

Characterization of mouse embryonic stem cell differentiation into the pancreatic lineage *in vitro* by transcriptional profiling, quantitative RT-PCR and immunocytochemistry

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ABSTRACT We have previously shown that mouse embryonic stem (ES) cells differentiate into insulin-positive cells via multi-lineage progenitors. Here, we used Affymetrix chips and quantitative RT-PCR analysis to determine transcriptional profiles of undifferentiated wildtype (wt) and *Pax4* expressing (*Pax4+*) ES cells and differentiated cells of committed progenitor and advanced stages. From undifferentiated to the committed stage, 237 (wt) and 263 (*Pax4+*) transcripts were 5- or more-fold up-regulated, whereas from the committed to the advanced stage, 28 (wt) and 5 (*Pax4+*) transcripts, respectively, were two- or more-fold up-regulated. Transcripts were classified into main subclasses including transcriptional regulation, signalling/growth factors, adhesion/extracellular matrix, membrane/transport, metabolism and organogenesis. Remarkably, endoderm-specific *Sox17* and early pancreas-specific *Isl1* transcripts were up-regulated at an earlier stage of multi-lineage progenitors, whereas highly up-regulated probe sets and transcripts of genes involved in endoderm, pancreatic, hepatic, angiogenic and neural differentiation were detected at the committed progenitor stage. *Pax4+* cells showed specific differences in transcript up-regulation and a lower amount of up-regulated neural-specific transcripts in comparison to wt cells, but no enhanced gene expression complexity. Immunocytochemical analysis of selected proteins involved in endoderm and pancreatic differentiation, such as chromogranin B, transthyretin, *Foxa1* and neuronatin revealed co-expression with insulin- or C-peptide-positive cells. The comparison of transcript profiles of ES cells differentiating *in vitro* with those of the embryonic and adult pancreas *in vivo* suggested that *in vitro* differentiated cells resemble an embryonal stage of development, supporting the view that ES-derived pancreatic cells are unable to complete pancreatic differentiation *in vitro*.

KEY WORDS: *mouse embryonic stem cells, transcriptional profiling, pancreatic differentiation, Pax4, Foxa1, chromogranin B, transthyretin, neuronatin*

Introduction

Insulin-producing cells have been generated from embryonic and adult stem and progenitor cells (Baetge, 2008; Bonner-Weir and Weir, 2005). However, so far no insulin-secreting cell population has been produced *in vitro* that would fulfil the requirements of therapeutic applications both with respect to the number of

insulin-producing cells and their functional competence, specifically, glucose-responsiveness (Serup, 2006).

Embryonic stem (ES) cells [rev. (Wobus and Boheler, 2005)] are characterized by almost unlimited proliferation and the ca-

Abbreviations used in this paper: ES, embryonic stem; *Pax4+*, *Pax4* overexpressing; qPCR, quantitative RT-PCR; wt, wildtype.

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capacity to differentiate into specialized progeny *in vitro*. Murine and human ES cells have been used to generate insulin-producing cells. Previous studies (Boyd *et al.*, 2008; D'Amour *et al.*, 2006; Hori *et al.*, 2002; Kroon *et al.*, 2008; Leon-Quinto *et al.*, 2004; Mfopou *et al.*, 2005; Miralles *et al.*, 2006; Soria *et al.*, 2000; Vaca *et al.*, 2006) and our own data (Blyszczuk *et al.*, 2003; Blyszczuk *et al.*, 2004; Schroeder *et al.*, 2006) have successfully shown the differentiation of ES cells into pancreatic cells with functional properties, thus demonstrating the proof-of-principle for the derivation of insulin-producing cells. However, strategies to select for nestin-positive cells (Lumelsky *et al.*, 2001) showed a lower and insufficient functional level of insulin-secretion (Hansson *et al.*, 2004; Rajagopal *et al.*, 2003; Sipione *et al.*, 2004) [for discussion, see (Boyd *et al.*, 2008; Kania *et al.*, 2004; Rolletschek *et al.*, 2006)].

Methods including transgene over-expression [e.g. (Blyszczuk *et al.*, 2003; Miyazaki *et al.*, 2004; Shiroy *et al.*, 2005)], lineage selection using pancreatic genes (Leon-Quinto *et al.*, 2004; Soria *et al.*, 2000), application of PI3-kinase inhibitors (Hori *et al.*, 2002), retinoic acid [e.g. (D'Amour *et al.*, 2006; Shi *et al.*, 2005)] and factors from fetal pancreas [e.g. (Vaca *et al.*, 2006)], have been used to induce ES cell differentiation into insulin-positive cells. However, high concentrations of activin A applied to human ES cells followed by sequential addition of pancreatic growth and differentiation factors have demonstrated the controlled activation of endoderm and pancreatic endocrine differentiation pathways [rev. (Bonafant-Haller and Herrera, 2008)] resulting in insulin-, glucagon-, somatostatin-, PP- and ghrelin-positive cells (D'Amour *et al.*, 2006); but the cells failed to respond to high glucose (D'Amour *et al.*, 2006). Only the transplantation of early endocrine progenitors into diabetic mice revealed the formation of functional beta-like phenotypes *in vivo* resulting in the normalization of high blood glucose level (Kroon *et al.*, 2008).

By using a three-step protocol for pancreatic differentiation, we have differentiated ES cells via embryoid bodies (EBs) and multilineage progenitors into committed progenitor cells and insulin-positive cells partially representing islet-like clusters (Blyszczuk *et al.*, 2004; Schroeder *et al.*, 2006). Differentiated insulin-positive cells co-expressed C-peptide indicating *de novo* insulin synthesis. However, only ES cells constitutively expressing the pan-

creas-specific developmental control gene *Pax4* (*Pax4*⁺) developed secretory granules (electron microscopy), showed a higher insulin level (ELISA), and ion channel activity similar to neonatal β -cells (electrophysiology). After transplantation, cells of islet-like clusters partially normalized the blood glucose level of STZ-treated diabetic mice (Blyszczuk *et al.*, 2003; Blyszczuk *et al.*, 2004; Blyszczuk and Wobus, 2004; Boyd *et al.*, 2008).

The aim of this study performed within the integrated European Union project 'FunGenES' was to elucidate the transcriptome of *Pax4*⁺ ES-derived cells in comparison to wild-type (wt) ES cells. By cDNA microchips and transcriptional profiling we identified transcripts up-regulated in committed progenitor cells potentially involved in endoderm and pancreatic development and cell function, but also in neurogenesis. Wt and *Pax4*⁺ cells showed some qualitative and quantitative differences of transcript levels. Undifferentiated ES and differentiated ES-derived cells were further analysed by quantitative RT-PCR and transcript levels compared to fetal and adult liver and pancreas, respectively. Proteins of selected transcripts including the endocrine cell-specific storage protein chromogranin B, the hepatic transporter protein transthyretin, the endoderm-specific transcription factor *Foxa1*, and neural-specific protein neuronatin were studied by immunocytochemistry and detected in ES-derived insulin- or C-peptide-positive cells.

Results

Microarray analysis demonstrated significant up-regulation of transcripts in cells at the committed progenitor stage

Three stages of wt and *Pax4*⁺ ES cells were comparatively investigated by hybridization to Affymetrix arrays: undifferentiated ES cells (stage 0d), cells at the committed stage (5+16d) and cells at the advanced differentiation stage (5+28d). The rationale for choosing these timepoints was the partial co-localization of C-peptide/cytokeratin 19, C-peptide/nestin and *Isl1*/nestin at the committed progenitor stage and the formation of nestin-negative C-peptide/insulin co-expressing clusters at day 5+28 [see (Blyszczuk *et al.*, 2004)].

After passing the quality control for each array (n=30), a set of robust multi-array analysis (RMA) normalized expression values were produced from wt and *Pax4*⁺ cells. To detect distinct expression differences between the conditions within the data sets, two ANOVA tests were performed. After conversion of Affymetrix probe set IDs to Genbank accessions, anonymous EST and 'RIKEN' labeled probes as well as redundancies were

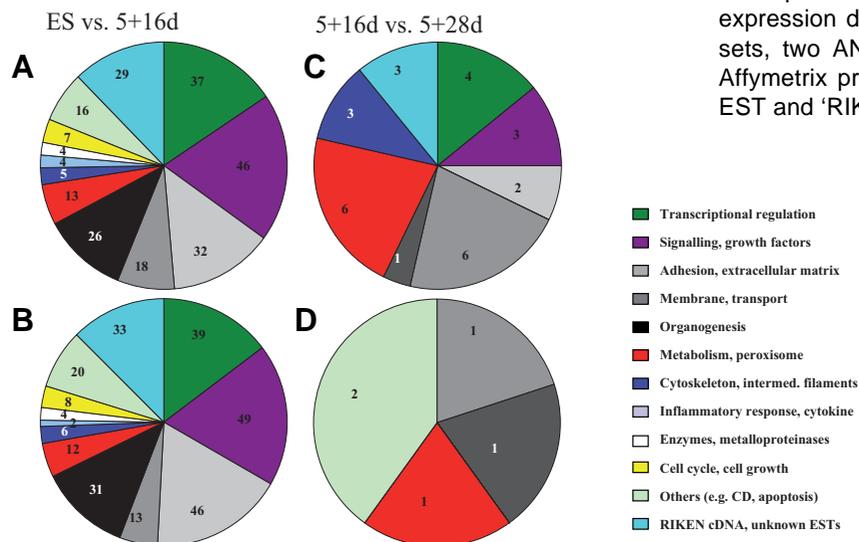


Fig. 1. Pie charts demonstrating up-regulated transcripts comparing three differentiation stages, clustered by biological function. (A) Transcripts of undifferentiated wt and **(B)** *Pax4*⁺ ES cells compared to the intermediate (committed) stage at 5+16d that were five- or more-fold up-regulated. **(C)** Transcripts of wt and **(D)** *Pax4*⁺ cells at the intermediate committed stage at 5+16d compared to the advanced differentiation stage at 5+28d that were two- or more-fold up-regulated. The absolute number of genes in each biological class is shown within each pie sector. For a complete list of up-regulated transcripts, see Suppl. Tables S2 and S4.

removed. Analysis of the differentially expressed genes resulted in identification of 3885 (wt) and 3653 (Pax4+) transcripts. By applying a P value $<10^{-6}$ data-filtering retained 243 (wt) and 244 (Pax4+) up-regulated transcripts (Suppl. Fig. S2).

To further analyse the temporal gene expression during ES cell differentiation, Student's t -test was applied (P value $<10^{-3}$, raw data are available at <http://www.ebi.ac.uk/arrayexpress/experiments/E-TABM-493>). In wt cells, we identified 1441 transcripts that were two- or more-fold up-regulated in the committed progenitor cells (5+16d) compared to undifferentiated cells (0d), whereas 38 genes were two- or more-fold up-regulated in cells at the terminal stage (5+28d) compared to stage 5+16d. In Pax4+ cells, 1387 transcripts were two- or more-fold up-regulated in committed progenitors (5+16d) compared to undifferentiated ES cells, whereas five transcripts were two- or more-fold up-regulated at stage 5+28d compared to stage 5+16d (Fig. 1).

In order to have a high confidence that selected genes are differentially expressed, we focused on those transcripts that exhibit at least a five-fold (0d vs. 5+16d) and a two-fold up-regulation (5+16d vs. 5+28d) between samples. We used the mouse chip annotation as provided by the Gene Ontology (GO) consortium and performed a literature database (PubMed) search to group genes.

Based on these criteria and after subtraction of redundancies, in wt committed progenitor cells 237 genes and in Pax4+ cells 263 genes at expression levels greater than five-fold were up-regulated at stage 5+16d compared to undifferentiated ES cells (Fig. 1; see Suppl. Tab. S2). Wild-type (Fig. 1A) and Pax4+ cells (Fig. 1B) showed up-regulation of transcripts involved in early embryogenesis and pattern formation, especially in the subclasses 'transcriptional regulation' (wt: $n=37$ / Pax4+: $n=39$), 'signalling/growth factors' ($n=46/n=49$), 'adhesion, extracellular matrix' ($n=32/n=46$) and organogenesis ($n=26/n=31$).

First, we searched for endoderm- and pancreas-specific transcription factors, but those transcription factors more than five-fold up-regulated were not identified at stage 5+16d. However, at the earlier stage of multi-lineage progenitors (5+9d), we found in wt cells up-regulated transcript levels of endoderm-specific Sox17 (3.2-fold up-regulated) and early pancreas-specific Isl1 (5.7-fold up-regulated) at the multi-lineage progenitor stage (5+9d, Suppl. Tab. S3, Pax4+ cells were not analysed) suggesting that endoderm-specific transcription factors are expressed at an earlier stage.

We continued our analysis to transcripts up-regulated in wt and Pax4+ cells at the committed progenitor stage (5+16d) and identified the following members of the groups 'transcriptional regulation': nuclear receptor subfamily 2, group F, member 1 (*Nr2f1*), (*Nr2f2*), forkhead box A1 (*Foxa1=HNF3 α*), limb-bud and heart (*Lbh*), and transcription factor 21

(*Tcf21*). Some transcripts were significantly up-regulated only in wt cells: zinc finger protein of the cerebellum 1 (*Zic1*), storkhead box 2 (*Stox2*), neuronal PAS domain protein 3 (*Npas3*), and oligodendrocyte transcription factor 1 (*Olig1*), others only in Pax4+ cells: myocyte enhancer factor 2C (*Me2c*), homeobox A3 (*Hoxa3*), and snail homologue 2 (*Drosophila*, *Snai2=slug*).

In the subclass 'signalling/growth factors', transcripts of genes encoding growth factors or other signaling-related molecules were differentially expressed in wt and Pax4+ cells: pleiotrophin (*Ptn*), endothelin receptor type B (*Ednrb*), insulin-like growth factor binding protein 4 (*Igfbp4*), *Igfbp5*, insulin-like growth factor 1 (*Igf1*), and *Igf2*. Transcripts of delta/notch-like EGF-related receptor (*Dner*) or secreted frizzled-related protein 1 (*Sfrp1*) were up-regulated in wt cells, whereas wingless-related MMTV integration site 5A (*Wnt5a*) or dickkopf homologue 2 (*X. laevis*, *Dkk2*) transcripts were found to be up-regulated only in Pax4+ cells.

A significant up-regulation of transcripts of genes involved in 'adhesion' or being part of the 'extracellular matrix' (ECM) was found for procollagens of type IV (*Col4a1*, *Col4a2*) or XI (*Col11a1*), decorin (*Dcn*), lumican (*Lum*), fibrillin 2 (*Fbn2*), cadherins 11 and 2 (*Cdh11*, *Cdh2*), microfibrillar-associated protein 2 (*Mfap2*), and matrix metalloproteinase 2 (*Mmp2*). Only in wt cells amyloid beta (A4) precursor protein (*App*) and hemicentin 1 (*Hmctn1*) and in Pax4+ cells procollagens of type I (*Col1a1*, *Col1a2*) and type V (*Col5a2*), tenascin C (*Tnc*) and vimentin (*Vim*) were detected.

A few transcripts of genes grouped into the class of 'membrane/transport'-related proteins such as fatty-acid binding protein 7 (*Fabp7*) and coatomer protein complex, subunit gamma 2 (*Copg2*) were identified in both cell lines. In wt cells transthyretin (*Ttr*), chromogranin B (*Chgb*) and secretogranin II (*Scg2*), and in Pax4+ cells very low density lipoprotein receptor (*Vldlr*) and chloride channel 4-2 (*Clcn4-2*) were significantly up-regulated.

Another subset of transcripts of genes involved in 'organogenesis' were significantly up-regulated in wt and Pax4+ cells, such

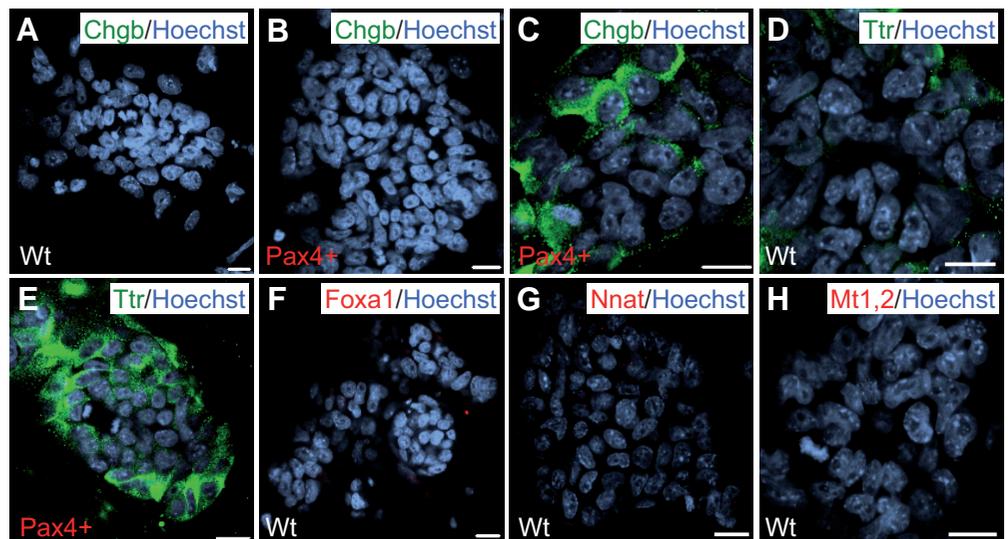


Fig. 2. Immunocytochemical analysis of five candidate markers in undifferentiated ES cells. (A) Chromogranin B (*Chgb*) immunoreactivity is absent in wt and most **(B)** Pax4+ cells, but detectable in some **(C)** Pax4+ ES cell colonies. **(D)** Transthyretin (*Ttr*) is almost absent in wt ES cells, but showed significant immunolabelling in **(E)** Pax4+ cells. Wt cells were negative for **(F)** *Foxa1*, **(G)** neuronatin (*Nnat*), and **(H)** metallothioneins *Mt 1, 2* (the antibody recognized both *Mt 1* and *2*). Bar, 20 μ m.

as neuropilin (*Nrp1*), endothelin receptor type A (*Ednra*) or quaking (*Qk*). Wt cells showed significantly up-regulated transcripts of neuronatin (*Nnat*) and delta-like 1 homologue (*Dlk1*), whereas Pax4+ cells showed significantly up-regulated transcripts of netrin1 (*Ntn1*) and selenoprotein N1 (*Seprn1*). The Suppl. Tables S2 summarize the up-regulated transcripts of all subgroups, including unknown ESTs and RIKEN cDNA.

Reasons for the differences in transcript levels between wt and Pax4+ cells may be due to statistics (P value $<10^{-3}$ and five- or more fold up-regulation between stages); and ESTs that do not fulfill these criteria would not be detected by the statistical analy-

sis.

Next, we compared transcript abundance of cells at the committed progenitor stage (5+16d) with cells at the advanced stage (5+28d). Remarkably, our data show that the gene expression complexity decreased with differentiation. In wt cells, 28 transcripts were two- or more-fold up-regulated at stage 5+28d in comparison to the progenitor stage at 5+16d (Fig. 1C, Suppl. Tab. S4A). Pax4+ cells at the advanced differentiation stage (5+28d) showed a two- or more-fold up-regulation of only a few transcripts ($n=5$) in comparison to the 5+16d stage (Fig. 1D, Suppl. Tab. S4B). The number of genes involved in transcriptional regulation decreased and genes involved in maintenance of cellular function became more abundant. Transcripts were detected in the subclasses 'membrane/transport' including aquaporin 4 (*Aqp4*) and ceruloplasmin (*Cp*), 'metabolism' including metallothioneins 1, 2 and 3 (*Mt1,2,3*), and exclusively in Pax4+ cells, in the subclass 'apoptosis' (*Nurr77*, *Ndg2*) (Suppl. Tab. S4).

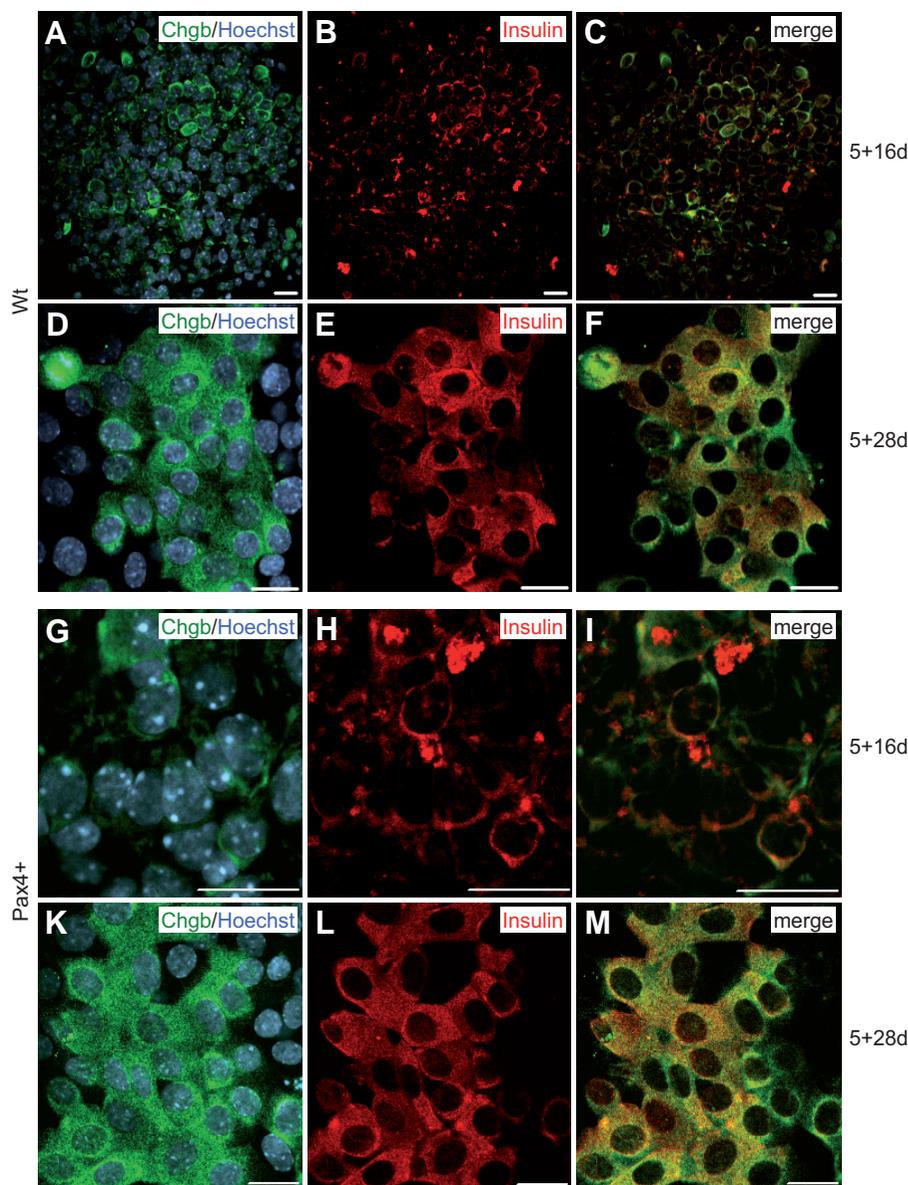


Fig. 3. Immunocytochemical analysis of chromogranin B (Chgb) in ES-derived cells at stages 5+16 (A-C, G-I) and 5+28d (D-F, K-M) in wt (A-F) and Pax4+ (G-M)-derived cells. (A) Positive immunofluorescence staining of wt cells at 5+16d and **(D)** of cells in an islet-like cluster at the advanced differentiation stage 5+28d coexpressing insulin **(B,E)**. **(C)** and **(F)** are merged images. **(G)** Positive immunoreactivity in few Pax4+ cells at stage of 5+16d and **(K)** in cells of an islet-like cluster at the advanced differentiation of 5+28d coexpressing insulin **(H,L)**. **(I,M)** Merged images. Bar, 20 μ m.

Identification of up-regulated transcripts at the committed progenitor stage in comparison to transcript data from embryonic, fetal and adult pancreas in vivo

To verify the biological significance of identified transcripts, the data of *in vitro* differentiated cells were compared to a transcriptome study done with cells of embryonic pancreas development (Gu *et al.*, 2004). A first comparison of ES-derived cells with early developing cells of the pancreatic lineage with respect to the spatial distribution of transcripts was performed. By comparing transcripts enriched in E7.5 endoderm with those of the ES-derived committed progenitor population, 20 (wt) and 21 (Pax4+) two- or more-fold up-regulated transcripts were detected (Suppl. Tab. S5A) including *Igf2*, *Igfbp5*, *Nrp1*, *Foxa1*, *Col4a*. A comparison of transcripts enriched in Pdx1+ pancreatic progenitor cells (E10.5) with ES-derived cells revealed 15 (wt) and 7 (Pax4+) two- or more-fold up-regulated transcripts (Suppl. Tab. S5B) including *Dlk1* and *Chgb*. Transcripts enriched in Ngn3+ endocrine progenitors (E13.5) identified by Gu *et al.* (2004) corresponded to 15 (wt) and 8 (Pax4+) two- or more-fold up-regulated transcripts in ES-derived cells, such as *Chgb*, *Cpe* and *Ctsf* (Suppl. Tab. S5C).

Next, we compared our data to the transcript profiles of early endoderm, Pdx1+ and Ngn3+ expressing cell stages with profiles of other stages as published by Gu *et al.* (2004). Transcripts enriched in Pdx1+ cells corresponded to 10 (wt) and 12 (Pax4+) up-regulated transcripts, including *Igfbp5* (wt, Pax4+), *Sfrp1* (wt), *Col1a2/5a2* (Pax4+), *Tnc* (Pax4+) and *Ptn* (wt, Pax4+; see Suppl. Tab. S5E). Comparative data of other stages are shown in

TABLE 1A

**TRANSCRIPT ANALYSIS OF UNDIFFERENTIATED ES CELLS COMPARED TO
CELLS OF THE COMMITTED PROGENITOR STAGE AT 5+16d**

Probe set ID	Gene symbol	Gene title	wt ES vs. 5+16d		Pax4+ ES vs. 5+16d	
			Pax4+ ES vs. 5+16d	Quant. PCR*	Fold change	Quant. PCR*
Transcriptional regulation						
1418496_at	Foxa1	forkhead box A1	7.3	+	6.0	+
1449397_at	Hoxb2	homeo box B2	3.0	+	6.2	+
Signaling, growth factors						
1448254_at	Ptn	Pleiotrophin	160.9	+	218.9	+
1423584_at	Igfbp7	insulin-like growth factor binding protein 7	-	+	20.1	+
1434413_at	Igf1	insulin-like growth factor	18.0	+	32.4	+
1448152_at	Igf2	insulin-like growth factor	10.5	+	14.1	+
1456482_at	Pik3r3	phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3 (p55)	6.4	+	-	+
1423104_at	Irs1	insulin receptor substrate 1	-	-(1.2x up)	6.7	+
Adhesion, extracellular matrix						
1427884_at	Col3a1	procollagen, type III, alpha 1	102.5	+	240.1	+
1423110_at	Col1a2	procollagen, type I, alpha 2	-	+	46.3	+
1449368_at	Dcn	Decorin	30.8	+	70.0	+
1416666_at	Serpine2	serine (or cysteine) proteinase inhibitor, clade E, member 2	3.8	+	5.8	+
Membrane, transport						
1455913_x_at	Ttr	Transthyretin	65.9	+	-	-
1415885_at	Chgb	chromogranin B	16.2	+	-	-
1417900_a_at	Vldlr	very low density lipoprotein receptor	4.7	+	10.7	-
Organogenesis						
1423506_a_at	Nnat	Neuronatin	39.4	+	-	+
1448943_at	Nrp1	neuropilin 1	10.2	-(1.3x up)	35.9	+
Metabolism, peroxisome						
1415949_at	Cpe	carboxypeptidase E	7.6	-(1.9x up)	10.0	+
1451019_at	Ctsf	cathepsin F	2.5	+	-	+
1417492_at	Ctsb	cathepsin B	-	+	7.2	+

TABLE 1B

**TRANSCRIPT ANALYSIS OF CELLS AT THE COMMITTED PROGENITOR STAGE 5+16d vs.
CELLS AT THE ADVANCED STAGE AT 5+28d**

Probe set ID	Gene symbol	Gene title	wt 5+16d vs. 5+28d		Pax4+ 5+16d vs. 5+28d	
			Fold change	Quant. PCR*	Fold change	Quant. PCR*
Metabolism, peroxisome						
1420575_at	Mt3	metallothionein 3	10.4	+	-	+
1422557_s_at	Mt1	Metallothionein 1	4.8	-(1.4x up)	-	+
1428942_at	Mt2	Metallothionein 2	4.7	-(1.7x up)	2.3	+
Cytoskeleton, intermediate filaments						
1426509_s_at	Gfap	Glial fibrillary acidic protein	46.4	+	-	+

Selected pancreatic-, hepatic and endothelial-specific transcripts that were two- or more-fold up-regulated in wt and Pax4+ ES-derived cells at stages 5+16d (0d vs. 5+16d) and 5+28d (5+16d vs. 5+28d) analysed by microarrays and real-time RT-PCR. (for a complete list of these genes, see Suppl. Tab. S2 and S4). * (+) More than two-fold up-regulated; (-) less than two-fold up-regulated or down-regulated (as indicated in brackets).

the Suppl. Tables S5D, F and G); and transcripts including Foxa1 (wt, Pax4+), Mafk (wt), Pik3r1 (wt, Pax4+), secretogranin II (wt) could be identified.

Because it is well known that signals/differentiation factors released by cells from other lineages have important effects on pancreatic development [rev. Bonal and Herrera, 2008; Soria, 2001] we also compared our data set with transcriptome data of other tissue samples as done by Gu *et al.* (2004). Comparable to the group of transcripts enriched in Pdx1-negative cells (i.e. mesoderm surrounding the pancreas, stomach and duodenum), 25 (wt) and 29 (Pax4+) two- or more-fold up-regulated transcripts were identified (Suppl. Tab. S6A) including Dlk1 (wt), Snai2

(Pax4+), Foxa1 (wt, Pax4+), Cdh11 (wt, Pax4+), Fbn2 (Pax4+), or Col1a subunits (wt, Pax4+). Corresponding to transcripts enriched in Ngn3- negative pancreatic cells (e.g. exocrine precursors, cells of mesodermal origin within the pancreas) 37 (wt) and 41 (Pax4+) two- or more-fold up-regulated transcripts including Igf1/ 2 (wt, Pax4+), Igfbp 4/5 (wt, Pax4+), collagen subunits (wt, Pax4+), Dcn (wt, Pax4+), Lum (wt, Pax4+), and Nrp (wt, Pax4+) were detected (Suppl. Tab. S6B).

In conclusion, our oligonucleotide array analysis performed with wt- and Pax4+-derived cells revealed the majority of up-regulated transcripts at the committed stage. Whereas up-regulated transcripts of definitive endoderm and early pancreas-

specific genes (Sox17, Isl1) were already detected at the multilineage progenitor stage, we identified a remarkable number of transcripts up-regulated at stage 5+16d that are associated with distinct mechanisms of endoderm and pancreas development and cell function. A number of those transcripts up-regulated in wt and Pax4+ cells corresponded to transcriptome data sets of embryonic pancreas development *in vivo*. However, quantitative variations of expression levels between wt and Pax4+ cells and also qualitative differences in transcript abundance were ob-

served between wt and Pax4+ cells.

Validation of microarray data by quantitative RT-PCR (qPCR)

A subset of genes associated with pancreatic, hepatic and endothelial cell development that showed significant up-regulation in cells of the committed and advanced stages was validated by qPCR and transcript levels were compared to those of fetal and adult pancreas, liver, and partially, brain tissue, respectively (see Table 1, Suppl. Fig. S3). In general, the data obtained by microarray analysis were confirmed by qPCR (see Tab. 1).

For specific genes we found differences in the transcript levels between wt and Pax4+ cells. Such differences in expression patterns were already evident in ES cells. Whereas most transcripts analysed showed no differences between wt and Pax4+ ES cells or were higher expressed in the wt (i.e. *Irs1*, *Pik3r3*, *Col1a2*, *Cpe* and *Nrp1*), Pax4+ ES cells revealed higher *Chgb*, *Nnat*, *Mt1,2,3*, *Ttr* and *Vldlr* transcript levels. This may reflect a correlation between Pax4-overexpression and a higher level of metabolic/secretory cell function and protection against stress-induced apoptosis in Pax4+ ES cells.

Some differences between Pax4+ and wt cells could be found at the committed stage (5+16d). Most of the transcripts analysed showed higher up-regulation in wt cells, whereas several transcripts, such as *Hoxb2*, *Ptn* and *Gfap* were more significantly up-regulated in Pax4+ cells, or were expressed at similar levels (i.e., *Nnat*, *Mt1,2*).

However, by comparing transcriptional changes from 5+16d to 5+28d, Pax4+ cells showed changes that more closely resemble the *in vivo* function of pancreatic cells, such as up-regulation of *Igf1*, *Igf1bp7*, *Ttr*, *Nrp1*, *Mt1,2*, or have similar levels (*Irs1*, *Chgb*, *Ctsb*, see Suppl. Fig. S3), whereas early endoderm markers (i.e. *Foxa1*, *Hoxb2*) and neural-specific (i.e. *Nnat*) transcripts were down-regulated.

Comparing fetal and adult pancreas tissue, the transcripts analysed in this study showed a broad variety. Most transcripts, including *Foxa1*, *Chgb*, *Mt1-3*, *Hoxb2*, *Igf2*, *Pik3r3*, *Ptn*, *Col1a2*, *Col3a1*, *Serpine2*, *Nrp1*, *Ctsf*, and *Cpe* decreased during maturation from fetal to adult pancreas. Transcripts like *Ttr*, *Nnat*, *Igf1bp7*, *Irs1*, *Ctsb*, and *Gfap* were up-regulated, while others (*Igf1*, *Dcn*, and *Vldlr*) were equally expressed in fetal and adult pancreatic tissue.

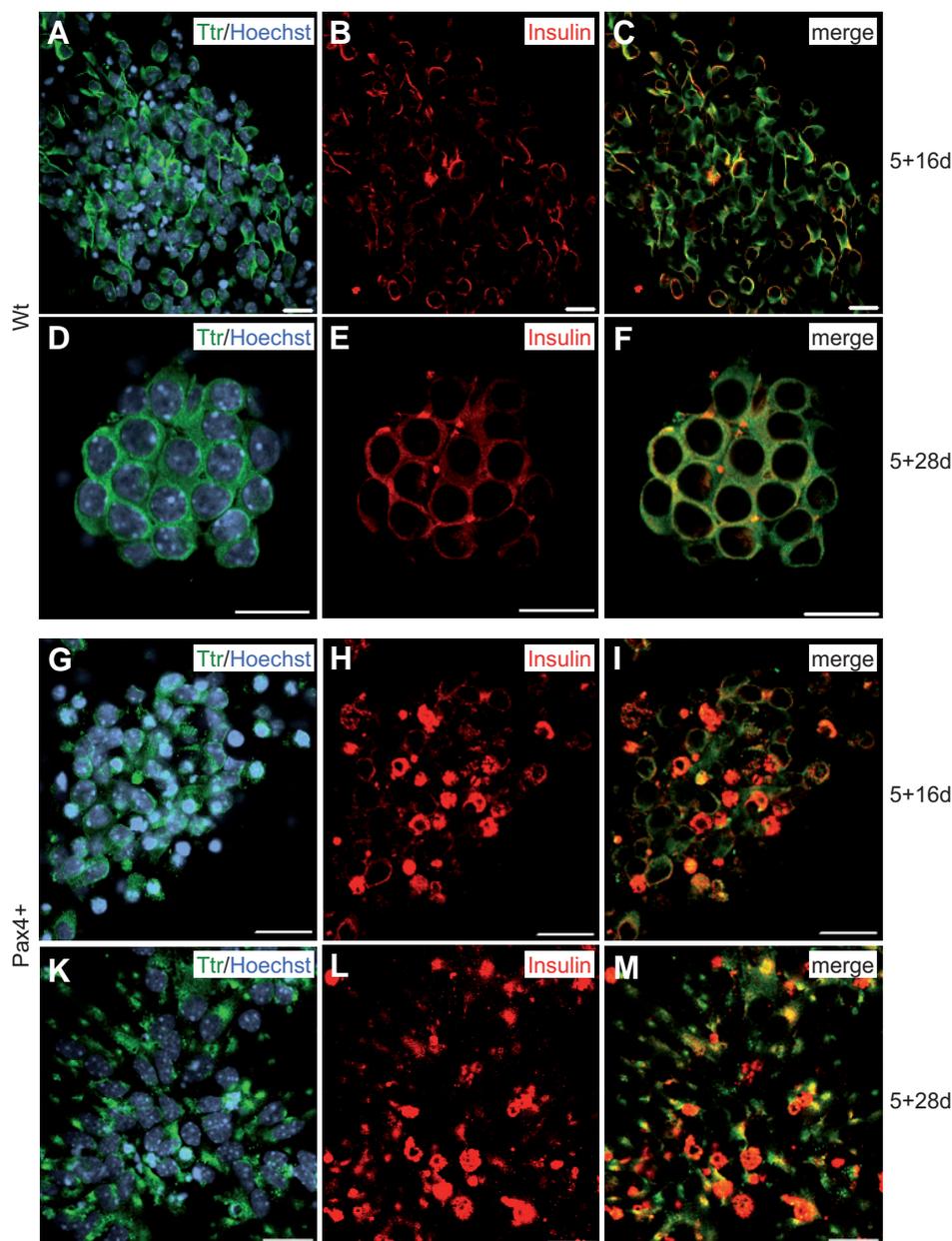


Fig. 4. Immunocytochemical analysis of transthyretin (Ttr) in ES-derived cells at stages 5+16 (A-C, G-I) and 5+28d (D-F, K-M) in wt (A-F) and Pax4+ (G-M)-derived cells. (A) Positive immunofluorescence staining of wt cells at 5+16d and **(D)** of cells in an islet-like cluster at the advanced differentiation stage 5+28d coexpressing insulin **(B,E)**. **(C,F)** Merged images. **(G)** Positive immunoreactivity in Pax4+ cells at stage 5+16d and **(K)** in cells at advanced differentiation of 5+28d coexpressing insulin **(H,L)**. **(I,M)** Merged images. Note cells with small and condensed nuclei displaying strong insulin staining, which represents apoptotic cells. Bar, 20 μ m.

Immunocytochemical analysis of selected candidate proteins in ES cells

Immunocytochemical analysis of five selected proteins was performed to verify the microarray and qPCR data and to proof their localization in C-peptide- or insulin-positive cells. The synaptic vesicle protein Chgb was selected, because *Chgb* is expressed in adult islets and Chgb is released by secretory granules. The hepatocyte-specific protein Ttr and transcription factor *Foxa1* were studied to verify its role in ES-derived pancreatic cells. The neural-specific protein Nnat annotated for neuronal differentiation and function [see (Chu and Tsai, 2005)] was selected to discriminate between neural and pancreatic progenitors. Metallothioneins (Mt) play a role in stress response and because of up-regulated transcript levels in cells of the advanced stage, were of interest. Moreover, *foxa1*, *ttr*, *chgb*, *nnat* expression has been detected in pancreas tissue or pancreas-derived cell lines, but not yet in ES-derived cells. Finally, for these five proteins, antibodies were available (lacking for other markers, such as collagen subunits, Pik3r3, serpine2 or Cpe).

Immunocytochemical analysis of Chgb in ES cells revealed its absence in undifferentiated wt (Fig. 2A) and most of Pax4+ ES cells (Fig. 2B), but localization in some Pax4+ ES cells at the periphery of colonies (Fig. 2C). Ttr was weakly detected in wt ES cells (Fig. 2D), but significantly present in Pax4+ ES cells (Fig. 2E), and *Foxa1* (Fig. 2F), Nnat (Fig. 2G) and Mt1,2 (Fig. 2H) were not found in ES cells (Mt3 proteins could not be analysed, because Mt3-specific antibodies were not available).

Immunocytochemical analysis of Chgb, Ttr, Foxa1, Nnat and Mt1,2 in insulin- or C-peptide-positive ES-derived progeny at 5+16d and 5+28 d

Next, we analysed the localization of the five proteins in insulin- or C-peptide-positive cells at committed progenitor (5+16d) and advanced (5+28d) stages of differentiation (Pancreas tissue immunolabelled by insulin- and C-peptide-positive antibodies served as positive control, see Suppl. Fig. S4).

Chgb was abundant in insulin-positive cells at both stages in wt (5+16d, Fig. 3 A-C) and Pax4+ (Fig. 3 G-I) cells. At stage 5+28d, Chgb and insulin co-localization was found in cells of islet-like clusters with no obvious morphological differences between wt (Fig. 3 D-F) and Pax4+ (Fig. 3 K-M) cells.

Fig. 5. Immunocytochemical analysis of Foxa1 in wt ES-derived cells at stages 5+16 (A-C) and 5+28d (D-F). (A) Positive immunofluorescence staining within the cytoplasm of cells at stage 5+16d and (D) of cells at the advanced differentiation stage of 5+28d coexpressing C-peptide (B,E). Cells in insert of (D) show immunostaining of Foxa1 also in the nuclei of a subset of cells. (C,F) Merged images. Bar, 20 μ m.

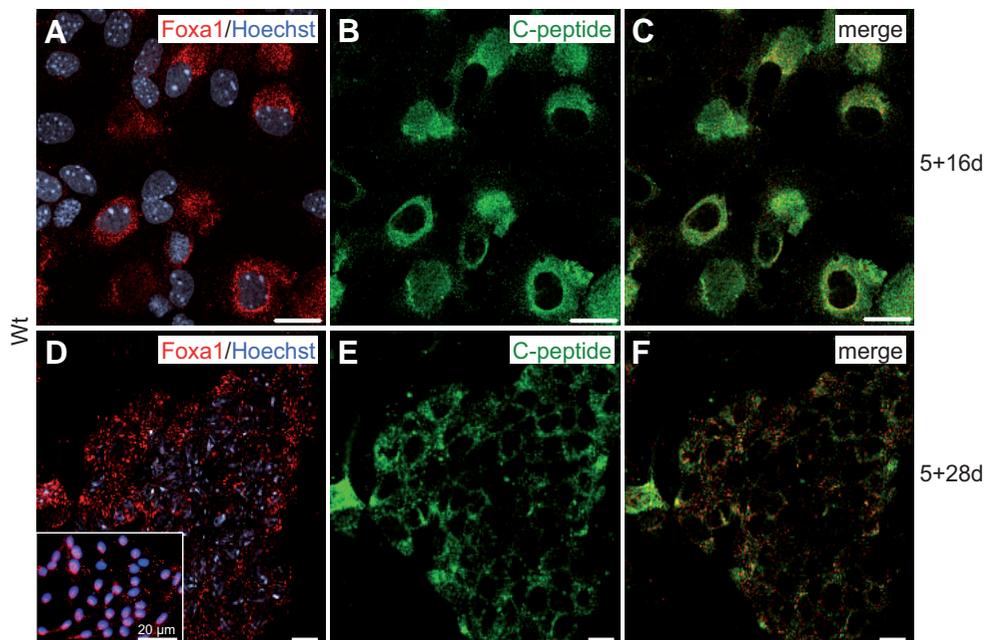


TABLE 2

NEURO-SPECIFIC TRANSCRIPTS THAT WERE TWO- OR MORE-FOLD UP-REGULATED IN WT AND PAX4+-ES-DERIVED CELLS AT THE COMMITTED STAGE (5+16D) COMPARED TO THE UNDIFFERENTIATED STAGE (0D)

Probe set ID	Gene	Gene title	wt	Pax4+
			ES vs 5+16d	ES vs 5+16d
			Fold change	
Transcriptional regulation				
1423477_at	Zic1	zinc finger protein of the cerebellum 1	77.0	-
1429138_at	Npas3	Neuronal PAS domain protein 3	7.0	-
Signaling, growth factors				
1448254_at	Ptn	Pleiotrophin	160.9	218.9
1437347_at	Ednrb	Endothelin receptor type B	76.8	64.9
1456379_x_at	Dner	delta/notch-like EGF-related receptor	20.0	-
1452251_at	Nbea	Neurobeachin	11.6	6.5
1451289_at	Dcamk1	Double cortin and alcium/calmodulin-dependent protein kinase-like 1	7.7	24.1
1455143_at	Nlgn2	neuroligin 2	3.6	3.9
Membrane, transpor				
1450779_at	Fabp7	fatty acid binding protein 7, brain	155.4	115.0
1415885_at	Chgb	chromogranin B	16.2	-
Organogenesis				
1423506_a_at	Nnat	Neuronatin	39.4	-
1448943_at	Nrp1	neuropilin 1	10.2	35.9
1424659_at	Slit2	slit homolog 2 (Drosophila)	8.4	11.3
1418285_at	Efnb1	ephrin B1	4.8	5.2
1454974_at	Ntn1	netrin 1	-	7.5
Inflammatory response, cytokine				
1448823_at	Cxcl12	chemokine (C-X-C motif) ligand 12	31.6	22.2
Enzymes, metalloproteinases				
1433855_at	Abat	4-aminobutyrate aminotransferase	9.5	-
Cell cycle, cell growth				
1423100_at	Fos	FBJ osteosarcoma oncogene	13.9	-

The list contains transcripts of genes implicated in neural differentiation and neuronal cell function, which were also detected in ES-derived cells following pancreatic differentiation via multi-lineage progenitor cells [according to (Blyszczuk *et al.*, 2004; Schroeder *et al.*, 2006)].

Ttr became abundant in insulin-positive wt cells at 5+16d (Fig. 4A-C), and co-localized with insulin-positive cells in islet-like clusters at the advanced stage (5+28d; Fig. 4D-F). Co-localization of Ttr and insulin was also found in Pax4+ committed progenitors (Fig. 4G-I) and cells at the advanced stage (Fig. 4K-M). However, Pax4+ cells showed signs of apoptosis with small condensed nuclei suggesting that these cells collected insulin from the medium. To verify that Ttr transcript up-regulation in ES-derived cells was not due to (parallel) formation of hepatic cells, immunofluorescence analysis was performed with α -fetoprotein- and albumin-specific antibodies in wt and Pax4+ ES-derived cells, but no colocalization of Ttr was found with these markers (data not shown).

Foxa1 was detected in wt cells as dot-like structures in the cytoplasm, but partially, was found also in nuclei (see insert in Fig. 5D) of C-peptide-positive cells at committed progenitor (5+16d, Fig. 5 A-C) and advanced (5+28d, Fig. 5 D-F) stages.

Nnat was clearly found in committed progenitors with characteristic round-shaped morphology and colocalized with insulin (Fig. 6 A-C). However, the fraction of immuno-positive cells decreased with differentiation; and at the advanced stage, Nnat was detected in only a few insulin-positive cells (Fig. 6 D-F). Significant Nnat immuno-reactivity was found in cells forming neuronal extensions representing neural phenotypes (see Fig. 6C, inset). Our data may suggest that Nnat (besides its abundance in neural cells) is also transiently expressed in a progenitor-like (bi-potential?) phenotype during pancreatic differentiation of ES cells.

The Nnat data presented here as well as the transcript data (see Table 2) demonstrate that both wt and Pax4+ cells contain a remarkable amount of neural cells suggesting that the differentiation protocol via multi-lineage progenitors induced also a neural

subpopulation. However, it is also evident that Pax4+ cells show a limited number and lower levels of neural transcript up-regulation in comparison to wt cells (see Table 2).

Mt1,2-specific proteins were not detected in C-peptide or insulin-positive cells, but found in the vicinity of C-peptide-positive clusters. At stage 5+16d, only a few Mt1,2-positive cells were present in C-peptide-negative cells (Fig. 7 A-C), whereas at the advanced stage, Mt1,2-positive cells were located in cells around C-peptide-positive clusters (Fig. 7 D-F).

Discussion

Transcriptional profiling of murine wt and Pax4+ ES cells induced to differentiate into pancreatic cells via multi-lineage progenitors revealed the maximum of up-regulated transcripts involved in endoderm, pancreatic, endothelial and neural differentiation at an intermediate stage of committed cells. Endoderm-specific transcription factors were up-regulated at the stage of multi-lineage progenitors. A remarkable number of neural transcripts was up-regulated, specifically in wt cells. Pax4+ cells did not show higher gene expression complexity in comparison to wt cells. Endoderm- and early pancreas-specific transcripts were detected and several proteins identified in insulin-/C-peptide-positive clusters. However the advanced stage of *in vitro* differentiated ES cells did not consistently allow complete differentiation into functional pancreatic cells.

Endoderm- and early pancreas-specific transcription factors were up-regulated at the early stage of multi-lineage progenitors

Based on our previous findings (Blyszczuk *et al.*, 2004), we expected to find up-regulated pancreas-specific transcription factors in the committed progenitor stage (5+16d). Reasons that we did not detect these transcription factors in the Affymetrix data set could be that (i) genes involved in early endoderm development were transiently up-regulated at lower levels, (ii) those transcripts were expressed at an earlier stage during ES cell differentiation, or (iii) transcripts were not present on the microchips. The latter explanation was true, i.e., for Pdx1 that could not be found in this study, because of absence on the Affymetrix chips. However, our previous analyses clearly demonstrated *Pdx1* expression in differentiated wt and Pax4+ cells (Blyszczuk *et al.*, 2003; Blyszczuk *et al.*, 2004).

Moreover, Sox17 and Isl1 up-regulated transcripts were detected at the earlier stage of multi-lineage progenitors. This is in agreement with findings that endoderm-specific *Sox17*, a Sry-related HMG box factor is required for early pancreas specification (Kanai-Azuma *et al.*, 2002) and late-stage differentiation of extra-embryonic endo-

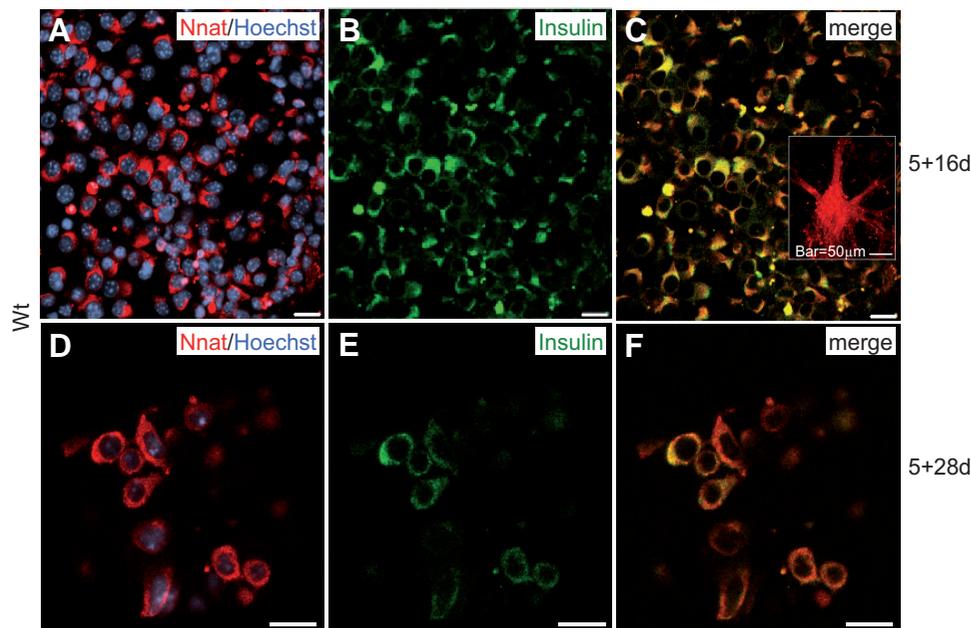


Fig. 6. Immunocytochemical analysis of neuronatin (Nnat) in wt ES-derived cells at stages 5+16 (A-C) and 5+28d (D-F). (A) Positive immunofluorescence staining of cells at stage 5+16d and (D) of cells at the advanced differentiation stage at 5+28d coexpressing insulin (B,E). Inset in (C) represents a group of neuronal cells with Nnat-labelled extensions. (C,F) Merged images. Bar, 20 μ m.

derm (Shimoda *et al.*, 2007); and pancreas-specific *Is1* regulating the differentiation of islets (Ahlgren *et al.*, 1997) is expressed in the developing pancreas and becomes restricted to endocrine cells in adult islets (Habener *et al.*, 2005). These data underline previous data of our (Blyszczuk *et al.*, 2004) and other (Boyd *et al.*, 2008) groups demonstrating that ES cells during *in vitro* differentiation via multi-lineage progenitors (at least partially) pass through the endoderm lineage.

The maximal number of up-regulated transcripts was present in cells at the committed progenitor stage

We determined transcript up-regulation of genes known to play a role in endoderm and pancreatic development and function, including *Hoxb2*, *Foxa1*, *Igf1,2*, *Irs1*, *Pik3r3*, procollagens *Col3a1*, *Col4a1*, *Dcn*, *Serpine2*, *Ttr*, *Chgb*, *Vldlr*, *Nnat*, *Nrp1*, *Cpe*, *Ctsb* and *metallothioneins* at the stage of committed progenitors (5+16d).

Hoxb2 and *Foxa1* expression suggests a progenitor-specific function in ES-derived cells. Up-regulated *Hoxb2* transcript levels in wt and Pax4+ cells comparable to *in vivo* levels of fetal pancreas and liver suggest that *Hoxb2* plays a role in ES-derived pancreatic differentiation. However, *Hoxb2*, a downstream target of retinoic acid, has been shown to be up-regulated also in pancreatic cancer (Segara *et al.*, 2005). The partial cytoplasmic localization of *Foxa1* in C-peptide-positive cells at stage 5+16d underlined the progenitor status of the cells. Transcription factors of the *Foxa* family are required for cell-specific activation of glucagon in alpha-cells (Kaestner *et al.*, 1999) and implicate a crucial role of *Foxa1* in the transcriptional control of glucose homeostasis (Kaestner *et al.*, 1999; Sharma *et al.*, 2005). A similar regulatory function in islets has been proposed for *Pik3r3*, known to play a major role in the suppression of glucose-stimulated insulin secretion (Eto *et al.*, 2002).

The transcriptome analysis detected up-regulated transcript levels of *Igf1,2* and *Igf1,2*, which are temporally and spatially expressed during pancreas development [rev. (Hill *et al.*, 1998)]. Protein abundance in beta cells supports an important role of the Igf/Igf1 system in endocrine pancreatic maturation, and the determination of beta-cell mass and insulin availability (Hill *et al.*, 1999). *Igf1,2*, are part of a complex system regulating beta-cell growth and function (Katz *et al.*, 1997). *Irs1* mediates metabolic and growth-promoting activity of insulin and *Igf1*, and the *Irs1* pathway modulates insulin secretion in pancreatic beta cells (Burks and White, 2001).

Within the group of adhesion and ECM proteins, transcripts of genes encoding procollagens (i.e. *Col3a1*, *Col4a1*) were up-regulated consistent with the *in vivo* situation, where collagens are the main ECM components of the islet microenvironment (Kaido *et al.*, 2004). Decorin (*Dcn*) belongs to the group of small leucine-rich proteoglycans expressed by sprouting endothelial cells. Decorin plays a role in regulating collagen fibril formation and stabilization, and modulates cell adhesion as well as transforming growth factor- β activity [for review see (Koning *et al.*, 2004; Kresse and Schonherr, 2001)]. *Dcn* transcripts were up-regulated in committed progenitor cells, but not in liver and pancreas. A functional role of *Dcn* in pathological mechanisms, i.e. inhibition of tumour cell growth, was described by Kresse and

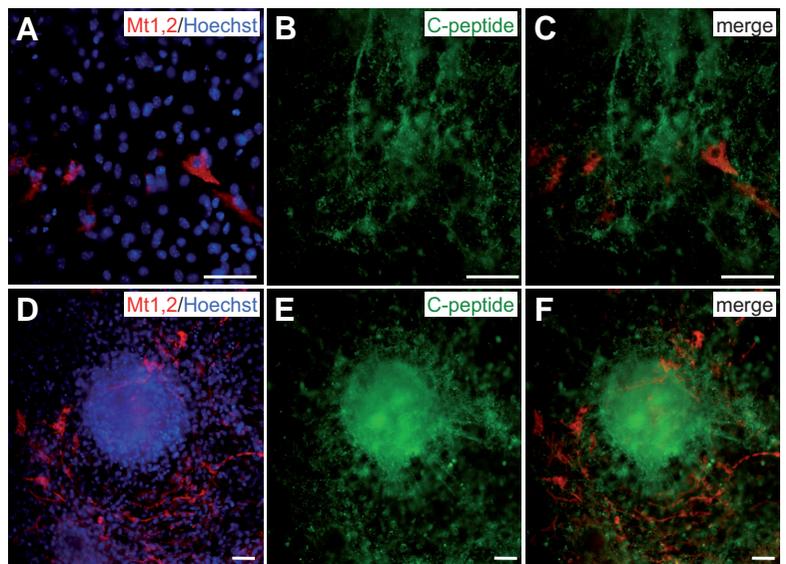


Fig. 7. Immunocytochemical analysis of metallothioneins Mt 1,2 in wt ES-derived cells at stages 5+16 (A-C) and 5+28d (D-F). (A) Positive immunofluorescence staining of Mt1,2 and (B,C) C-peptide-negative cells at stage 5+16d and (D-F) of cells at the advanced stage of 5+28d. (E,F) Mt1,2 positive cells were detected in C-peptide-negative cells around C-peptide-positive clusters. Bar, 20 μ m.

Schonherr (2001) and Koning *et al.* (2004).

Serpine2 transcripts were slightly up-regulated in committed progenitor cells, whereas significant levels were detected in adult pancreas. *Serpine2* regulates protease activity in smooth muscle and prostate cancer cells and is an important modulator of tumor-host interaction in pancreatic cancer (Buchholz *et al.*, 2003), but we have no explanation for the high *serpine2* levels in adult pancreas.

The transcript up-regulation of *Ttr* in wt cells of the committed progenitor stage was unexpected, because the gene has been annotated for hepatic differentiation and liver function, but not yet for ES-derived pancreatic cells. *Ttr*, a transport protein for thyroxine or retinol is synthesized in the liver, brain and endocrine pancreas (Jacobsson *et al.*, 1989; Kato *et al.*, 1985). A potential role of *Ttr* in pancreatic cells has been described by Refai *et al.* (2005) who demonstrated a promoting effect for glucose-induced increase of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) and insulin release due to direct *Ttr* effects on glucose-induced electrical activity (Refai *et al.*, 2005). The authors postulated a protective role of *Ttr* against beta cell apoptosis. The presence of *Ttr* in undifferentiated Pax4+ ES cells (Fig. 2E) and cells of the advanced stage (see Fig. 4; Suppl. Fig. S3) may suggest a similar role in ES cells *in vitro*.

We further identified characteristic proteins of the pancreatic endocrine secretory pathway (Karlsson, 2001), such as *Chgb* and secretogranin II (*ScgII*= chromogranin C) in *in vitro* differentiated ES cells. *Chgb* is known to bind calcium (Gorr *et al.*, 1989), exert trophic activity in neurons (Chen *et al.*, 1992) and mediate the sorting of peptide hormones to the secretory granules (Natori and Huttner, 1996). The presence of variable *Chgb* epitopes in endocrine pancreatic cell types suggests that different *Chgb* epitopes are expressed in different cell types (Portela-Gomes and Stridsberg, 2002), and besides *Chgb*, secretory granule-specific *ScgII* levels were up-regulated in wt cells. *ScgII* is highly expressed in alpha cells (Boonen *et al.*, 2007) and shown to be

enriched in mature islets (see Suppl. Tab. S5G; Gu *et al.*, 2004). Scg11 and Chgb up-regulation and Chgb colocalization with insulin in both wt and Pax4+ ES-derived cells suggests that the cells attained, at least partially, a pancreatic fate *in vitro*.

The very low density lipoprotein receptor (*Vldlr*) has been detected in pancreatic islets (Roehrich *et al.*, 2003) and correlated with the survival of beta cells (Cnop *et al.*, 2002), but we have no explanation for high *Vldlr* levels in Pax4+ ES cells.

Neuronatin (*Nnat*) and neuropilin (*Nrp1*), members of the subgroup "organogenesis" were also up-regulated in ES-derived committed cells *in vitro*, consistent with *in situ* hybridization data showing *Nnat* expression in the pancreas of mouse E14.5 and E16.5 embryos (Chu and Tsai, 2005). Although *Nnat* is highly expressed in postmitotic neurons and in neonatal and adult brain (Joseph *et al.*, 1994) [rev. (Gavazzi, 2001)], *Nnat* is also expressed in other adult tissues, such as pituitary and adrenal glands, lung, uterus, skeletal muscle, ovary and pancreas (Arava *et al.*, 1999; Niwa *et al.*, 1997). *Nnat* was determined as a downstream target of NeuroD/BETA2 in the pancreas playing a role in glucose-mediated insulin secretion (Chu and Tsai, 2005). The presence of *Nnat* protein in insulin-positive committed progenitor cells suggests a role of *Nnat* in ES cell differentiation. But, transcript and protein abundance at the advanced stage and presence in neural cells points also to the induction of neural phenotypes during ES cell differentiation via multi-lineage progenitors [see (Blyszczuk and Wobus, 2004; Rolletschek *et al.*, 2006)].

Three members present in the secretory compartment of islets were also detected in ES-derived cells, such as cathepsin B [Ctsb; (Kuliawat *et al.*, 1997)], cathepsin F [Ctsf; (Gu *et al.*, 2004)] and carboxypeptidase E [Cpe; (Marzban *et al.*, 2005)]. The metabolic enzyme Cpe is involved in processing of pro-islet amyloid polypeptide in beta cells (Marzban *et al.*, 2005), whereas Ctsb is involved in proteolytic cleavage within secretory granules (Conlon *et al.*, 1995). The presence of cathepsins and carboxypeptidase suggests that mechanisms involved in insulin and IAPP processing *in vivo* may play a role in ES-derived pancreatic progenitors.

Metal-binding metallothioneins (*Mt*)^{1,2} are expressed in acinar, ductal (De Lisle *et al.*, 1996) and beta (Chen *et al.*, 2001) cells. Remarkably, *Mt*^{1,2,3} transcripts were up-regulated in Pax4 undifferentiated ES cells, and *Mt*^{1,2} transcripts were increased in committed progenitors, but specifically in cells at the advanced stage (whereas the neural-specific *Mt*³ was detected at lower level in Pax4+ cells). In the pancreas, metallothioneins are present in acinar cells, pancreatic ducts (De Lisle *et al.*, 1996) and beta cells (Chen *et al.*, 2001) and are supposed to play a role in zinc homeostasis and protection against reactive oxygen species [ROS; (Chen *et al.*, 2001)]. The up-regulation of metallothioneins and their close localization around C-peptide-positive clusters (see Fig. 7 D-F) suggests a role in support of beta-cell-specific functions and/or a role in (ROS-dependent?) stress-response.

The significant up-regulation of the intermediate filament protein GFAP, a characteristic cytoskeletal protein of astrocytes [rev. (Eng *et al.*, 2000)] would suggest that ES-derived cells at the advanced stage are GFAP-positive astrocytic cells and partially represent neural cell types (see below). However, this population may also represent a type of stellate-like cells present in (adult) pancreas and (fetal) liver. Stellate cells are expressed in response to tissue damage (Bachem *et al.*, 1998) and are positive

for mesenchymal and neural markers.

Neural-specific transcripts were up-regulated in wt cells, but at lower level in Pax4+ cells

The microarray analysis demonstrated a significant up-regulation of transcripts involved in neurogenesis and neuronal cell function including the zinc finger protein of the cerebellum1 (*Zic1*), neuronal PAS domain protein3 (*Npas3*), *Nnat*, pleiotrophin (*Ptn*), endothelin receptor type B (*Ednrb*), neuropilin2 (*Nlgn2*), *Nrp1* and *Gfap* (see Tab. 2). However, only 11 neural-specific transcripts were up-regulated in Pax4+ cells, instead of 17 in wt cells, suggesting that Pax4+ cells are more committed to a pancreatic fate than wt cells.

The expression of neural-specific developmental control genes during pancreas development (i.e. *Isl1*, *ngn3*, *NeuroD1*, *Pax6*) is relevant for ES cells differentiating into pancreatic and neural lineages (Roche *et al.*, 2005; Rolletschek *et al.*, 2006; Soria, 2001). The neural stem/progenitor cell-specific protein nestin was also detected during *in vitro* differentiation of ES cells [i.e. (Lumelsky *et al.*, 2001); see (Blyszczuk *et al.*, 2003; Blyszczuk *et al.*, 2004)] and islets (Zulewski *et al.*, 2001), but selection of ES-derived nestin-positive cells was not efficient in generating islet-like cells (Hansson *et al.*, 2004; Kania *et al.*, 2004; Rajagopal *et al.*, 2003). Whereas *in vivo* development assures gene expression in a spatially and temporally regulated manner, during *in vitro* differentiation of ES cells, cell types of all somatic lineages (= multi-lineage progenitors) are present in the EB outgrowths. This could explain the insufficient pancreatic differentiation of ES cells via neural cell types [see (Hansson *et al.*, 2004; Sipione *et al.*, 2004)] and requires the application of specific selective differentiation methods as recently presented by D'Amour *et al.* (2006) and Kroon *et al.* (2008).

Lineage tracing studies demonstrated, however that neural progenitor cells may have the capacity to differentiate into both neural and pancreatic lineages (Seaberg *et al.*, 2004) and would suggest the idea of a pancreatic progenitor cell type defined by both neural (ectoderm) and pancreas (endoderm)-specific properties.

Pax4+ cells revealed some diversity in comparison to wt cells and showed signs of apoptosis at the advanced stage

The present data revealed differences between the transcriptomes of wt and Pax4+ that were up-regulated from ES cells to the committed stage. For example, in many subgroups (transcriptional regulation; signalling/growth factors; adhesion, ECM; organogenesis) a higher number of transcripts were up-regulated in Pax4+ compared to wt cells (see Fig. 1). However, the verification of transcriptome data by qPCR revealed only *Hoxb2*, *Ptn* and *Mt*^{1,2} (genes involved in pancreas development/function) as up-regulated transcripts in Pax4+ cells, whereas generally, more up-regulated transcripts were found in wt cells. Reasons for these dissimilarities may be: (i) Pax4+ cells expressed less neuronal transcript numbers (see Table 2), (ii) transcripts had a P value >10⁻³ in Pax4+ cells [i.e. *Chgb* (p=0.003), *Ttr* (p=0.02), *Scgb* (p=0.002), *Itm2a* (p=0.0012)], and therefore, did not fulfill the cutoff value of statistical analysis, and (iii) Transcripts were less than five-fold up-regulated, e.g. in wt cells *Vldlr* (4.7 fold up), *Cln4-2* (4.2 fold up).

However, our study also clearly demonstrated a relatively low

number of up-regulated transcripts from the committed to the advanced stage. We may speculate about fate-restricted conditions of cells at the advanced stage, specifically in Pax4+ cells (see Fig. 1), whereas cells at 5+16d represent a progenitor cell population. Specifically, Pax4+ cells showed no signs of higher maturation, but rather signs of apoptosis, such as transcript up-regulation of the Nurr77 downstream gene 2 [*Ndg2*; (Rajpal *et al.*, 2003)], cytochrome c testes (*Cyc*) and apoptotic phenotypes (see Fig. 4 G-M). As demonstrated by Brun and coworkers, Pax4 over-expression in beta-cells caused an increase of proliferation paralleled by increased Bcl-xL (anti-apoptotic), c-myc and Id2 mRNA levels, while Bcl-xL activity resulted in altered mitochondrial calcium level and impaired glucose-induced insulin release (Brun *et al.*, 2004). In our studies, Pax4+-derived cells showed signs of metabolic starvation at the advanced stage presumably because of higher proliferation of the cells [see (Blyszczuk *et al.*, 2003; Blyszczuk *et al.*, 2004), although daily medium changes were performed during advanced cultivation stages.

Comparison of transcriptomes of ES-derived cells and of tissue samples suggest an embryonic/ fetal developmental stage

The comparison of expression levels of *in vitro* differentiated ES-derived cells with transcript levels of fetal and adult pancreas revealed that many genes, such as *Foxa1*, *Hoxb2*, *Ttr*, *Igf1*, *Irs1*, *Chgb*, cathepsin B (*Ctsb*) and F (*Ctsf*), carboxypeptidase E (*Cpe*) and *Mt1* and *2* were expressed at levels comparable to levels of the fetal pancreas. The comparison of our data with a transcriptome analysis of cells isolated from pancreas during development (Gu *et al.*, 2004) revealed partial overlapping gene functions. Comparable to the group of genes enriched in E7.5 endoderm *in vivo*, we detected *Igf2*, *Igf1*, *Nrp1*, *Foxa1*, *Pem*, *Col4a1*; within the group of Pdx1-positive E10.5 pancreatic progenitor cells *Dlk1* and *Chgb*; and in the group of E13.5 Ngn3-positive endocrine progenitors *Chgb*, *Cpe* and *Ctsf*.

Although we have to consider that in the study by Gu *et al.* (2004) other microarray chips have been used, our data suggest that ES-derived cells after *in vitro* differentiation show some endoderm- and pancreas-specific markers, but do not represent mature pancreatic cells.

While ES-derived cells differentiating via multi-lineage progenitors (our study) attained partial functional competence *in vivo* after transplantation into diabetic mice as shown by Blyszczuk *et al.* (2004) and Boyd *et al.* (2008), the present data as well as recent studies (Kroon *et al.*, 2008) led us to conclude that ES-derived cells are unable *in vitro* to pass through terminal differentiation stages into functional islet-like cells. The formation of glucose-responsive insulin-producing cells was only shown to proceed *in vivo*, if pancreatic endocrine progenitor cells were transplanted (Kroon *et al.*, 2008). Obviously, to differentiate into functional beta-cells, endocrine and pancreatic progenitors need additional (not yet identified) factors that are not available for *in vitro* differentiation.

Material and Methods

Cell culture and differentiation

ES cells of line R1 [wt, (Nagy *et al.*, 1993)] and R1 ES cells constitutively expressing Pax4 [Pax4+, (Blyszczuk *et al.*, 2003)] were cultured on

embryonic fibroblast feeder layers in Dulbecco's modified Eagle's medium and supplements as described (Blyszczuk *et al.*, 2004; Wobus *et al.*, 2002). The cells were passaged at least three times without feeders before use in the experiments to minimize the feeder cell content. The three-step protocol of pancreatic differentiation (Suppl. Fig. S1) included (i) the formation of embryoid bodies (EBs), (ii) after EB plating on adhesive substrates the expansion and spontaneous differentiation to form multi-lineage progenitors and (iii) after dissociation of progenitor cells, differentiation induction into the pancreatic lineage and formation of islet-like clusters (Blyszczuk *et al.*, 2004; Schroeder *et al.*, 2006).

In short, Pax4+ and wt ES cells (n=600 cells/20 μ l) were plated on the lids of Petri dishes (\varnothing 10 cm), cultured as 'hanging drops' for 2 days and on bacteriological plates (\varnothing 6 cm) in suspension for 3 days to form EBs. At day 5, EBs were plated in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Karlsruhe, Germany) supplemented with 20% FCS (selected batches, Invitrogen), 2 mM L-glutamine (Invitrogen), non-essential amino acids (1:100 of stock solution, Invitrogen), α -monothioglycerol (final concentration 450 μ M; Sigma, Steinheim, Germany) and penicillin/streptomycin (1:100 of stock solution, Invitrogen) (differentiation medium I). For spontaneous differentiation into multi-lineage progenitors, the EB outgrowths were cultured for another 9 days in differentiation medium I. At day 5+9, cells were dissociated by 0.1% trypsin (Invitrogen): 0.08% EDTA (Sigma) in PBS (1:1) for 1 min, collected by centrifugation and replated onto poly-L-ornithine/laminin-coated tissue culture dishes in 'N2 medium + NA' containing DMEM/F12 (Invitrogen) supplemented by 20 nM progesterone, 100 μ M putrescine, 1 μ g/ml laminin, 25 μ g/ml insulin, 30 nM sodium selenite, 10 mM nicotinamide (NA) (all from Sigma), 50 μ g/ml transferrin, B27 media supplement (dilution 1:50) (all from Invitrogen) and 15% FCS (= differentiation medium II). At day 5+10, the medium was replenished and cells were cultivated in differentiation medium II without FCS up to the stage 5+16d (stage of committed progenitors) and 5+28d (islet-like clusters) for analysis [see (Blyszczuk *et al.*, 2004; Schroeder *et al.*, 2006)].

cRNA probe generation and hybridization to Affymetrix microarray chips

Total RNA of Pax4+ and wt cells from five independent experiments was isolated from undifferentiated ES cells (0d), multi-lineage progenitor cells (5+9d; only wt cells), committed progenitor cells (5+16d) and cells at the terminal differentiation stage (5+28d), respectively. The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions with on-column DNase treatment (RNase-free DNase Set; Qiagen) for 60 min to avoid genomic DNA contamination. RNA quality was measured as a function of the ratio of absorbance at 260 and 280 nm. Furthermore, all RNA samples were analysed using agarose gel electrophoresis and stained to check for integrity of 18S and 28S RNA. Biotinylated cRNA was prepared according to the standard Affymetrix protocol (Expression Analysis Technical Manual, 1999; Affymetrix, Santa Clara, USA). Double-stranded cDNA was synthesized from 10 μ g total RNA using the SuperScript Choice System (Invitrogen) and the T7-(dT)24 primer (Affymetrix). Following a phenol/chloroform extraction and ethanol precipitation, the cDNA was transcribed into biotin-labelled cRNA using the Retic Lysate IVT™ kit (Ambion Inc., Austin, TX, USA). The cRNA product was purified using the RNeasy kit (Qiagen) and fragmented to an average size of 30-50 bases according to Affymetrix recommendations. Fifteen micrograms of fragmented cRNA were hybridized for 16 hr at 45°C on the Mouse Genome 430 2.0 Array. The arrays were washed and stained in the Affymetrix Fluidics Station 450 and further scanned using the GeneChip® Scanner 3000 (Affymetrix).

Data normalization and expression profiling

Quality control was performed by using the GCOS 2 software package, its noise and background estimation and the 3' to 5' expression ratios of the housekeeping genes GAPDH and β -actin. After passing the quality control a set of robust multi-array analysis (RMA) normalized expression

values was produced using RMAexpress 0.2 (Bolstad *et al.*, 2003). An outlier in the sets of five biological replicates per condition was detected and removed applying the Nalimov test at $P < 10^{-3}$. ANOVA were performed to detect general expression differences dependent on differentiation stages. Expression profiles were examined by hierarchical cluster analysis using Cluster version 2.11 (Eisen *et al.*, 1998) applying mean-centring and normalization of genes and arrays before average linkage clustering with un-centred correlation. A Student's *t*-test (unpaired, assuming unequal variances) P -value $< 10^{-3}$ and a fold change > 2 between two conditions was applied to define differential expression. In the case of multiple gene to probe set assignment (redundancy), the probe set with the highest maximum signal value over all tested arrays was selected. An intersection of up-regulation in committed progenitors (5+16d) compared with undifferentiated ES cells (0d) (five-fold, *t*-test P -value $< 10^{-3}$) and compared with cells at the terminal differentiation stage (5+28d) (two-fold, *t*-test P -value $< 10^{-3}$) was used to identify up-regulated transcripts at advanced stages of pancreatic differentiation.

Quantitative (q) RT-PCR analysis

Total RNA of a separate set of experiments ($n=3$) was isolated by the RNeasy Mini Kit method as described above, and reverse transcribed using previously published protocols (Schroeder *et al.*, 2006; Wobus *et al.*, 2002). In addition, total RNA was prepared from fetal (E16 d p.c.) and adult (2 months age) tissue of liver, pancreas and brain. Changes in mRNA levels of specific genes were quantified using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany). A set of specific oligonucleotide primers (Suppl. Tab. S1) was designed using Primer Express software 2.0 (Applied Biosystems). Briefly, amplification of the target genes was monitored via SYBRTM green fluorescence signals generated during each PCR cycle. Fluorescence signals were regarded positive if the fluorescence intensity reached 10x the standard deviation of the baseline fluorescence (threshold). The amplification mix was prepared following the SYBR green PCR master mix (Applied Biosystems, Warrington, UK) protocol. Primers were used in a concentration of 100 nM. Each reaction was performed in triplicate in 96-well optical reaction plates under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension). Relative mRNA levels were calculated using the standard curve method (see User Bulletin #2; Applied Biosystems).

Immunohistochemistry and immunofluorescence analysis

Whole pancreas tissue of eight weeks old mice were fixed in 4% paraformaldehyde (PFA) in PBS overnight. After fixation, samples were dehydrated in graded alcohol for 2 hr each (70%, 80%, and 96% ethanol, isopropanol, and xylene) and embedded in hot paraffin (60°C). Pancreas were sectioned in 5µm strength using a microtome (Medim, Zug, Switzerland) and mounted on silanized slides. For immunofluorescence, the sections were de-paraffinized overnight at 60°C and rehydrated through a series of graded alcohols.

Tissue sections, ES cells, and EB-derived cells growing on cover slips were fixed by 4% PFA or 0.1% glutaraldehyde in 4% PFA at room temperature (RT) for 20 min, respectively, and processed for immunofluorescence analysis as described (Schroeder *et al.*, 2006). Hoechst 33342 (5 µg/ml, RT, 10 min) was used to label the nuclei. The following primary antibodies (dilution 1:100) were used: Mouse anti-insulin IgG (clone K36AC10, Sigma-Aldrich, Hamburg, Germany), sheep anti-C-peptide, rabbit anti-chromogranin B (both from Acris, Hiddenhausen, Germany), goat anti-neuronatin (S-14, Santa Cruz Biotechnology, Heidelberg, Germany), sheep anti-prealbumin (= transthyretin, Biotest, Saco, ME, USA), rabbit anti-Foxa1 (Chemicon, Hampshire, UK), mouse anti- α fetoprotein, mouse anti-metallothionein (Mt) IgG (specific for Mt subunits 1,2; Invitrogen) and rabbit anti-albumin (Abcam, Cambridge, UK).

The following secondary antibodies were used according to the manufacturers' recommendations: Cy3 anti-mouse IgG, Cy3 anti-rabbit, Cy3 anti-goat (all from Jackson ImmunoResearch Laboratories, West

Grove, PA, USA), Alexa 488 anti-mouse IgG, Alexa 488 anti-sheep, Alexa 488 anti-rabbit (all from Molecular Probes, Karlsruhe, Germany). Samples were analysed by the fluorescence microscope ECLIPSE TE300 (Nikon, Japan) and the confocal laser scanning microscope LSM 510 META (Carl Zeiss, Jena, Germany).

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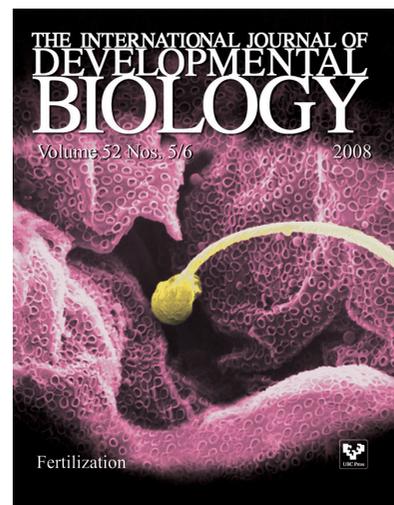
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