). Moreover, NeuroD1-ESC-derived clusters contained a high number of insulin-positive cells (Fig. 6B). In contrast, WT-ESC-derived clusters displayed occasional insulin+ cells, which seldom formed typical clusters at stage 5+16d. With WT-ESC, the typical clusters were only observed at the terminal stage of 5+28d (see Blyszczuk *et al.*, 2004; Schroeder *et al.*, 2006). Together, these observations indicate that in NeuroD1-ESC the formation of C-peptide+/ins+ clusters is accelerated.

Discussion

The aim of this study was to examine the ability of engineered mouse ES cells to differentiate into pancreatic endocrine cells following a differentiation protocol previously optimized for differentiation into insulin-producing cells (Blyszczuk *et al.*, 2004; Schroeder *et al.*, 2006). This protocol was confirmed to be efficient in generating functional glucose-responsive insulin-producing cells (Boyd *et al.*, 2008) and to induce the generation of other cell types of the islet of Langerhans, such as α- and δ-cells (Schroeder *et al.*, 2006; Boyd *et al.*, 2008). Following this protocol,

a fraction of the cell population differentiates into pancreatic precursor-like cells, evidenced by the co-expression of the neurofilament nestin and the endocrine-specific transcription factor Isl-1. At a later stage, they further differentiate into insulin-producing cells organized in clusters. The fraction of cells adopting a β-like phenotype can be significantly increased by over-expressing the transcription factor Pax-4 (Blyszczuk *et al.*, 2004; Schroeder *et al.*, 2006), which suggests that expression of endogenous Pax-4 is rate-limiting for β-cell differentiation.
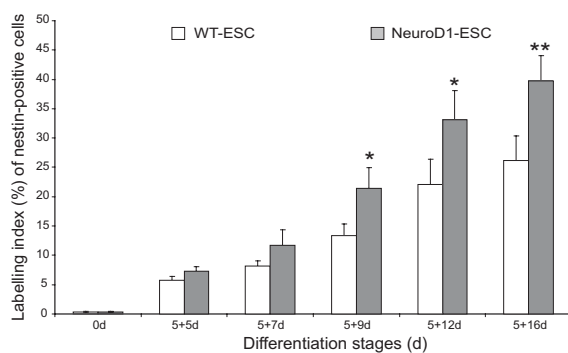
The rationale of our experimental approach relies on the assumption that the differentiation protocol does not allow efficient activation of endocrine differentiation programs because some critical transcription factors are present in limiting amounts. Forced expression of these factors would then facilitate the activation of an endocrine differentiation program by providing additional transcriptional activities. To this aim, a number of ES cell lines were engineered to over-express transcription factors involved in the determination of the pancreatic epithelium (Pdx-1, Jonsson *et al.*, 1994; Gu *et al.*, 2002), of the endocrine lineage (Ngn-3, Gradwohl *et al.*, 2000), and of the β-cell lineage (NeuroD1, Nkx2.2, Nkx6.1, Naya *et al.*, 1997; Sussel *et al.*, 1998; Sander *et al.*, 2000). Because endocrine differentiation is known to result from the coordinated action of several transcription factors (Jensen, 2004) (Murtaugh, 2007), we sought to generate ES cell lines expressing multiple factors to further enhance the yield of endocrine differentiation. Real-time PCR analysis of the α, β and δ cell markers, *Ins, Glc* and *Sst*, respectively, revealed that each cell line showed a specific pattern of gene expression. ES cell lines over-expressing Nkx2.2 and Nkx6.1, either alone or in combination with Pdx1, showed no increase in insulin gene expression compared to control cells, despite Nkx2.2 and Nkx6.1 were previously shown to regulate β-cell differentiation (Sussel *et al.*, 1998; Sander *et al.*, 2000). Similarly, over-expression of Pdx-1 had no effect on the yield of β-cell differentiation despite Pdx-1 is known both to be required *in vivo* for the development of all pancreatic cell types (Jonsson *et al.*, 1994) (Gu *et al.*, 2002), and to be a critical regulator of *ins* gene expression in mature β-cells (Ahlgren *et al.*, 1998; Holland *et al.*, 2002). It was previously reported that over-expression of Pdx-1 had only little effect on the yield of β-cell differentiation from mouse (Blyszczuk *et al.*, 2003; Miyazaki *et al.*, 2004)
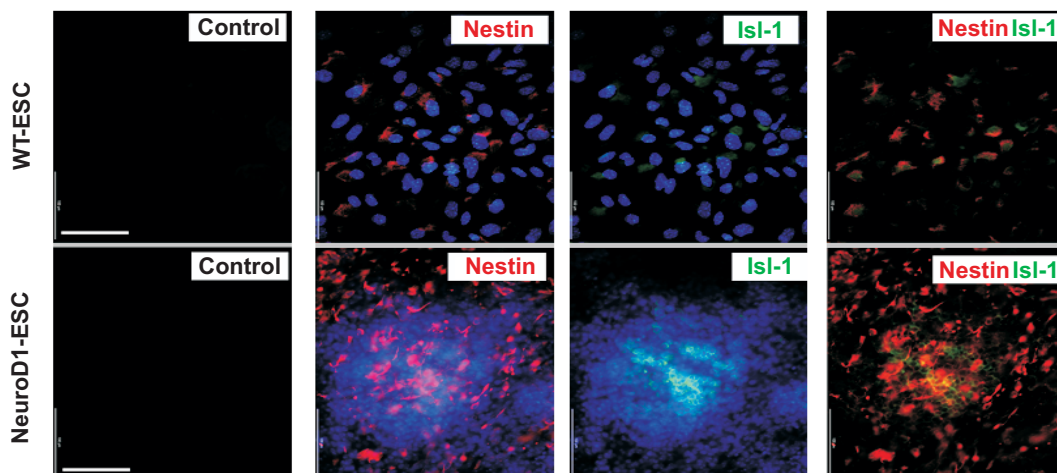

A

B

C

WT ND1+
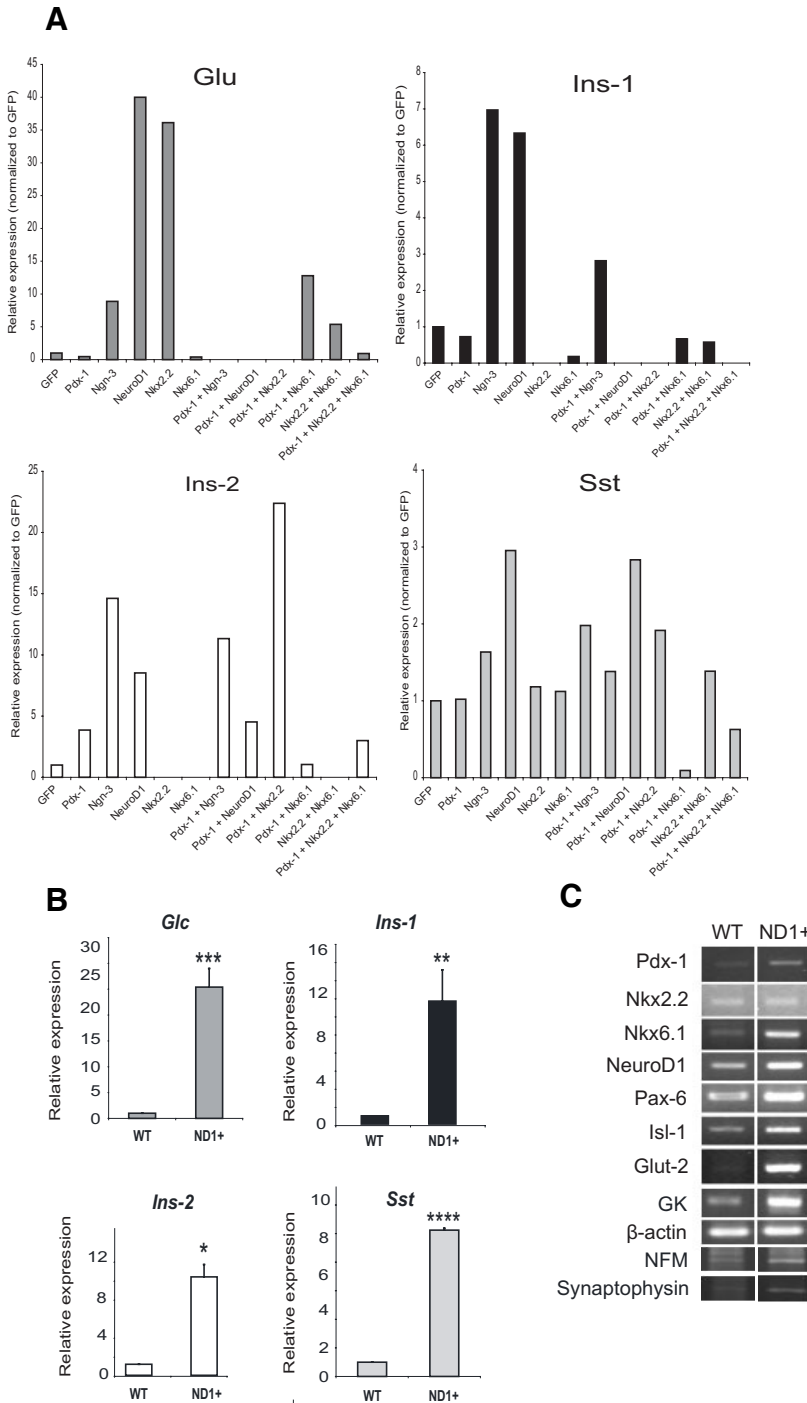
Fig. 4. Expression of endocrine markers *ins-1, ins-2, Glc* and *Sst* in the engineered ES cell lines. (A,B) *Real-time PCR analysis of* ins-1, ins-2, Glc and Sst *mRNA levels at differentiation stage 5+28d. mRNA levels measured in ES cell lines expressing transcription factors were subsequently normalized to mRNA levels measured in the GFP* **(A)** *or WT* **(B)** *control lines. (B) Histograms represent means and standard errors calculated in three replicates (statistical significance was determined with the unpaired two-sided t test. p<0.2 = *; p<0.01 = **; p<0.001 = ***).* **(C)** *Semi-quantitative RT-PCR analysis of endocrine transcription factors, β-cell and neuronal marker expression in NeuroD1-ESC and WT-ESC.*

(Takayama *et al.*, 2008) and human ESCs (Lavon *et al.*, 2006). Together, these observations indicate that none of the three transcription factors Pdx-1, Nkx2.2, and Nkx6.1 is rate limiting during β-cell differentiation induced in our protocol. By contrast, over-expression of Ngn-3 and NeuroD1 increased the expression of α, β and δ cell markers, NeuroD1 showing the strongest effect of both. This result is in agreement with gain-of-function studies which revealed that Ngn-3 and NeuroD1 induced endocrine differentiation when expressed ectopically *in vitro* (Heremans *et al.*, 2002) (Gasa *et al.*, 2004) (Treff *et al.*, 2006) or *in vivo* (Apelqvist *et al.*, 1999; Schwitzgebel *et al.*, 2000; Grapin-Botton *et al.*, 2001; Kojima *et al.*, 2003; Kaneto *et al.*, 2005). Surprisingly, ES cell lines over-expressing Pdx-1 and NeuroD1 showed no increase in endocrine markers. We can speculate that forced expression of Pdx-1 prevents ectopic NeuroD1 from properly activating the transcription of endocrine genes in differentiating cells. Alternatively, it may be inferred that the Pdx-1/NeuroD1-ESC expresses NeuroD1 at a lower level than the NeuroD1-ESC that would make NeuroD1-associated transcriptional activity unable to activate an endocrine differentiation program in Pdx-1/NeuroD1-ESC.
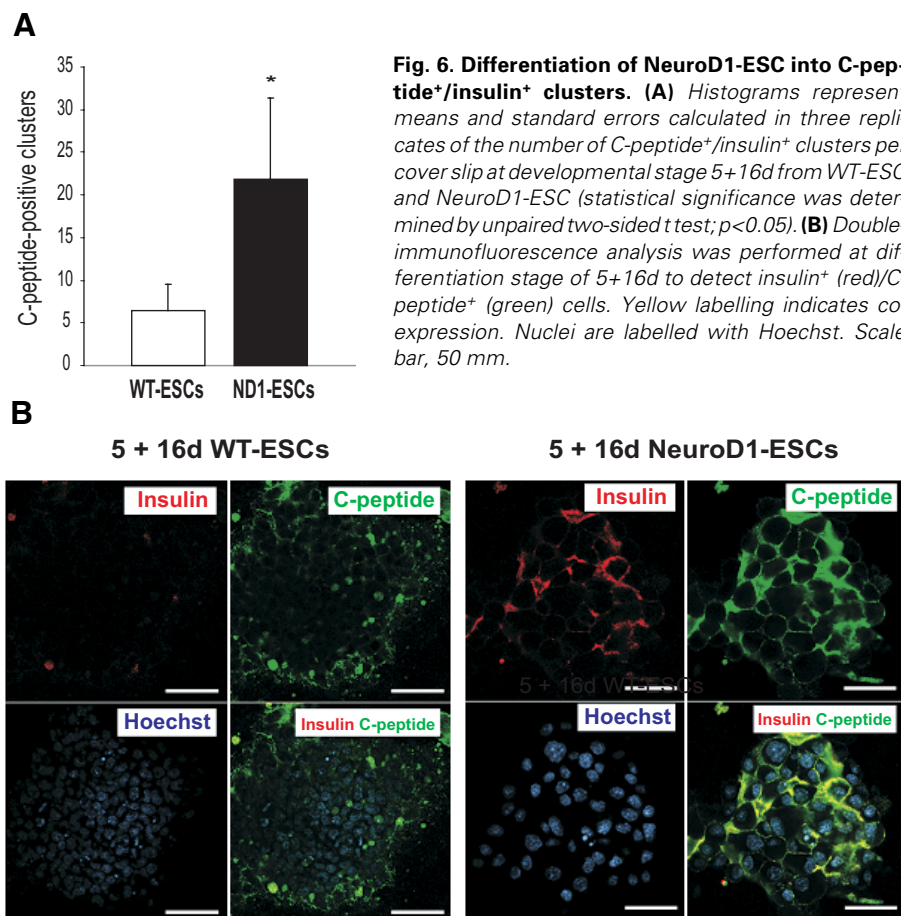
In the second part of this work, we focused our study on NeuroD1-ESC that showed a vastly improved capacity for endocrine differentiation compared to all other engineered ES cell lines, including differentiation to the β-cell lineage. The influence of NeuroD1 on β-cell differentiation was evidenced by a strong increase in the expression of genes encoding Pdx-1, Nkx6.1, Pax-6, Isl-1, the glucose receptor Glut-2 and GK. These observations are in line with - and extend - the previous observations of Saitoh *et al.* who showed that forced expression of NeuroD1 *via* adenoviral vector infection restores the expression of insulin lost upon passaging of Pdx-1-expressing ES cells (Saitoh *et al.*, 2007). Together, these observations indicate that NeuroD1 is a rate-limiting factor during *in vitro* differentiation of ES cells into β-like cells.

How enforced NeuroD1 expression stimulates endocrine and β-cell differentiation is not yet clear. First, we showed that NeuroD1-ESC generates more nestin[+]/Isl-1^{+} precursors than WT-ESC at the early differentiation stage 5+9d. This suggests that, at this multilineage progenitor stage, more cells representing progenitors of all three primary germ layers, including multilineage pancreatic progenitors, are formed. This might result from an increased proliferation rate, as it was observed in the whole nestin[+] cell population. However, whether the proliferation of the definitive endocrine progenitor population is accelerated remains to be determined. Second, NeuroD1-ESC was found to produce insulin[+]/C-peptide[+] clusters as early as stage 5+16d, therefore approximately 12 days earlier than WT-ESC. Premature differentiation into insulin[+]/C-peptide^{+} cells might result from the premature differentiation of nestin[+]/Isl-1^{+} multilineage precursors at stage 5+9d. Alternatively, it can be speculated that over-expression of NeuroD1 increases the proliferation rate of differentiating β-like cells. This hypothesis is supported by the observation that homozygous NeuroD1 *null* mice have a strong reduction in the number of insulin-producing β-cells. Islet morphogenesis appeared to be arrested between E14.5 and E17.5, a period characterized by major expansion of the β-cell population (Naya *et al.*, 1997). Mentionable is the dramatic growth rate that characterizes NeuroD1-ESC cultures at final stages of differentiation (beyond stage 5+16d). It results in massive cell overgrowth paralleled by high metabolic activity and subsequent failure to analyse insulin[+]/C-peptide[+] clusters at stage 5+28d. However, the elevation of β-cell marker expression (*Glut-2*, *GK*, *ins-1* and *ins-2*) at 5+28d indicates that NeuroD1 over-expression increased the yield of β-like cell differentiation at late stages. This could result either from an increase in the fraction of cells adopting a β-like phenotype, and acceleration of the differentiation process into glucose-producing

A

B

Fig. 5. Differentiation of NeuroD1-ESC into nestin[+]/Isl1^{+} progenitors and analysis of proliferation. (A) *Proliferation analysis of nestin^{+} cells in undifferentiated (0d) NeuroD1-ESC and WT-ESC, and at differentiation stages of 5+5d, 5+7d, 5+9d, 5+12d and 5+16d, revealed by double-immunofluorescence for expression of nestin and incorporation of BrdU. Histograms represent means and standard errors calculated in four replicates. Statistical significance was tested by the Anova test with 85% of confidence interval (p<0.05 = *; p<0.01 = **).* **(B)** *Double-immunofluorescence analysis of nestin (red)- and Isl-1 (green)-positive cells, performed at differentiation stage of 5+9d of NeuroD1-ESC and WT-ESC. Nuclei are labelled with Hoechst. Scale bar, 100 mm.*

A

Fig. 6. Differentiation of NeuroD1-ESC into C-pep-tide⁺/insulin⁺ clusters. (A) *Histograms represent means and standard errors calculated in three replicates of the number of C-peptide⁺/insulin⁺ clusters per cover slip at developmental stage 5+16d from WT-ESC and NeuroD1-ESC (statistical significance was determined by unpaired two-sided t test; p<0.05).* **(B)** *Double-immunofluorescence analysis was performed at differentiation stage of 5+16d to detect insulin⁺ (red)/C-peptide⁺ (green) cells. Yellow labelling indicates co-expression. Nuclei are labelled with Hoechst. Scale bar, 50 mm.*

B

cells. Further modification of the differentiation protocol is needed to study the maturation of insulin⁺/C-peptide⁺ clusters at terminal stages *in vivo* (Kroon *et al.*, 2008), in particular measure insulin release upon glucose stimulation.

Materials & Methods

Plasmid construction and lentiviral vector production

pEFs-eGFP harbors the sequence encoding the enhanced green fluorescent protein driven off the minimal version of the human EF1α promoter (EFs) (Fluckiger *et al.* 2006). *pEFs-Pdx-1, pEFs-Ngn-3, pEFs-NeuroD1, pEFs-Nkx2.2* and *pEFs-Nkx6.1* lentiviral vectors were generated from *pEFs-eGFP* by replacing the fragment containing the eGFP sequence by the full-length cDNAs encoding mouse Pdx-1 (5'-SalI(blunt)/BglII-3' fragment from *pZL1-Pdx1*), HA-tagged mouse Ngn-3 (5'-HindIII(blunt)/EcoRV-3' fragment from *pcDNAI/Amp/HA-Ngn-3*), Myc-tagged mouse NeuroD1 (5'-BamHI/XhoI-3' fragment from pCS2+MT-NeuroD1), hamster Nkx2.2 (5'-AvrII(blunt)/XhoI-3' fragment from *pBAT12-shNkx2.2*), and hamster Nkx6.1 (5'-XhoI(blunt)/XbaI(blunt)-3' fragment from *pBAT12-Nkx6.1* (Rudnick *et al.*, 1994)). The method for producing SIV-based vectors in 293T cells is fully described elsewhere (Negre *et al.*, 2000) (Mangeot *et al.*, 2000). Briefly, 293T cells were transfected with a mixture of DNAs containing 10 μg of the *pGRev* plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) envelope, 10 μg of the *pSIV3⁺* plasmid encoding the gag, pol, tat and rev proteins, and 13 μg of the *pEFs* plasmid encoding the cDNA of interest, using the calcium phosphate precipitation technique. The following day, cells were refed with 7 ml of DMEM and further cultured for 24 hours. The supernatant was then collected, cleared by centrifugation (3000 RPM, 15 minutes) and passed through a 0.8 μm filter.

ES cell culture and gene transduction

Undifferentiated murine ES cells from the feeder-independent CGR8 cell line were routinely cultured in Glasgow's modified Eagle's medium (GMEM; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 10% foetal calf serum (FCS; Biowest, Nuaillé, France, www.biowest.net), 2 mM L-glutamine (Invitrogen), 100 μM non-essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 100 U/ml penicillin-100 μg/ml streptomycin (Invitrogen), 100 μM β-mercaptoethanol (Sigma, St. Louis, http://www/sigmaaldrich.com) and 1000 U/ml of Leukemia Inhibitory Factor (LIF) as previously described (Savatier *et al.*, 1996). 10⁵ CGR8 ES cells were infected for 5 hours with 1 ml of lentiviral supernatant supplemented with LIF, and further cultured for 48 hours. The infected cells were sub-cloned in 96 well plates. Individual colonies were amplified and transgene expression analysed by immunoblotting.

In vitro *differentiation of ES cells*

The protocol used for *in vitro* differentiation of ES cell lines was previously described (Blyszczuk *et al.*, 2004; Schroeder *et al.*, 2006). Differentiation of ES cell lines was induced by the formation of embryoid bodies (EB) using the "hanging drop" method (600 ESCs/EB) in ES cell medium without LIF. After 2 days, EBs were collected and further grown in suspension for 3 days. At day 5 of differentiation (5d), EBs were plated on gelatine-coated 6-well plates (10 EBs/well) in "Differentiation Medium I" (see Schroeder *et al.*, 2006) and further cultured for 9 days. At day 14 of differentiation (5+9d), EB outgrowths were cultured in "Differentiation Medium II" supplemented with 15% FCS for 24 hours. At day 15 of differentiation (5+10d), cells were refed with "Differentiation Medium II" without FCS until 33 days of differentiation (5+28d).

For immunofluorescence analysis EBs (5d) were plated on gelatine-coated 60 mm dishes (20 EBs/dish). At the stage of 5+9d, EB outgrowths were dissociated by incubation in a solution of 0.1% trypsin (Invitrogen):0.08% EDTA (Sigma) in PBS (1:1) for 90 seconds at room temperature, collected by centrifugation (300 rpm, 3 min), and replated onto poly-L-ornithine/laminin-coated cover slips placed in 60 mm dishes.

Semi-quantitative and real-time PCR analysis

Total RNA was extracted using RNAeasy kits with on-column DNAse digestion (Qiagen, http://www.qiagen.com). Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega Corp., Madison, WI, http://www.promega.com) and Random Primers (MWG-Biotech AG, Ebersberg, Germany, http://www.mwg-biotech.com), according to the manufacturer's recommendations. PCR reactions were performed with a Perkin-Elmer thermal cycler, operating on a regimen of 94°C for 30 sec, 55 to 62°C (according to primers) for 30 sec and 72°C for 60 sec. Primers, annealing temperatures and number of cycles are:
Pdx-1: 5'-ACCATGAACAGTGAGGAGCA-3' (forward),
    5'-TCCTCTTGTTTTCCTCGGGT-3' (reverse), 58°C, 35 cycles;
Nkx2.2: 5'-AACCGTGCCACGCGCTCAAA-3' (forward),
    5'-AGGGCCTAAGGCCTCCAGTCT-3' (reverse), 58°C, 34 cycles;
Nkx6.1: 5'-TCTTCTGGCCCGGGGTGATG-3' (forward),
    5'-AGCCGCGTGCTTCTTCCTCCA-3' (reverse), 62°C, 34 cycles;
NeuroD1: 5'-CTTGGCCAAGAACTACATCTGG-3' (forward),
    5'-GGAGTAGGGATGCACCGGGAA-3' (reverse), 58°C, 34 cycles;
Pax-6: 5'-GCTTCATCCGAGTCTTCTCCGTTAG-3' (forward),

5'-CCATCTTGCTTGGGAAATCC G-3' (reverse), 60°C, 30 cycles;
Isl-1: 5-AGATATGGGAGACATGGGCGAT-3' (forward),

5'-ACACAGCGGAAACACTCGATG-3' (reverse), 55°C, 35 cycles;
Glut-2: 5'-CGG TGGGACTTGTGCTGCTGG-3' (forward),

5'-CTCTGAAGACGCCAGGAATTCCAT-3', 60°C, 40 cycles;
GK: 5'-TGGATGACAGAGCCAGGATGG-3' (forward),

5'-ACTTCTGAGCCTTCTGGGGTG-3' (reverse), 60°C, 35 cycles;
Nfm: 5'-TATTGTGACTGAGGGCTG-3' (forward),

5'-GAGGCACTAAGGAG-3' (reverse), 60°C, 45 cycles;
Syp: 5'-TACCGAGAGAACAACAAAGGGC -3' (forward),

5'-GCCTGTCTCCTTGAACACGAAC-3' (reverse), 60°C, 45 cycles.
β-actin: 5'-TGAAACAACATACAATTCCATCATGAAGTGTGA-3' (forward),

5'-AGGAGCGATAA TCTTGATCTTCATGGTGCT-3' (reverse),
58°C, 20 cycles.

Quantitative PCR was performed using the LightCycler™ 1.5 system and the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Applied Science, Indianapolis, http://www.roche-applied-science.com) according to the manufacturer's instructions. Reactions were carried out in a total volume of 20 µl, comprising 0.4 µM of each primer, 4 mM $MgCl_2$, 2 µl SYBR Green, and 5 µl of diluted cDNA. Primers are:
ins-1: 5'-CCAGCTATAATCAGAGACCA-3' (forward) and

5'-GTGTAGAAGAAGCCACGCT-3' (reverse);
ins-2: 5'-AGCAGGAAGCCTATCTTCCA-3' (forward) and

5'-GGACATGGGTGTGTAGAAGAA-3' (reverse);
Glc: 5'-CAGAGGAGAACCCCAGATCA-3' (forward) and

5'-TGACGTTTGGCAATGTTGTT-3' (reverse);
sst: 5'-GAGGCAAGGAAGATGCTGTC-3' (forward) and

5'-CAGCAGCTCTGCCAAGAAGT-3' (reverse).

Amplification and online monitoring was performed using the LightCycler™ 1.5 system. Following 40 amplification cycles, melt-curve analyses were performed to verify only the desired PCR product had been amplified. PCR efficiency of both the target and reference genes was calculated from the derived slopes of standard curves by the LightCycler software (Roche Molecular Biochemicals LightCycler Software, Version 4.0). These PCR efficiency values were used to calculate the relative quantification values for calibrator-normalised target gene expression by the LightCycler relative quantification software (Version 4.0). In all cases target genes were normalised to *β-actin*.

Immunofluorescence analysis

ES cells and differentiated cells growing on cover slips were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 20 minutes at room temperature and processed for immunofluorescence analysis as described (Wobus *et al.*, 2002). The following primary antibodies and dilutions were used: rabbit IgG anti-Pdx-1; 1:100; mouse IgG anti-Myc (Santa-Cruz Biotechnology, Santa Cruz, CA, http://scbt.com) 1:100; rabbit IgG anti-HA (Upstate, http://www.upstate.com) 1:100; mouse IgG anti-Nkx2.2 (Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww/) 1:50; rabbit IgG anti-Nkx6.1 (Dr Ole Madsen, Gentofte, Denmark) 1:1000; mouse IgG anti-nestin (Developmental Studies Hybridoma Bank) 1:3; rabbit IgG anti-Isl-1 (Abcam, http://www.abcam.com) 1:50 guinea pig IgG anti-C-peptide (Linco Research, St. Charles, MO, http://www.lincoresearch.com) 1:100; and mouse IgG anti-insulin (Sigma) 1:100. The following secondary antibodies were used according to the manufacturer's recommendations: FITC-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG (both from Molecular Probes Inc., Eugene, OR, http://www.probes.in vitrogen.com); FITC-conjugated anti-guinea pig IgG and Cy3-conjugated anti-mouse IgG (all from Jackson ImmunoResearch Laboratories, West Grove, PA, http://www.jackson immuno.com). Cells were observed with a Leica DMRE (Leica, Heerbrugg, Switzerland, http://www.leica.com) and a LSM 510 META confocal laser scanning microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, http://www.zeiss.com).

Flow cytometry

Cells were dissociated with 0.25% trypsin-EDTA, fixed with 2% PFA in PBS at 4°C for 1 hour, and permeabilized in Tris Buffer Saline (TBS) + 0.1% Triton X-100. Non-specific binding was blocked with 10% normal goat serum for 20 min at room temperature. Cells were incubated overnight at 4°C with mouse IgG anti-Oct4 (Santa-Cruz Biotechnology) 1:1000 and with rabbit IgG anti-Nanog (e-bioscience, http://www.ebioscience.com) 1:500. After several rinses in TBS, cells were exposed to affinity-purified goat anti-mouse or anti-rabbit IgG conjugated to FITC (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature. Cells were analysed using FACS Canto II (Beckton-Dickinson). Fluorescence was measured at 488 nm. Data were recorded and analysed using DiVa software.

Immunoblotting

ES cells were washed and scraped in ice-cold PBS, centrifuged and frozen at - 80°C. Cell pellets were lysed in 20 mM Hepes pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.5% NP40, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and cocktail of protease inhibitor (Roche Diagnostic) for 1 hr at 4°C. Protein lysates were cleared by centrifugation (14,000 RPM for 20 min). For immunoblotting, 50 µg of total proteins were resolved by SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. After overnight treatment with blocking buffer (50 mM Tris-Hcl pH 7.6, 150 mM NaCl, 0.1% tween, 5% dry milk), the membranes were probed with primary antibodies at the following dilutions: anti-Pdx1 (1:500); anti-HA (1:500); anti-Myc (1:600); anti-Nkx2.2 (1:150) and anti-Nkx6.1 (1:10,000). Blots were incubated with horseradish peroxidase-coupled anti-mouse or anti-rabbit and developed using enzymatic chemiluminescence reagents (ECL, Amersham, Buckinghamshire, U. K., http://www.amersham.com).

Proliferation assay by BrdU incorporation

To assess their proliferation capacity cells were incubated with 10µM 5-Bromo-2'-deoxyuridine (BrdU, Roche, Mannheim, Germany) for 24h. Cover slips were washed in PBS, incubated in 2 N HCl at room temperature for 30 min, and fixed in methanol:acetone (7:1, v/v) at -20°C for 10 min. After washing three times in PBS, 1% bovine serum albumin (BSA) in PBS was applied as a blocking agent for 30 min, followed by incubation with primary rat anti-BrdU (clone BU1/75 [ICR1], Abcam Ltd., Cambridge, UK) and primary mouse anti-nestin antibodies (clone Rat 401, Developmental Studies Hybridoma Bank, Iowa City, IA, diluted 1:3 in PBS) for 90 min, then secondary rabbit anti-rat (Cye-labeled, 1:800 in 1% BSA in PBS, Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa 488-labeled goat anti-mouse secondary antibodies (diluted 1:100 with 1% BSA in PBS, Molecular Probes, Eugene, OR) for 60 min. Nuclei were stained by Hoechst 33342, and the cover slips were mounted in Dako Cytomation fluorescent mounting medium (Dako, Hamburg) on SuperFrost glass slides (Menzel, Braunschweig, Germany). BrdU-positive and nestin-positive cells in the EB outgrowths (n=10 fields) were estimated as percentage of Hoechst 33342-labeled cells using the fluorescence microscope ECLIPSE E600 (Nikon) and the confocal laser scanning microscope LSM510 META (Zeiss, Munich, Germany).

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