

# Embryonic stem cells differentiate into insulin-producing cells without selection of nestin-expressing cells

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**ABSTRACT** We present a new strategy for the differentiation of embryonic stem (ES) cells into insulin-producing cells via a multi-step process without selection and induction of nestin-positive cells. During ES cell differentiation, transcript levels of genes characteristic of early and mature beta cells including Pdx1, Pax4, insulin and islet amyloid pancreatic peptide are up regulated. Islet-like clusters are characterized by expression of C-peptide, insulin and partially cytokeratin 19 as well as by ion channel activity similar to that found in embryonic beta cells. Cells of islet-like clusters show glucose-dependent insulin release at terminal stage. At an intermediate stage, nestin is partially co-expressed with C-peptide and cytokeratin 19, whereas islet-like clusters at the terminal stage are nestin-negative. We conclude that expression of nestin and cytokeratin 19 is a normal property of ES cells preceding differentiation into C-peptide/insulin-producing cells without any selection for nestin-positive phenotypes.

**KEY WORDS:** mouse embryonic stem cell, differentiation, C-peptide, insulin-producing cell, nestin

## Introduction

A potential source of transplantable cells for the treatment of diabetes are embryonic stem (ES) cells, which have an almost unlimited proliferation capacity, while retaining the potential to differentiate *in vitro* into cells of all three primary germ layers [for review, see (Czyz *et al.*, 2003)]. Insulin-expressing cells have been generated from mouse (Soria *et al.*, 2000; Leon-Quinto *et al.*, 2004) and human (Assady *et al.*, 2001) ES cells. Insulin-positive cells were also derived from mouse ES cells via selection of progenitor cells expressing the intermediate filament (IF) protein nestin (Lumelsky *et al.*, 2001). However, it was recently suggested that insulin-positive cells derived from ES cells absorbed and concentrated the hormone from the medium rather than producing insulin (Rajagopal *et al.*, 2003; Hansson *et al.*, 2004); whereas other reports described ES cell differentiation into transplantable insulin-producing cells *via* nestin-positive cells by treatment with a phosphoinositide 3-kinase inhibitor (Hori *et al.*, 2002) and by *Pax4* transgene expression (Blyszczuk *et al.*, 2003), respectively.

*In vivo*, nestin expression during pancreas development was only detected in mesenchymal and not in pancreatic epithelial cells (Selander and Edlund, 2002). Whereas cytokeratin 19 (CK19) was found to be expressed in the developing pancreas

and in pancreatic duct cells, where potential pancreatic stem cells reside (Bouwens, 1998; Bonner-Weir *et al.*, 2000; Brembeck *et al.*, 2001), nestin was not identified as a specific marker of beta-cell precursors (Humphrey *et al.*, 2003). However, after cultivation of pancreatic islets *in vitro*, nestin-positive cells were detected (Zulewski *et al.*, 2001). The controversial findings substantiated our studies to characterize nestin and CK 19 expression during differentiation of ES cells into C-peptide/insulin-positive cells and To this aim, we established a new protocol for the differentiation of ES cells into the pancreatic lineage without pre-selection of nestin-positive cells, determined the abundance of C-peptide-, CK19- and nestin-positive cells and analyzed the functional capacity of differentiated cells. We found a co-expression of nestin with C-peptide and CK19 at intermediate stage, but not in

*Abbreviations used in this paper:* EB, embryoid body; ES, embryonic stem; bFGF, basic fibroblast growth factor; CK, cytokeratin; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; IAPP, islet amyloid polypeptide; IF, intermediate filament; IMDM, Iscove's modified Dulbecco's medium; ITSFn, insulin, transferrin, selenium, fibronectin; PBS, phosphate buffered saline; PFA, paraformaldehyde; RT-PCR, reverse transcriptase-polymerase chain reaction; SEM, standard error of the mean; STZ, streptozotocin; wt, wild type.

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islet-like clusters. Our data let us to conclude that nestin and CK 19 are involved in pancreatic differentiation *in vitro* and that cells differentiating along these pathways acquire some characteristics of pancreatic beta-like cells.

## Results

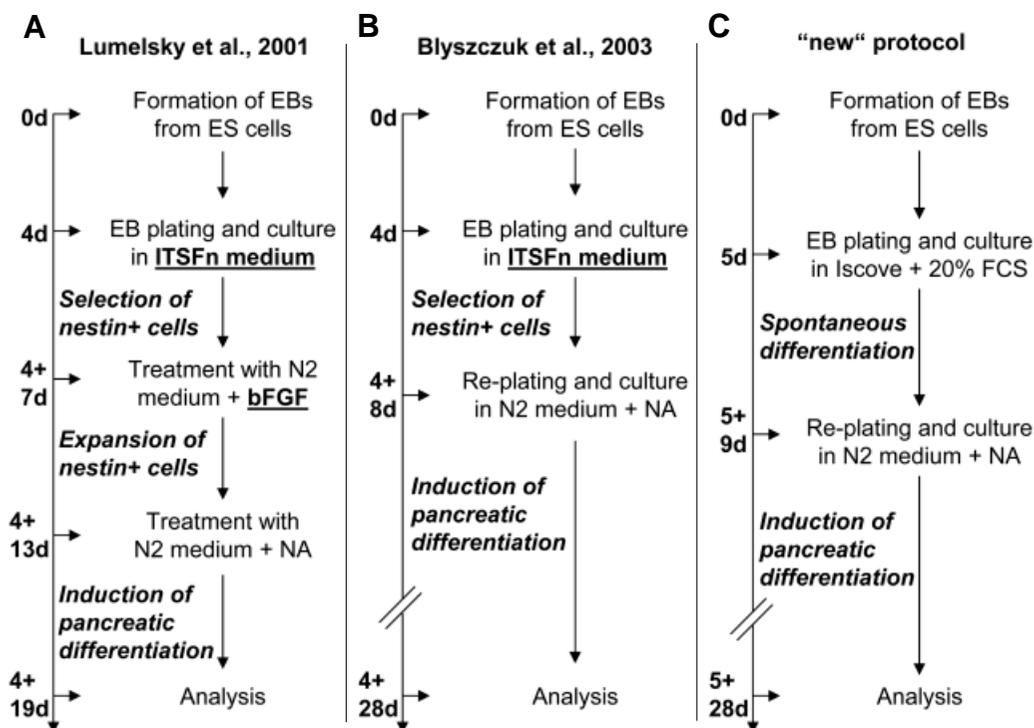
### **Transcript levels of pancreatic genes are upregulated in ES-derived cells differentiating into insulin-producing cells without selecting nestin-positive cells**

Our new differentiation protocol for the generation of ES-derived insulin-producing cells (Fig. 1C) includes the spontaneous generation of multi-lineage progenitor cells and their differentiation induction into islet-like clusters. No selection for nestin-positive cells is performed at any time of ES cell differentiation. In brief, ES cells were cultured in 'hanging drops' and in suspension for 5 days to form embryoid bodies (EBs) followed by 9 days culture after EB plating (= 5+9d) for differentiation into cells representing derivatives of all three primary germ layers [for details see (Rohwedel *et al.*, 2001; Wobus *et al.*, 2002; Blyszczuk *et al.*, 2003)]. At this stage, cells were re-plated onto poly-L-ornithine/laminin-coated culture dishes [see (Rolletschek *et al.*, 2001)] into a 'pancreatic differentiation medium' (= N2 medium + nicotinamide, NA, see Fig. 1C) and cultured until stage 5+28d. Cells were analyzed for pancreatic differentiation markers at stages 5+9d, 5+16d and 5+28d. In the present study and in

previous reports (Lumelsky *et al.*, 2001; Hori *et al.*, 2002; Moritoh *et al.*, 2003; Rajagopal *et al.*, 2003; Blyszczuk *et al.*, 2003), the same 'pancreatic differentiation medium' was used to induce pancreatic differentiation. However, in contrast to Lumelsky *et al.* (2001), our new protocol avoids the enrichment of nestin-positive cells in ITSFn medium [see Lee *et al.* (2000), used also in our former study (Blyszczuk *et al.*, 2003); see Fig. 1B] and the proliferation induction of nestin-positive cells by bFGF [according to Lumelsky *et al.* (2001); compare Fig. 1 A,C]. Therefore, our new protocol allows the characterization of cells during pancreatic differentiation of ES cells without enrichment of specific subpopulations that may affect commitment and differentiation into the pancreatic lineage.

Employing RT-PCR and immunofluorescence techniques we show that by applying our new protocol ES cells differentiate into the pancreatic lineage. Upon induction of pancreatic differentiation at day 5+9, development into pancreatic endocrine cells is demonstrated by the up-regulation of Pdx1, Pax4, insulin and IAPP mRNA levels (Fig. 2A) resulting in the formation of self-assembling islet-like clusters, which are labelled by insulin and C-peptide following differentiation (Fig. 2B).

Pdx1 transcripts were detected at low level at day 5+9, showed no Pdx1 signals at the intermediate stage (5+16d), but increased significantly at the terminal stage (5+28d). Lack or low Pdx1 expression at early stages *in vitro* confirms also recent findings which show that Pdx1 is not required for the induction of pancreatic differentiation from ES cells *in vitro* (Moritoh *et al.*, 2003); although it plays a key role in early pancreas development (Jonsson *et al.*, 1994) as well as in terminal differentiation



**Fig. 1. Schematic representation of protocols used to generate functional islet-like clusters from ES cells *in vitro*.** (A-C) The protocols developed by (A) Lumelsky *et al.* (2001), (B) that employed in our former study [Blyszczuk *et al.* (2003)] and (C) that of the present study ("new" protocol), differ in the generation of progenitor cells, but all protocols use the same pancreatic differentiation medium. All protocols generate progenitor cells from ES cells via EB formation, but protocols (A) and (B) enrich nestin-positive cells in ITSFn supplemented medium, whereas according to the "new" protocol (C), EB-derived cells differentiate spontaneously. A further expansion of nestin-positive cells by bFGF is performed only in protocol (A), whereas in methods (B) and (C) the selective expansion of nestin-positive cells is omitted. According to our "new" protocol (C), a progenitor population representing cells of all three primary lineages is generated by spontaneous differentiation of ES cells followed by the induction of pancreatic differentiation.

and functionality of pancreatic cells (Ahlgren *et al.*, 1998).

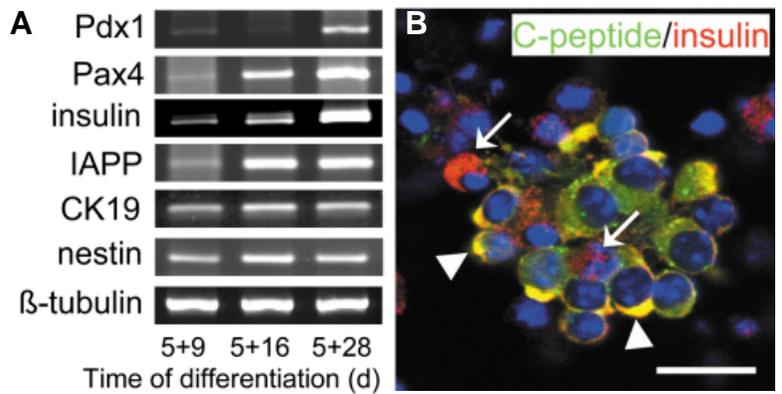
Another evidence for the activation of a pancreatic differentiation program in ES cells by following our protocol, is the up-regulation of insulin and IAPP, a peptide almost exclusively expressed in beta-cells and co-secreted with insulin (Fehmann *et al.*, 1990).

Pax4 transcripts were up regulated upon induction of pancreatic differentiation at 5+16d and 5+28d. *In vivo*, Pax4 is essential for proper beta-cell development (Sosa-Pineda *et al.*, 1997) and its expression is restricted to the endocrine progenitor cell fate (Dohrmann *et al.*, 2000). We have recently reported that constitutive expression of Pax4 in ES cells significantly promotes pancreatic differentiation (Blyszczuk *et al.*, 2003).

To test whether ES-derived insulin-positive cells produce or only absorbed insulin from the medium [see (Rajagopal *et al.*, 2003)], we analysed co-expression of insulin and C-peptide. The formation of C-peptide (part of proinsulin when converted into insulin) demonstrates *de novo* insulin production and excludes those cells that only concentrate insulin from the medium. The number of C-peptide-labelled cells increased after induction of pancreatic differentiation up to about 3 fold at terminal stage (5+28d, Table 1). A high fraction of insulin-positive cells (about 85%) co-expressed C-peptide (see arrowheads in Fig. 2B) and were organized in islet-like clusters at terminal stage of 5+28d (Fig. 2B), whereas only a low fraction (about 10- 15%) of cells were labeled by insulin, but not by C-peptide (see arrows in Fig. 2B). These cells were eventually characterized by small, condensed nuclei suggesting an apoptotic pathway as described (Rajagopal *et al.*, 2003). In contrast to Rajagopal *et al.* (2003), we showed co-expression of insulin and C-peptide in most of the ES-derived cells indicating *de novo* insulin production and, as demonstrated in Fig. 2A, high insulin mRNA levels were determined at terminal stages.

**Nestin is coexpressed in C-peptide-positive cells after induction of pancreatic differentiation**

To answer the question, whether nestin is involved in the generation of insulin-producing cells during ES cell differentiation,



**Fig. 2. Transcript levels and an islet-like cluster after pancreatic differentiation of ES cells. (A)** mRNA levels of Pdx1, Pax4, insulin, IAPP, cytoke-  
 ratin (CK)19, nestin and  $\beta$ -tubulin (housekeeping) genes of ES-derived cells during *in vitro* differentiation are shown at stages 5+9d, 5+16d and 5+28d. **(B)** Immunofluorescence of C-peptide and insulin co-expressing cells in an islet-like cluster at terminal stage of differentiation (5+28d). Arrowheads indicate insulin/C-peptide co-expressing cells, whereas arrows indicate insulin-positive and C-peptide-negative cells with small and condensed nuclei suggesting apoptosis. Nuclei are labelled by Hoechst 33342. Bar, 20  $\mu$ m.

we analysed transcript abundance and co-localization of nestin and C-peptide by immunostaining at stages 5+9d, 5+16d and 5+28d. Nestin mRNA levels were detected at all stages (see Fig. 2A) and nestin-positive cells were present at constant level during differentiation between day 5+9 and 5+28 (Table 1).

A high co-expression of nestin and C-peptide was found exclusively at stage 5+16d (Fig. 3G, Tables 2A and 2B). Continued differentiation up to 5+28d resulted in C-peptide expressing islet-like clusters negative for nestin (Fig. 3K). Our findings demonstrate that nestin is transiently involved in ES-derived pancreatic differentiation.

To determine, if nestin-positive cells co-express desmin [another IF protein detected in mesenchymal cells and in pancreatic stellate cells, see (Lardon *et al.*, 2002)] and whether such cells are involved in pancreatic differentiation of ES cells, we performed double immunostaining with desmin- and nestin-specific antibodies. Partial co-expression of nestin and desmin was detected at

TABLE 1

**NUMBER OF NESTIN-, C-PEPTIDE-, CYTOKERATIN (CK) 19, CARBONIC ANHYDRASE II- (CARB. ANH. II), DESMIN- AND ISLET-1 (ISL-1)-IMMUNOREACTIVE CELLS AS A PERCENTAGE OF HOECHST 33342-LABELLED CELLS DURING PANCREATIC DIFFERENTIATION OF R1 ES CELLS**

Stage	Number of positive cells						
	Nestin (%)	C-peptide (%)	CK19 (%) MeOH:Ac	CK19 (%) 4% PFA	Carb. anh. II (%)	Desmin (%)	Isl-1 (%)
5+9d	23.7 $\pm$ 5.3	9.9 $\pm$ 1.7	8 $\pm$ 2.2	< 1.0	n.d.	32.5 $\pm$ 5.9	3.1 $\pm$ 1.9
5+16d	21.7 $\pm$ 4.8	32.4 $\pm$ 3.3	< 1.0	31.6 $\pm$ 6.2	32.2 $\pm$ 4.5	20.1 $\pm$ 3.7	15.4 $\pm$ 7.1
5+28d	21.4 $\pm$ 4.3	26.6 $\pm$ 7.7	< 1.0	21.3 $\pm$ 1.3	26.6 $\pm$ 8.0	n.d.	8.7 $\pm$ 2.9

Cells of each immunopositive fractions partially overlap. Differentiation stage 5+9d represents spontaneous differentiation, whereas stages 5+16d and 5+28d represent 7 and 19 days, respectively, after induction of pancreatic differentiation. Immunostaining for CK19 was performed separately after fixation with methanol-acetone (MeOH:Ac) and 4% paraformaldehyde (PFA). Each value represents mean  $\pm$  SEM, n.d. = not determined, n  $\geq$  3 experiments.

TABLE 2

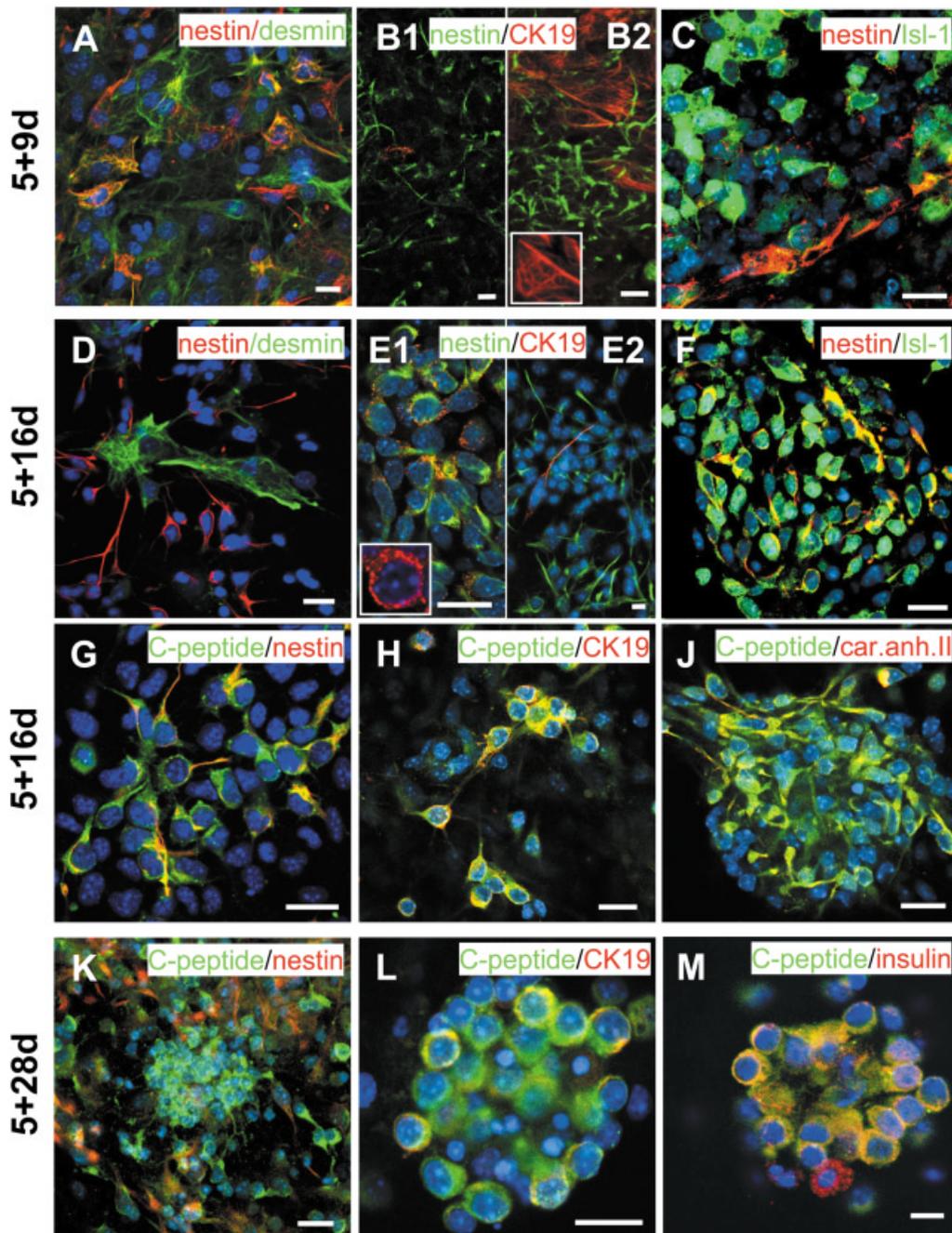
**DOUBLE IMMUNOFLUORESCENCE ANALYSIS OF ES-DERIVED INTERMEDIATE CELL STAGES REPRESENTING NESTIN (A) AND C-PEPTIDE (B) CELL POPULATIONS**

(A)	Stage	Nestin (%)	C-peptide (%)	Isl-1 (%)	Desmin (%)
	5+9d	100	34.1 $\pm$ 14.5	1.0 $\pm$ 0.9	46.1 $\pm$ 10.3
	5+16d	100	83.9 $\pm$ 7.1	60.4 $\pm$ 13.7	15.0 $\pm$ 6.2
	5+28d	100	41.0 $\pm$ 6.7	8.5 $\pm$ 3.6	n.d.

Number (in %) of C-peptide-, Islet-1 (Isl-1)- and desmin-labelled cells determined within the fraction of nestin-positive cells (nestin-positive cells were set to 100%). Each value represents mean  $\pm$  SEM, n.d. = not determined, n  $\geq$  3 experiments.

(B)	Stage	C-peptide (%)	Nestin (%)	Carb. anh. II (%)	CK19 (%)
	5+16d	100	82.2 $\pm$ 6.9	99.8 $\pm$ 0.3	97.6 $\pm$ 0.8
	5+28d	100	30.7 $\pm$ 6.7	99.7 $\pm$ 0.2	73.0 $\pm$ 1.4

Number (in %) of nestin-, carbonic anhydrase II (carb. anh. II)- and cytoke-  
 ratin (CK) 19-labeled cells as a percentage of the fraction of C-peptide-positive cells (C-peptide-positive cells were set to 100%). Each value represents mean  $\pm$  SEM, n  $\geq$  3 experiments.



**Fig. 3. Double-immunofluorescence analysis of ES-derived cells at intermediate and terminal stages following pancreatic differentiation.** (A–F) Analysis of nestin-labelled cells for co-expression with desmin (A,D), cytokeratin (CK) 19 [fixed with paraformaldehyde, (B1, E1) and methanol: acetone (B2, E2)], Isl-1 (C,F), both fixed with paraformaldehyde, at early (5+9d, A–C) and intermediate (5+16d, D–F) stages of differentiation. Note that not all cells show nuclear Isl-1 labelling in (3F). (G–M) Immunofluorescence analysis of C-peptide-labelled cells for co-expression with nestin (G,K), CK19 [fixed with paraformaldehyde, (H,L)] carbonic anhydrase II (carb. anh. II, (J)) and insulin (M) at intermediate (5+16d, G–J) and terminal (5+28d, K–M) stages of differentiation. Quantitative analysis of the immunofluorescence signals is presented in Tables 1 and 2, respectively. Nuclei are labelled by Hoechst 33342. Bar, 20  $\mu$ m.

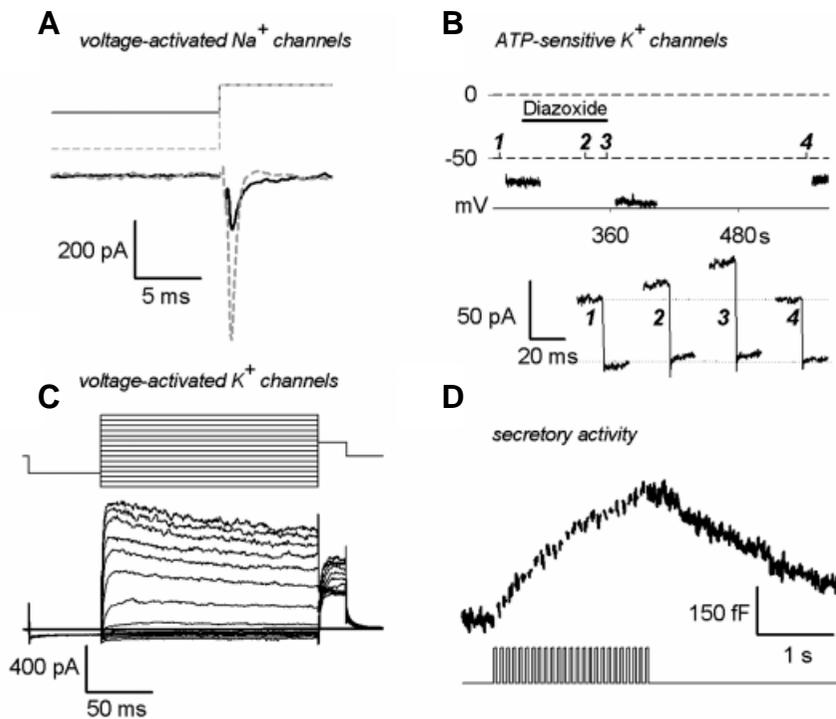
stage 5+9d (Fig. 3A). Whereas upon induction of pancreatic differentiation, only a low cell fraction co-expressed nestin and desmin (Table 2A), the remaining nestin- and desmin-positive cells showed clear differences in the morphology (see Fig. 3D). Moreover, the total number of desmin-positive cells decreased after induction of pancreatic differentiation (see Table 1). These findings suggest that desmin is not involved in the terminal differentiation of pancreatic cells.

**Cytokeratin 19 is expressed during ES-derived pancreatic differentiation, but is not organized into filaments**

Because cytokeratin 19 (CK19) is expressed in developing pancreatic epithelium, where the definitive pancreatic progenitor

reside and in adult pancreatic duct cells (Brembeck *et al.*, 2001), where potential pancreatic stem cells may be located (Bouwens, 1998; Bonner-Weir *et al.*, 2000; Ramiya *et al.*, 2000), next we asked whether CK19 is involved in the generation of C-peptide/insulin-positive cells during ES cell differentiation.

CK19 transcripts were detected at all stages of pancreatic differentiation (see Fig. 2A) suggesting the involvement of CK19 during ES cell differentiation. However, immunofluorescence analysis demonstrated specific differences in the organization of CK19-positive intermediate filaments by applying different fixation methods. Conventional fixation with methanol/acetone was suitable for detection of intermediate filaments (Fig. 3B2, E2) and we found a low fraction of CK19-positive cells at stage 5+9d,



**Fig. 4. Results of whole-cell patch-clamp analysis of wt and Pax4+ ES-derived cells from islet-like clusters at stage 5+30d.** (A) Inactivation of voltage-activated  $\text{Na}^+$  currents at sub-physiological membrane potentials. Depolarisation to  $-20$  mV from  $-75$  mV and  $-150$  mV generated inward currents of 210 and 640 pA, respectively. (B) Voltage-activated  $\text{K}^+$  currents induced by a family of depolarising pulses in steps of 15 mV. (C) Current-clamp recording of a cell treated with diazoxide (100  $\mu\text{M}$ ). At points indicated by numbers,  $\text{K}^+$  conductance was measured using square hyperpolarization from  $-60$  mV to  $-110$  mV. Cell hyper-polarization during diazoxide superfusion is associated with significant increase in membrane conductance. (D) Typical increase in membrane capacitance triggered by a train of depolarising pulses from  $-80$  mV to  $+10$  mV indicates  $\text{Ca}^{2+}$ -dependent exocytosis.

whereas after pancreatic differentiation induction (5+16d), almost no CK19-positive cells were found (Table 1). In addition, the cells showed no co-expression of CK19 and nestin at early (5+9d) and intermediate (5+16d) stages (Fig. 3 B2, E2). After fixation with 4% paraformaldehyde, CK19-labelled dot-like structures were detected in the cytoplasm of a few cells at stage 5+9d (Fig. 3B1), however upon induction of pancreatic differentiation (stage 5+16d), CK19 labeling was found in 31.6% cells (Table 1) and was highly co-expressed with nestin (Fig. 3E1, Table 2A). After continued differentiation (stage 5+28d), the number of CK19-positive cells was reduced (21.3%, Table 1). Similar dot-like structures were described for another IF protein, vimentin, after fixation with paraformaldehyde (Prahla *et al.*, 1998). We conclude that the different fixation properties reflect conformational changes (intermediate filaments versus dot-like structures) of the IF proteins during differentiation.

Next, we determined, whether CK19-positive cells are present in the fraction of C-peptide-positive cells that are involved in the formation of islet-like clusters *in vitro*. Double immunofluorescence analysis showed that 97.6% (5+16d) and 73% (5+28d) of C-peptide positive cells co-expressed CK19 (Fig. 3 H,L, Tab. 2B). These data suggest that CK19 protein is abundant during pancreatic differentiation, but the protein is not organized in intermediate filaments. The decrease of CK19-labeling in C-peptide-positive cells at terminal stage may indicate a certain maturation of ES-derived cells during differentiation. This would be in agreement with the finding that mature beta cells *in vivo* are negative for CK19 (Bouwens *et al.*, 1997).

#### Isl-1 is expressed in ES-derived progenitor cells

The data presented so far have shown that nestin and CK19 are partially and transiently co-expressed in C-peptide/insulin-producing cells. Next, we asked whether Isl-1, a LIM homeodomain

protein that controls cell-fate decisions required for the differentiation of islet cells (Ahlgren *et al.*, 1997) is expressed during ES cell differentiation. We found only a low fraction of Isl-1-positive cells at the early differentiation stage of 5+9d and almost no cells co-expressed Isl-1 and nestin (Fig. 3C, Tables 1 and 2A). However, after induction of pancreatic differentiation, 60.4% of nestin-positive cells co-expressed Isl-1 and only occasionally cells positive for Isl-1 and negative for nestin were found (Table 2A). Continued differentiation resulted in a decreased number of Isl-1-positive cells at stage 5+28d (Table 1). These findings suggest that Isl-1 is transiently expressed in the nestin-positive subpopulation of committed pancreatic cells expressing nestin and C-peptide (at stage 5+16d). The data are in agreement with recent findings, which showed that Isl-1 expression is restricted to a specific subpopulation of pancreatic stem cells during pancreas development (Chiang and Melton, 2003). Our immunofluorescence analyses suggest that Isl-1 is activated in nestin-positive cells committed to the pancreatic lineage, because a remarkable number of cells co-expressing Isl-1 and nestin were detected at day 5+16d (see Fig. 3F, Table 2A).

#### Carbonic anhydrase II is involved in pancreatic differentiation *in vitro*

Another protein expressed in pancreatic duct cells is carbonic anhydrase II (Githens *et al.*, 1992). We investigated the abundance of carbonic anhydrase II in C-peptide-positive ES-derived cells and found that upon induction of pancreatic differentiation nearly all C-peptide-positive cells co-expressed carbonic anhydrase II (Fig. 3J, Table 3). *In vivo*, mature pancreatic beta-cells express carbonic anhydrase V instead of carbonic anhydrase II (Parkkila *et al.*, 1998) suggesting that *in vitro* generated ES-derived cells represent an immature pancreatic cell population, rather than definitive mature pancreatic beta-cells.

### Insulin-producing cells functionally express beta-cell-specific properties

Next, we investigated functional properties of ES-derived wt cells in comparison to ES cells constitutively expressing Pax4 (Pax4+). We have reported that Pax4+ cells promote pancreatic differentiation (Blyszczuk *et al.*, 2003) and confirmed this by applying our new differentiation protocol. Pax4+ cells formed C-peptide-positive islet-like clusters similar as described for wt cells, but showed a higher level of C-peptide-labelled cells (not shown). The functional status of both cell lines was analysed by electrophysiological studies using the patch-clamp technique on cells found within islet-like clusters and by ELISA. Electrophysiological measurements showed that ES-derived pancreatic cells were excitable and revealed characteristic properties of beta-cells with respect to voltage-activated Na<sup>+</sup> and K<sup>+</sup> currents (Fig. 4 A,B, Table 3). In some Pax4+ cells, K<sub>ATP</sub> channels were responsive to the specific channel opener diazoxide (Fig. 4C, Table 3) and secretory activity was induced via activation of voltage-activated Ca<sup>2+</sup> channels (Fig. 4D, Table 3). The characteristics of voltage-activated Na<sup>+</sup> channel inactivation, voltage-activated K<sup>+</sup> channel properties and the Ca<sup>2+</sup>-dependent secretory activity in ES-derived pancreatic cells (Table 3) suggest a similarity of ES-derived pancreatic cells to embryonic beta-cells. The presence of cells responsive to K<sub>ATP</sub> channel agonists and glucose-induced secretory activity of Pax4+ ES-derived cells represent a developmentally advanced status in comparison to wt cells.

To further determine the functional status of insulin-producing cells, we analyzed glucose-dependent insulin release by ELISA. We found that both cell lines released higher insulin levels in response to 27.7 mM glucose compared to 5.5 mM glucose (Fig. 5A). Pax4+ cells showed higher insulin levels, but the ratio of released/intracellular insulin level was similar for wt and Pax4+ cells (Fig. 5B). The increased intracellular insulin level and insulin release of Pax4+ cells in comparison to wt cells correlated with higher levels of C-peptide expression in Pax4+ cells.

### Insulin-secreting cells generated from Pax4+ cells normalize blood glucose levels in diabetic mice

We tested the *in vivo* potential of ES-derived insulin-producing cells by transplanting differentiated (stage 5+28d) wt and Pax4+ cells (n = 4 - 6 x 10<sup>6</sup>), respectively, under the kidney capsule and into the spleen of streptozotocin (STZ)-induced diabetic mice. Cells were transplanted into hyperglycemic mice with blood

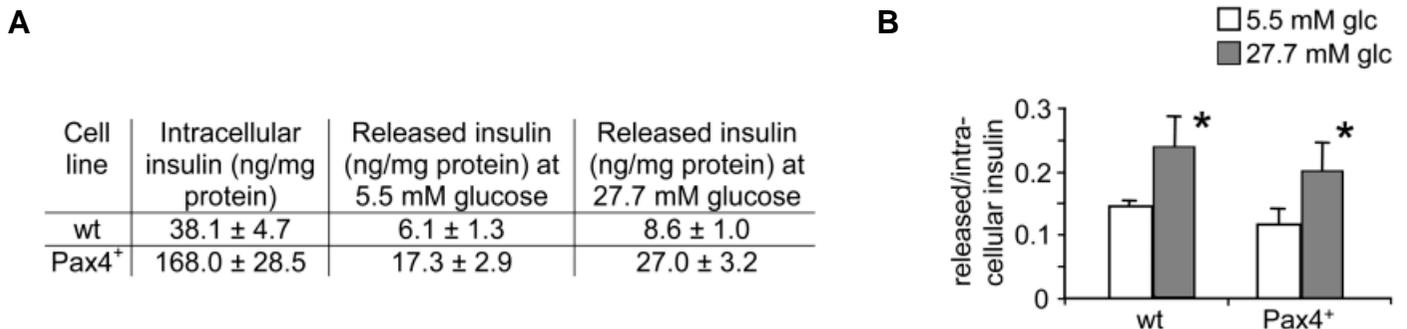
glucose levels above 15 mM and blood glucose levels and body weight were monitored over a period of about 5 weeks. Mice transplanted with wt cells and sham-transplanted control animals remained hyperglycemic at levels of about 25- 30 mM (Fig. 6A) and suffered from diabetic weight loss (Fig. 6B). Animals transplanted with Pax4+ cells reduced blood glucose levels 2 weeks after transplantation and attained a normal glycemia status by 5 weeks (Fig. 6A). Weight loss was attenuated in mice engrafted with Pax4+ cells (Fig. 6B).

In two mice engrafted with Pax4+ cells, partial graft removal by unilateral nephrectomy was performed 26 days after transplantation. These mice showed an increase in blood glucose level in contrast to non-nephrectomized mice (Fig. 6A). Some mice transplanted either with wt or Pax4+ cells developed tumours.

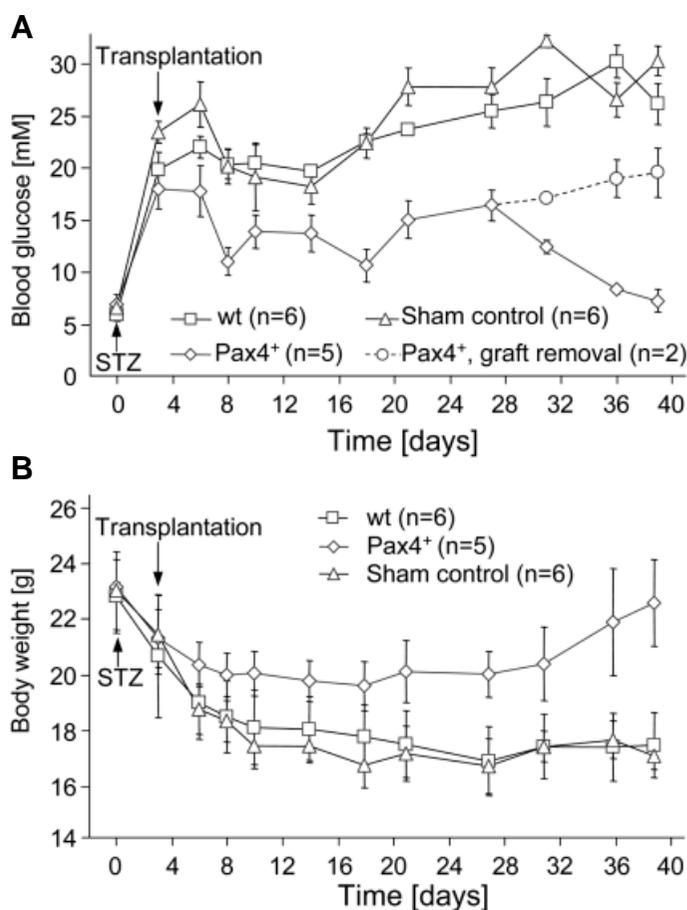
## Discussion

The application of a new protocol to generate insulin-producing cells without selecting for nestin-positive cells enabled the formation of islet-like clusters that express Pdx1, Pax4, IAPP and insulin. The cells released insulin in response to glucose, showed beta-cell-specific ion channel activity and are able to rescue diabetes in an animal model. C-peptide expression in insulin-producing cells and glucose-dependent insulin release presents evidence that the cells produced and released insulin, whereas only a low fraction of cells may uptake insulin from the medium. We show that the differentiation of ES cells without selection of nestin-positive cells generates islet-like clusters. In principle, the formation of insulin-producing cells was described by several authors (Lumelsky *et al.*, 2001; Hori *et al.*, 2002; Blyszczuk *et al.*, 2003; Moritoh *et al.*, 2003; Miyazaki *et al.*, 2004), however, the specific properties of ES-derived pancreatic cells may be different. It was presented that insulin-positive cells derived from ES cells (*via* selecting nestin-positive progenitors!) concentrate insulin from the medium (Rajagopal *et al.*, 2003; Sipione *et al.*, 2004) and/or that the differentiated cells show an artifactual insulin release (Hansson *et al.*, 2004). These findings would suggest that pancreatic differentiation from ES-derived nestin-positive cells generated by selective culture in ITSFn medium and in the presence of bFGF is irreproducible and shows no or very limited evidence of pancreatic differentiation.

Our data show that the selection of nestin-positive cells with ITSFn and bFGF is neither obligatory nor profitable for successful



**Fig. 5. Determination of insulin levels in ES-derived cells after pancreatic induction by ELISA. (A)** Levels of intracellular and released insulin in response to 5.5 mM and 27.7 mM glucose, respectively, determined after 15 min of static incubation of wt and Pax4+ ES-derived cells at stage 5+28d. **(B)** Representation of glucose-dependent insulin release by the ratio of secreted and intracellular insulin levels. Each value represents mean ± SEM. Statistical significance was tested by the Student *t* test: \*, *P* < 0.05.



**Fig. 6. Transplantation of wt and Pax4+ ES-derived cells at stage 5+28d into STZ-treated diabetic mice and body weight of animals after transplantation. (A)** Non-fasted blood glucose levels decreased in animals transplanted with Pax4+ insulin-producing cells as compared to animals receiving wt cells or sham transplants. In two mice transplanted by Pax4+ cells, unilateral nephrectomy was performed 26 days after transplantation to remove substantial parts of the graft. **(B)** Transplantation of Pax4+ ES-derived cells ameliorates weight loss in diabetic mice in contrast to wt cells and sham transplants.

pancreatic differentiation and does not promote the generation of specific pancreatic progenitors when applied to ES-derived cells. Previously, we have shown that the expansion of nestin-positive cells in the presence of bFGF is not required for the activation of pancreatic differentiation [(Blyszczuk *et al.*, 2003), see Fig. 1B]. Therefore, in our present study, all selective steps, which support the growth and maintenance of nestin-positive cells, were avoided. Nevertheless, we show that nestin-expressing cells develop *in vitro* from ES cells following the induction of pancreatic differentiation.

Nestin is unequivocally accepted as a marker of neural progenitor cells, however the expression of nestin during pancreatic development remained controversial. Nestin was found in a subset of (insulin-negative) cells in pancreatic islets, suggesting that it might be a marker of pancreatic progenitor cells (Hunziker and Stein, 2000); however, Selander and Edlund did not find nestin in the pancreatic ductal epithelium (Selander and Edlund, 2002), where the potential progenitor cells reside (Bouwens,

1998; Bonner-Weir *et al.*, 2000), instead, nestin was detected in mesenchymal cells, called pancreatic stellate cells (Lardon *et al.*, 2002; Klein *et al.*, 2003). Recent lineage-tracing studies using *nestin* regulatory elements to drive Cre-mediated activation of reporter genes in the developing pancreatic epithelium showed that nestin was transiently expressed in pancreatic epithelial progenitor cells in E10.5 mouse embryos (Esni *et al.*, 2004). Later in development, nestin expressing cells were found to contribute mainly to the exocrine lineage (Delacour *et al.*, 2004). In human pancreatic tissue, nestin was detected only at low levels in endocrine cells (Street *et al.*, 2004). But, when cultivated *in vitro*, islet-derived cells express nestin, proliferate and differentiate into pancreatic endocrine phenotypes (Zulewski *et al.*, 2001). Recent data confirmed that isolated and *in vitro* cultured adult pancreatic progenitors co-express neural and pancreatic markers including nestin (Seaberg *et al.*, 2004). Here, we show that nestin is transiently expressed in ES-derived pancreatic cells. After induction of pancreatic differentiation, nestin is highly co-expressed with C-peptide at an intermediate stage, but at the terminal stage, C-peptide positive clusters were negative for nestin. These results show that *in vitro*, nestin is transiently expressed in ES-derived pancreatic progenitors committed to differentiation, similar to ES-derived neuronal [see Lee *et al.* (2000); Rolletschek *et al.* (2001)] and hepatic [see Kania *et al.* (2003)] lineages, but nestin is not detected in terminally differentiated pancreatic (as well as differentiated neuronal and hepatic) cell types. Moreover, before induction of pancreatic differentiation, a fraction of cells co-expressing nestin and desmin was found. Together, these data suggest that nestin expression in ES-derived progeny represents a property of multi-lineage progenitors characterized by immaturity and differentiation capacity (Wiese *et al.*, 2004).

We demonstrate that ES-derived cells at the intermediate stage express C-peptide, CK19, carbonic anhydrase II and partially Isl-1. These properties suggest that the ES-derived progenitor population shows similarities with developing pancreatic beta-cells and pancreatic duct epithelial cells. CK19 is expressed in the duct epithelium in proposed pancreatic stem cells (Bouwens, 1998; Brembeck *et al.*, 2001), but not in functional islets (Bouwens *et al.*, 1997). *In vitro*, CK19 was not organized in intermediate filaments as demonstrated for pancreatic duct cells *in vivo* [see (Bouwens *et al.*, 1995; Bouwens, 1998)]. Functional cytokeratins form heteropolymeric filaments composed of CKs of subclasses I and II (Herrmann and Aebi, 2000). Therefore, we may argue that the CK19 filament organization is defective in ES-derived cells, because of inappropriate intermediate filament association (or lack of intermediate filament associated proteins) due to insufficient or not optimal culture conditions. But, similar to the *in vivo* situation, CK19 is down regulated in ES-derived (C-peptide-positive) islet-like clusters at the terminal differentiation stage. Here, we firstly show that ES-derived pancreatic cells reveal at least, some functional properties of embryonic beta-like cells. The characteristic voltage-activated Na<sup>+</sup> channel inactivation (Göpel *et al.*, 1999), voltage-activated K<sup>+</sup> channel properties and the Ca<sup>2+</sup>-dependent secretory activity in ES-derived pancreatic cells suggest a similar excitability of ES-derived pancreatic cells compared to embryonic beta-cells. These cells additionally are characterized by the presence of K<sub>ATP</sub> channels (Ashcroft and Rorsman, 1989) that were found in Pax4+, but not in wt cells. These and the other properties of ES-derived pancreatic islet-like clusters, such as partial expres-

TABLE 3

**ELECTROPHYSIOLOGICAL PARAMETERS OF WT AND PAX4+ ES-DERIVED CELLS (N= 16)  
FROM ISLET-LIKE CLUSTERS AT STAGE 5+30d**

Parameter	Wt cells		Pax4+ cells		t-test P $\geq$	embryonic $\beta$ -cells		adult $\beta$ -cells amplitude
	amplitude	n	amplitude	n		amplitude	n	
Cell capacitance (pF)	4.7 $\pm$ 0.6	14	6.7 $\pm$ 0.9	11	0.08	3.63 $\pm$ 0.33	10	7.4 $\pm$ 0.3
V <sub>m</sub> (start) (mV)	-37.8 $\pm$ 3.2	12	-39.9 $\pm$ 2.8	14	0.61	-50.5 $\pm$ 7.6	6	-60/ -80
Na <sup>+</sup> current (pA)	-1310 $\pm$ 511	6/15	-938 $\pm$ 317	7/11	0.42	-365 $\pm$ 31	4/9	-392 $\pm$ 10
Half inact. Na <sup>+</sup> (mV)	-86.0 $\pm$ 5.1	5/6	-84.2 $\pm$ 4.1	5/7	0.28	-100 $\pm$ 7	4	-104 $\pm$ 1
K <sup>+</sup> steady (pA)	+508 $\pm$ 70	13/16	+598 $\pm$ 106	14/16	0.34	1176 $\pm$ 156	8	+ 950
Gap-junction conductance (nS)	1.6 $\pm$ 0.2	5/5	1.6 $\pm$ 0.4	4/5	0.89	0.9 $\pm$ 0.3	9	1.4 $\pm$ 0.2
K <sub>ATP</sub> conductance (nS)		0/5	0.28 $\pm$ 0.06	3/5		1.78 $\pm$ 0.50	9	2.6
Diazoxide ind. $\Delta$ V <sub>m</sub> (mV)	-13	1/5	-12.5 $\pm$ 2.1	4/5		38.7 $\pm$ 5.6	5/5	
Capacitance (fF)	48	1/15	170 $\pm$ 95	3/13		30 $\pm$ 5	4/6	165 $\pm$ 39

Each value represents mean  $\pm$  SEM. Statistical significance was tested by the Student T-test. The values reported for embryonic beta-cells are from Meneghel-Rozzo *et al.* (2004). The values for adult beta-cells are from Göpel *et al.* (1999) and Speier and Rupnik (2003).

sion of CK19 and relatively low insulin levels, demonstrate that the cells are comparable to embryonic beta cells and do not represent definitive islet cells. This would be in agreement with recent data of quantitative gene expression levels in ES-derived hepatocyte-like cells that corresponded to a fetal developmental stage (Jochheim *et al.*, 2004). The rescue of the diabetic phenotype by differentiated Pax4+ cells indicates that ES cells expressing *Pax4* have the potential to function as beta-like cells. The higher efficiency of Pax4+ cells *in vivo* corresponds to the *in vitro* electrophysiological properties showing that Pax4+ cells represent a higher maturation stage in comparison to wt cells. Because also three other independently transfected Pax4+ clones showed increased insulin synthesis (unpublished data), we exclude clonal variation as explanation for the improved functional capacity of Pax4+ cells. Since there was a delay in blood glucose improvement for two weeks after transplantation, we speculate that *in vivo*, engrafted cells would mature further into a more functional cell type with physiological properties comparable to beta-like cells. Such *in vivo* maturation has been also observed in porcine neonatal pancreatic cell clusters transplanted into diabetic mice (Omer *et al.*, 2003). In the future, a more detailed characterization of the transplanted cells will be necessary to assess the influence of the *in vivo* environment on the maturation of insulin-producing cells.

In summary, our model represents a new strategy to generate pancreatic progenitor and early pancreatic endocrine cells by a procedure that avoids all selection steps, which might critically affect ES cell differentiation into insulin-producing cells. Our *in vitro* differentiation protocol allows the investigation of genetic and epigenetic modifications in ES cells as well as the analysis of differentiation factors and signalling mechanisms necessary for the generation and maturation of pancreatic progenitors *in vitro*.

## Materials and Methods

### Cell culture and differentiation conditions

ES cells of line R1 (wild type, wt) and cells constitutively expressing *Pax4* (Pax4+) were cultured as described (Wobus *et al.*, 2002; Blyszczuk *et al.*, 2003). Cells were differentiated in embryoid bodies (EBs, 600 ES cells/EB) using the "hanging drop" method (Wobus *et al.*, 2002). At day 5, EBs were plated in Iscove's modified Dulbecco's medium (IMDM, Gibco, Germany) supplemented with 20% FCS, L-glutamine, non-essential amino acids and  $\alpha$ -

monothioglycerol [see (Wobus *et al.*, 2002)] and cultured for 9 days. At day 5+9, EB outgrowths were dissociated by 0.1% trypsin (Gibco): 0.08% EDTA (Sigma, Germany) in PBS (1:1) for 1 min, collected by centrifugation and re-plated onto poly-L-ornithine/laminin-coated tissue culture dishes in "N2 medium + NA" containing DMEM/F12 (Gibco) supplemented by 20 nM progesterone, 100  $\mu$ M putrescine, 1  $\mu$ g/ml laminin, 25  $\mu$ g/ml insulin, 30 nM sodium selenite, 10 mM nicotinamide (NA) (all from Sigma), 50  $\mu$ g/ml transferrin, 5  $\mu$ g/ml fibronectin, B27 media supplement (dilution 1:50) (all from Gibco) and 15% FCS. At day 5+10, the medium was changed and cells were cultivated in the differentiation medium without FCS until stages of analysis.

### Immunofluorescence analysis

ES-derived cells growing on cover slips were fixed by 4% paraformaldehyde in PBS

for 20 minutes at room temperature and with methanol: acetone (7:3) for 10 minutes at -20°C, respectively and processed for immunofluorescence analysis as described (Wobus *et al.*, 2002). Hoechst 33342 (5  $\mu$ g/ml, 10 minutes, 37°C) was used to label the nuclei. The following primary antibodies and dilutions were used: guinea pig IgG anti-C-peptide (Linco, USA) 1:100, mouse IgG anti-nestin (Developmental Studies Hybridoma Bank, USA) 1:3, rabbit IgG anti-desmin (Dako, Denmark) 1:100, mouse IgM anti-cytokeratin19 (Cymbus, UK) 1:100, rabbit IgG anti-Isl-1 (Abcam, UK) 1:200, rabbit IgG anti-carbonic anhydrase II (Abcam) 1:200 and mouse IgG anti-insulin (Sigma) 1:40. The following secondary antibodies were used according to the manufacturer's recommendations: FITC anti-mouse IgG, Cy3 anti-mouse IgG, Cy3 anti-mouse IgM, FITC anti-mouse IgG, FITC anti-guinea pig IgG, FITC anti-mouse IgG, FITC anti-rabbit IgG, Cy3 anti-rabbit IgG (all from Jackson ImmunoResearch Laboratories, USA). Samples were analysed by the fluorescence microscope ECLIPSE TE300 (Nikon, Japan) and the confocal laser scanning microscope LSM 510 META (Carl Zeiss, Germany). For quantitative evaluation of each experiment, at least 1000 Hoechst-positive cells were analysed.

### RT-PCR analysis

Cells were suspended in lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M  $\beta$ -mercaptoethanol). Total RNA was isolated by the single step extraction method according to (Chomczynski and Sacchi, 1987). mRNA was reverse transcribed using PolyT tail primer Oligo d(T)<sub>16</sub> (PerkinElmer, Überlingen, Germany) and cDNA was amplified using the following primers: (name of the analysed gene, forward and reverse primer sequence and the length of the amplified fragment are given): *CK19* 5'-ctg cag atg act tca gaa cc-3', 5'-ggc cat gat ctc ata ctg ac-3', 299 bp; *IAPP*, 5'-tga tat tgctgc ctc gga cc-3', 5'-gga gga ctg gac caa ggt tg-3', 233 bp; *insulin*, 5'-gtggatgcttctctgcccctg-3', 5'-atg ctg gtc cag cac tga-3', 288 bp; *nestin* 5'-cta cca gga cgc cgt ggc-3', 5'-tcc aca gcc agc tgg aac tt-3', 220 bp; *Pax4*, 5'-acc aga gct tgc act gga ct-3', 5'-ccatttcagcttctctg-3' 300 bp; *Pdx1*, 5'-ctt tcc cgt gga tga aat cc-3', 5'-gtc aag ttc aac atc act gcc-3', 230 bp;  *$\beta$ -tubulin*, 5'-tca ctg tgc ctg aac tta cc-3', 5'-gga aca tag ccg taa act gc-3' 317 bp.

One third of each PCR reaction was electrophoretically separated on 2% agarose gels containing 0.35  $\mu$ g/ml of ethidium bromide. Gels were illuminated with UV light and the ethidium bromide fluorescence signals of gels were stored by the E.A.S.Y. system (Herolab GmbH, Wiesloch, Germany).

### Electrophysiological measurements

The electrophysiological methods used were similar to those described for primary pancreatic tissue slices (Speier and Rupnik, 2003). Clusters at stage 5+30d were resuspended into low gelling point agarose (Seaplaque GTG agarose, BMA, Walkersville, MD, USA; 1.9% w/v in extracellular

solution), cooled and sliced in ice-cold solution using a vibratome (VT 1000 S, Leica, Nussloch, Germany). The slices, 100  $\mu\text{m}$  thick, were stored at room temperature in extracellular solution bubbled with carbogen (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ). Standard whole-cell patch-clamp was used in current- and in voltage-clamp mode to measure the following electrophysiological parameters: membrane potential and membrane capacitance, currents through voltage-activated  $\text{Na}^+$  and  $\text{K}^+$  channels and ATP-sensitive  $\text{K}^+$  ( $\text{K}_{\text{ATP}}$ ) channels. Glucose (20 mM) and classical agonists and antagonists of insulin secretion [tolbutamide (100  $\mu\text{M}$ ) and diazoxide (200  $\mu\text{M}$ )] were used to modify the electrical properties of the cells. A patch-clamp lock-in amplifier (SWAM II, Celica, Ljubljana, Slovenia) operating at 1.6 kHz lock-in frequencies was used. Data were transferred to a PC via an A/D converter (PCI-6035E, National Instruments, Austin, Tex., USA) and acquired by WinWCP software (John Dempster, University of Strathclyde, UK). Borosilicate glass capillaries (GC150F-15; WPI, Sarasota, Fla., USA) were pulled (P-97; Sutter Instruments, Novato, CA, USA) to a resistance of 3–4 M $\Omega$  in our pipette solution. During patch-clamp experiments, the slices were superfused at 35°C with extracellular solution and held on the bottom of the recording chamber by a platinum-frame with nylon fibre. The perfusion chamber was mounted on an inverted microscope (40w, NA 0.8 and 60w, NA 1.0, Eclipse E600FN; Nikon, Japan). The extracellular solution contained (in mM): 125 NaCl, 2.5 KCl, 1.25  $\text{Na}_2\text{HPO}_4$ , 26  $\text{NaHCO}_3$ , 2 Na-pyruvate, 0.5 ascorbic acid, 3 myo-inositol, 5 lactic acid, 1  $\text{MgCl}_2$ , 2  $\text{CaCl}_2$ , 3 glucose. The pipette solution contained (in mM): 150 KCl, 10 HEPES, 2  $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ , 0.05 EGTA, 2  $\text{ATPNa}_2$ , pH 7.2 (adjusted with KOH) (all from Sigma).

#### Quantitative insulin determination by ELISA

To estimate total cellular and secreted insulin levels, differentiated wt and Pax4+ cells were pre-incubated for 90 minutes at 37°C in Krebs Ringer Bicarbonate Hepes (KRBH) buffer containing 118 mM sodium chloride, 4.7 mM potassium chloride, 1.1 mM potassium dihydrogen phosphate, 25 mM sodium hydrogen carbonate (all from Carl Roth), 3.4 mM calcium chloride (Sigma), 2.5 mM magnesium sulphate (Merck, Germany), 10 mM Hepes (Gibco) and 2 mg/ml bovine serum albumin (Gibco) supplemented with 2.5 mM glucose. For induced insulin release, cells were further incubated in KRBH buffer supplemented with 27.7 mM glucose and alternatively with 5.5 mM glucose and 10  $\mu\text{M}$  tolbutamide (Sigma) for 15 minutes at 37°C. The control was incubated with 5.5 mM glucose. Proteins were extracted from the cells with acid ethanol at 4°C overnight, followed by cell sonification. Determination of cellular and secreted insulin was performed using an insulin ELISA kit (Mercodia AB, Sweden). Protein was determined by the Bradford assay (Bio-Rad).

#### Transplantation into Streptozotocin (STZ) induced diabetic mice

Transplantations were essentially carried out as described (Blyszczuk *et al.*, 2003). In brief, 8–10 week old male BALB/c mice ( $n = 25$  each for wt and Pax4+ cells,  $n = 14$  for sham transplantation) were treated with a single i.p. injection of 200 mg/kg body weight STZ (Sigma). The diabetic status was determined by the presence of weight loss, polyuria and hyperglycaemia. For surgery, mice were anaesthetized with avertin (2.5% in PBS, 15  $\mu\text{l/g}$  body weight). Through a lumbar incision, the left kidney and the spleen were exposed. Using a blunt 30g-needle, approximately  $4\text{--}6 \times 10^6$  cells were injected under the kidney capsule and into the spleen. Alternatively, culture medium was applied for sham transplants as control. Non-fasted blood glucose levels were measured between 8:00 and 10:00 a.m. using a OneTouch glucometer. For unilateral nephrectomy, the ureter and all vessels connecting to the left kidney were ligated using 5-0 size suture and the whole kidney was removed from the anaesthetized animal. After 39 days,  $n = 10$  Pax4+ cell-transplanted animals were alive. Out of these, 5 mice responded to transplantation with a normalization of blood glucose levels, as shown in Fig. 6. In contrast, from  $n = 11$  surviving mice that were transplanted with wt cells and  $n = 8$  surviving sham-transplanted mice, none showed improvement of blood glucose levels. Mice of each of these control groups ( $n = 6$ ) were chosen on the basis of comparable hyperglycaemic status prior to transplantation.

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