Diabetologia (2006) 49:2541–2545
DOI 10.1007/s00125-006-0441-y

FOR DEBATE

Generation of pancreatic insulin-producing cells from embryonic stem cells — 'Proof of principle', but questions still unanswered

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Received: 11 November 2005 / Accepted: 28 August 2006 / Published online: 20 September 2006

Keywords Embryonic stem cells · Insulin-producing cells

Abbreviations

E	embryonal day
EB	embryoid body
EMT	epithelial–mesenchymal transition
ES	embryonic stem

The generation of insulin-producing cells from differentiating mouse embryonic stem (ES) cells was described some years ago [1], but subsequent studies could not replicate the results. Instead, using the same differentiation protocol, it was found that insulin immunoreactivity occurred as a consequence of insulin uptake from the medium [2], neuronal cells were formed [2–4], or insulin was released as an artefact from differentiated ES cells [2, 3]. Functional pancreatic cells, however, were successfully generated using lineage selection strategies based on pancreas-specific promoters [5, 6], by modified protocols in combination with transgene expression [7–9], or by addition of a phosphoinositol-3 kinase inhibitor [10]. The differentiated cells showed properties of (neonatal) beta cells, such as insulin transcripts and C-peptide/insulin co-expression, insulin-secretory granules, ion channel activity of embryonal beta cells, and normalisation of blood glucose level after transplantation into diabetic mice [5–10]. Most of the differentiation protocols required a long cultivation period, including 4–5 days of embryoid body (EB) formation, followed by 3–4 weeks of differentiation, and some protocols required genetic manipulation. Recently, a relatively short procedure of pancreatic differentiation by a three-step experimental approach was published [11]. The strategy is based on the combined treatment by activin A, all-*trans*-retinoic acid, and other factors such as basic fibroblast growth factor (bFGF), which induced murine ES cells to differentiate into insulin-producing cells within 2 weeks. The authors presented results on insulin transcripts, C-peptide/insulin co-expression, glucose-induced insulin release, and the normalisation of glycaemia following transplantation into diabetic mice.

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Neuronal vs pancreatic differentiation in vivo and of ES cells in vitro

On looking more closely at the C-peptide-positive cells differentiated according to the protocol of Shi et al. [11], it became obvious that both pancreatic and neuronal differentiation had been induced. In part, the clusters showed the typical morphology of grape-like structures, but also neuronal cell types (Fig. 1a). Immunohistochemical stainings revealed varying levels of co-expression of C-peptide, a byproduct of pro-insulin synthesis (used as a marker for insulin-producing cells), and the neuron-specific beta-III tubulin (Fig. 1b–g). Such ectoderm-derived insulin-producing (C-peptide-positive) cells have been generated from ES cells via EB formation, as well as from monolayer cultures

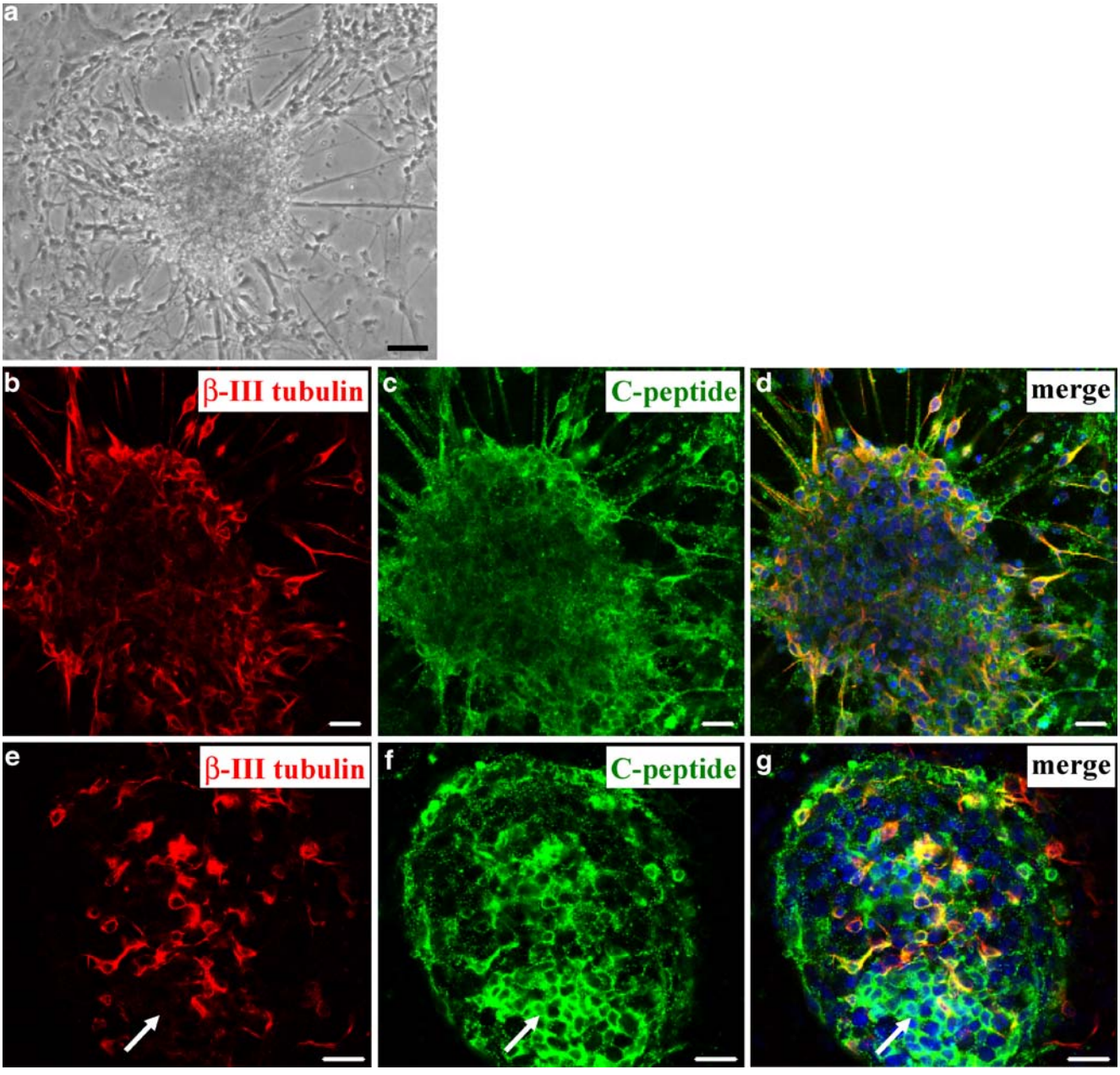

Fig. 1 Morphological and immunocytochemical characterisation of ES (R1) cell-derived derivatives following pancreatic differentiation, according to Shi et al. [11]. **a** Cluster morphology (phase contrast) after pancreatic differentiation including treatment with activin A and retinoic acid at day 2+12. Co-expression of beta-III tubulin (**b**, **e**, *red*) with C-peptide (**c**, **f**, *green*) appears yellow (**d**, **g**). *Arrows* indicate C-peptide-positive cells of the cluster without beta-III tubulin staining representing potential pancreatic insulin-producing cells. For immunolabelling see [40] and [8]. Bar=50 μm (**a**), 20 μm (**b**–**g**)

following exposure to pancreatic differentiation factors (see [12]).

Similarities between pancreatic beta cells and neurons have long been known (Table 1). During embryogenesis in vivo, the same transcription factors regulate both neuroectoderm and pancreatic differentiation processes in a spatially and temporally controlled manner. During pancreatic differentiation, *neurog3* mRNA has been detected in pancreatic buds as early as embryonic day (E) 9.5 [13] and the neurog3 protein has been found at E11.5 in epithelial cells of the pancreatic buds [14]. Similar expression patterns were described for *Pax6* and *Isl1* ([13], for review see [15]). In parallel, during neuronal and glial cell development, *Isl1*, *Pax6*, and *neurog3* mRNA and proteins could be detected as early as E10.5, when neurogenesis begins ([16, 17], see Table 1). It has also been found that the intermediate filament protein nestin—suggested as marker of neural stem or progenitor cells—is transiently

Table 1 Major similarities between pancreatic beta cells and neuronal cells

Category	Name	References
Transcription factors	Isl1	[16, 41]
	Neurog3	[13, 17]
	Pax6	[42]
	Pax4	[43]
Proteins of the secretory pathways	Chromogranin B, Synaptophysin	[44–46]
Metabolic enzymes	Tyrosine hydroxylase	[47, 48]
Excitation	K_{ATP} channel, voltage-dependent L-type Ca^{2+} channel	[49]
Gap junction protein	Connexin-36	[50, 51]
Transient formation of intermediate filament proteins in vitro	Nestin	[1, 8, 20, 52]

produced during neuronal [18] and pancreatic [1, 7–9, 19] differentiation of ES cells, respectively (see [19–22]).

In contrast to embryogenesis, pancreas-specific transcription factors are not expressed in a spatially controlled pattern during ES cell differentiation in vitro. As a consequence, exogenously applied activin A would induce ES cells to differentiate in parallel into pancreatic beta-like cells and into neuronal phenotypes (see Fig. 1). Previous studies have shown that activin A induces the formation of neuronal extensions and neurofilament proteins in PC12 cells [23] and enhances the activity of neuron-specific L-type Ca^{2+} channels in neuroblastoma cells [24], suggesting the involvement of activin A in neural differentiation. Consequently, activin A treatment in ES-derived EBs containing a population of multi-lineage progenitor cells will induce neuroectodermal progenitors into neural phenotypes, and endoderm progenitors into pancreatic phenotypes. A more sophisticated application schedule of activin A treatment and serum removal is clearly necessary to enrich endoderm progenitor cells, as recently presented by D'Amour et al. [25]. Neuronal cells, however, were not analysed in this study.

Major differences between endoderm- and ectoderm-derived insulin-producing cells are based on different functions of insulin. In ectodermal cells, insulin acts as a growth factor, whereas in endoderm-derived pancreatic tissues, insulin is involved in hormonal regulation of nutrient homeostasis. Due to these different functions, two main problems arise with the generation of ectoderm-derived insulin-producing cells in tissue culture systems: (1) the level of insulin release is much lower than that of pancreatic beta cells; and (2) release is not regulated by high glucose levels. Glucose-responsive insulin-producing cells generated from brain-derived human neural progenitor cells released 0.5–1% of the C-peptide level secreted by beta cells of the pancreas [26]. Application of such cell populations for transplantation purposes remains questionable and insufficient.

Role of nestin in epithelial–mesenchymal transitions during in vitro differentiation?

Another aspect important for the development of pancreatic cells is epithelial–mesenchymal transition (EMT) [27, 28]. Pancreas development and, in particular, exocrine differentiation, are critically dependent on the presence of factors secreted from mesenchyme [29–31]. Follistatin and fibroblast growth factors represent some of the key mesenchymal factors that actively promote pancreatic development [32–34]. In addition, in the endocrine compartment of the developing pancreas, glucagon and glucagon-like peptide 1-producing alpha cells play an important role in the differentiation of early insulin-producing cells [35]. All these signalling factors have specific functions in pancreas development, but will also be important for pancreatic differentiation of ES cells [36]. Moreover, soluble factors of the blood vessel endothelium within the islets of Langerhans are known to induce endocrine pancreas development [37], and the participation of the vascular endothelium is necessary to regulate hormonal interactions between cells of the islets. It has been postulated that it is not only epithelial–mesenchymal and intra-epithelial interactions, but also endothelial–mesenchymal transitions that may play a role in proper pancreas development [21, 31].

There has been speculation that nestin expression/re-expression is involved in these processes [20]. Following in vitro culture, nestin and vimentin are formed as a consequence of EMT-dedifferentiation into 'stem-like' mesenchymal cells (see [21]). This would imply that nestin cannot be regarded as a tissue-specific marker, but plays a general role in maintaining stem/progenitor cell properties by unequal partitioning of cytoplasmic components during cell division (see [38]). The finding that nestin-positive cells are generally formed in early differentiated ES cell derivatives in vitro [20], however, requires culture conditions that do not lead to the differentiation induction of neuronal cell types.

In summary, whereas present studies show that ES-derived pancreatic cells produce and release insulin in response to high glucose concentrations in functional assays, the differentiated cell clusters do not currently represent islets (see [39]). Neuronal cells are present, whereas endothelial cells are not integrated into islet-like clusters. As a next step, it will be necessary not only to establish efficient ES cell differentiation strategies to increase the yield of insulin expression, synthesis and release, but also to avoid the presence of artificially developed cell types in the islet-like clusters.

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