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Special Contribution

The generation of insulin-producing cells from embryonic stem cells - a discussion of controversial findings

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ABSTRACT The derivation of insulin-producing cells from embryonic stem (ES) cells has been controversially described. Whereas several authors showed successful differentiation of mouse ES cells into islet-like clusters, others could not confirm the results. Here, we present a detailed comparison of the various strategies used to generate pancreatic cells with respect to protocols and differentiation factors and give an explanation of the contradictory findings. It is suggested that the selection or enrichment of ES-derived nestin-positive cells should be avoided, since these cells are already committed to a neural fate before pancreatic differentiation is induced.

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

Introduction

The generation of insulin-producing cells from differentiated embryonic stem (ES) cells by a four-step protocol was described some years ago (Lumelsky *et al.*, 2001), but subsequent studies could not confirm these results. Instead, it was demonstrated that: (i) insulin immunoreactivity could occur as a consequence of insulin uptake from the medium (Rajagopal *et al.*, 2003), (ii) neuronal cells could be formed (Rajagopal *et al.*, 2003; Hansson *et al.*, 2004; Sipione *et al.*, 2004), or (iii) insulin could be released artifactually from differentiated ES cells (Rajagopal *et al.*, 2003; Hansson *et al.*, 2004). Other authors, using modified protocols with or without *Pax4* transgene expression (Blyszczuk *et al.*, 2003; Blyszczuk *et al.*, 2004) or addition of a specific PI3K inhibitor (Hori *et al.*, 2002) reported the generation of pancreatic cells characterized by glucose-responsive insulin release and some functional properties of pancreatic cells. However, until now, there has been no explanation of these contradictory findings and a critical discussion of the pancreatic differentiation protocols is lacking.

Comparison of the differentiation systems

In Protocols 1 to 4 (Fig. 1), embryoid body (EB) outgrowths were cultured in medium supplemented by insulin, transferrin, selenium and fibronectin (= ITSFn). Culture in ITSFn medium was originally developed to enrich nestin-positive cells before induction into the neuronal lineage (Okabe *et al.*, 1996). In Protocols 1 to 3, proliferation of nestin-positive cells was supported by addition of bFGF (= FGF-2), but after the induction of pancreatic differentiation the FGF-2 was removed. In Protocol 2 (Miyazaki *et al.*, 2004), pancreatic differentiation had already been induced at stage 4 (with additional factors), in contrast to Protocols 1 and 3 (see Fig. 1).

In Protocols 4 (Blyszczuk *et al.*, 2003) and 5 (Blyszczuk *et al.*, 2004), the culture medium was not supplemented with FGF-2 and the differentiation time was extended to between 28 and 32 days. These cells showed insulin transcripts and C-peptide/insulin co-expression (Blyszczuk and Wobus, 2004; Blyszczuk *et al.*, 2004). ES cells constitutively expressing *Pax4* showed insulin-secretory granules (Blyszczuk *et al.*, 2003), ion channel activity of embryonal beta cells and normalization of blood glucose level after transplantation into diabetic mice (Blyszczuk *et al.*, 2004). These properties were not observed in cells which differentiated according to Protocols 1 and 3 (Fig. 1). This led us to question the role of FGF-2 in pancreatic differentiation of ES cells *in vitro.*

Pancreatic *vs.* neural differentiation

Following differentiation according to Protocols 1, 2 and 3, both pancreatic and neuronal cells were generated from ES cells (Lumelsky *et al.*, 2001; Hori *et al.*, 2002; Sipione *et al.*, 2004; Rajagopal *et al.*, 2003; Hansson *et al.*, 2004; Miyazaki *et al.*, 2004). As mentioned above, culture in

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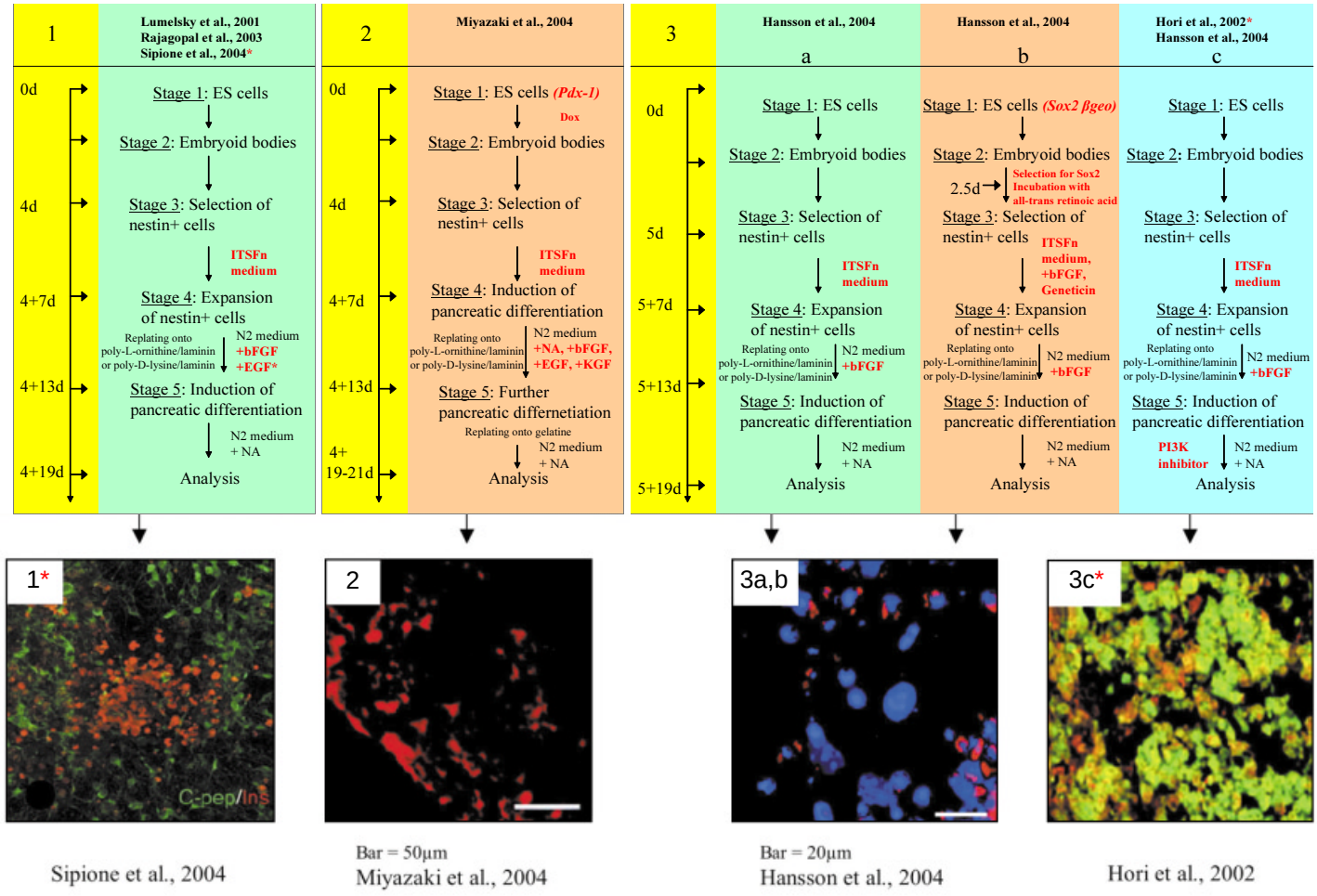

ITSFn- and FGF-2-supplemented medium induced commitment of cells to a neuronal fate. Also, the culture of early ES-derived cells, with a high concentration of all-trans retinoic acid (RA, 10^{-6} mol, Protocol 3b), definitely promotes *neuronal* differentiation of ES cells (Wobus *et al.*, 1994), whereas constitutive expression of Sox2 inhibited neuronal differentiation, but maintained properties of neural progenitors (Graham *et al.*, 2003). When cultured in the presence of FGF-2, also cells expressing the pancreatic developmental control gene *pdx-1* differentiated into the neuronal lineage (Miyazaki *et al.*, 2004).

There are several indications of a close relationship between neural and pancreatic cell types. It is well known that the transcription factors Isl-1, Ngn3, Pax6 and Pax4, neuropeptide-processing enzymes and glucose transporters are expressed in both cultured neural and endocrine pancreatic cells (Edlund, 1998; Edlund, 2001). Recently, multipotent precursor cells have been clonally isolated from adult pancreatic tissue and, when induced to differentiate, the cells developed into neural and pancreatic cell types, including neuronal and glial cells, pancreatic endocrine beta-, alpha- and delta-cells and pancreatic exocrine and stellate cells (Seaberg *et al.*, 2004). These findings suggest a close relationship of neural and pancreatic cells. We therefore speculate that the addition of ITSFn and FGF-2 before induction of pancreatic differentiation would induce commitment of cells to a neuronal fate. However, once the cells have become committed to a neuronal fate, the procedure for induction of pancreatic differentiation eventually would activate apoptotic pathways.

FGF-2 is known to play a critical role in development *in vivo* (e.g. Joseph-Silverstein *et al.*, 1989; Kalcheim and Neufeld, 1990). *In vitro*, FGF-2 supports the proliferation and differentiation of brain-derived neural progenitor cells (Gritti *et al.*, 1995). FGF-2 is used to form neurospheres from adult neural stem cells (Reynolds and Weiss, 1992) and to generate nestin-positive cells from ES cells (Okabe *et al.*, 1996). A recent study shows that neural stem cells cultured as neurospheres in the presence of FGF-2 lose their original fate specification, resulting in modified transcript levels of regulatory genes (Hack *et al.*, 2004).

During pancreatic development, the prepancreatic endoderm is sensitive to FGF-2 concentration (Deutsch *et al.*, 2001). Whereas higher doses elicit a hepatic program, lower doses or lack of FGF-2 allow pancreatic differentiation (Kim and MacDonald, 2002). This suggests that local concentrations of FGF-2 determine the choice between a hepatic or a pancreatic fate (Rossi *et al.*, 2001) or more

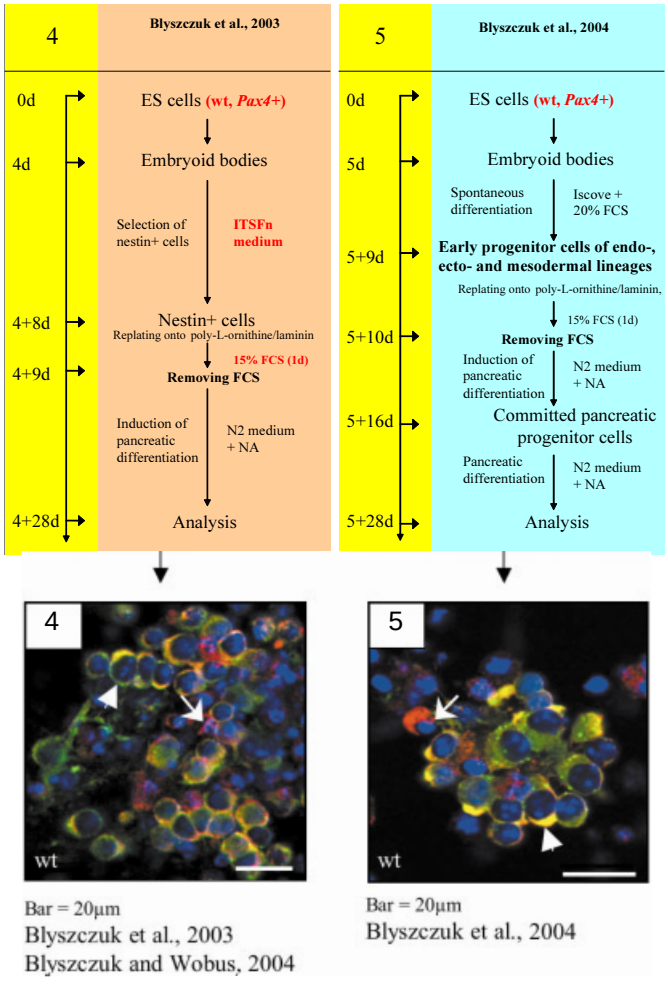

Fig. 1. Comparison of Protocols 1 to 5 used for the differentiation of ES cells into insulin-producing islet-like clusters. *Images shown below show immunofluorescence labeling of insulin or C-peptide /insulin according to the following protocols:* **(Protocol 1)** *Based on Lumelski* et al. *(2001). No coexpression of C-peptide (green) with insulin (red) was found; instead, there are distinct cell populations positive either for C-peptide or for insulin (see Sipione et al., 2004).* **(Protocol 2)** *Based on Miyazaki* et al. *(2004). After pancreatic differentiation at stage 5, C-peptide-positive clusters (red) were detected, but no double labeling with insulin was obtained (Dox, doxycycline).* **(Protocol 3a,b)** *Based on Hansson* et al. *(2004). No C-peptide staining was found and insulin staining (red) was restricted to apoptotic cells.* **(Protocol 3c)** *Based on Hori* et al. *(2002). C-peptide (green)/insulin (red) co-expression (yellow) was found after the addition of the PI3K inhibitor to the differentiation medium, contrary to Hansson* et al. *(2004).* **(Protocol 4)** *Based on Blyszczuk* et al. *(2003). Co-expression of C-peptide (green) with insulin (red) was observed. Arrowheads indicate C-peptide/insulin co-expression (yellow), whereas arrows indicate insulin-positive and C-peptide-negative cells (red) with small and condensed nuclei, suggesting apoptosis. These cells may represent those that take up insulin from the medium (see Blyszczuk and Wobus, 2004).* **(Protocol 5)** *Based on Blyszczuk* et al., *(2004). Coexpression of C-peptide (green) with insulin (red) was demonstrated. Arrowheads indicate C-peptide/insulin co-expression (yellow), whereas arrows indicate insulin-positive and C-peptide-negative cells (red). These cells may represent apoptotic cells, but the number of apoptotic cells in the islet-like structures was reduced compared with Protocol 4.*

generally, that different progenitor cells respond differently to FGF-2.

Protocol 5 was designed to avoid the use of ITSFn and FGF-2, so that selection or enrichment of specific cell types before induction of pancreatic differentiation would not occur. ES cells spontaneously differentiated via EBs into multi-lineage progenitor cells and following induction of pancreatic differentiation by serum-free medium containing niacinamide and laminin, functional beta-like cells were generated (Fig. 1, Protocol 5; Table 1; Blyszczuk *et al.*, 2004).

Insulin uptake and induction of apoptosis

The selection of nestin-positive cells according to Protocol 1 resulted in the activation of apoptotic pathways (Rajagopal *et al.*, 2003; Miyazaki *et al.*, 2004) and the ES-derived cells were C-peptide-negative and showed artifactual insulin release (Rajagopal *et al.*, 2003; Hansson *et al.*, 2004, see Fig. 1, Protocol 3a). C-peptide labeling of cells is an indication of proinsulin synthesis and can be demonstrated in cells that differentiate without FGF-2 according to Protocol 4 (Blyszczuk and Wobus, 2004). Only 10

TABLE 1

COMPARISON OF DIFFERENT PROTOCOLS AND PARAMETERS OF PANCREATIC DIFFERENTIATION OF MOUSE ES-DERIVED CELLS

Differentiation Protocol (see Fig. 1)	References	Insulin mRNA	C-peptide /insulin co-expression	*In vitro* glucose response	*In vitro* C-peptide secretion	Rescue of diabetes in animal models	Electrophysiological studies	ELM studies (insulin granules)
Protocol 1	(Lumelsky *et al.*, 2001)	+	n.d.	+	n.d.	-	n.d.	n.d.
	(Rajagopal *et al.*, 2003)	-	-	-	n.d.	n.d.	n.d.	n.d.
	(Sipione *et al.*, 2004)	+	+ different cell populations were stained	+	n.d.	-	n.d.	-
Protocol 2	(Miyazaki *et al.*, 2004)	+	+ (C-peptide single staining)	-	n.d.	n.d.	n.d.	-
Protocol 3 a,b,c	(Hansson *et al.*, 2004)	-	-	+	-	n.d.	n.d.	n.d.
Protocol 3c	(Hori *et al.*, 2002)	+	+	+	n.d.	+	n.d.	n.d.
Protocol 4	(Blyszczuk *et al.*, 2003)	+	+	+	n.d.	+	n.d.	+
Protocol 5	(Blyszczuk *et al.*, 2004)	+	+	+	n.d.	+	+	n.d.

n.d., not done

to 15% of the insulin-positive cells were not labelled by C-peptide (see Fig. 1, Protocol 4, arrow) and the cells showed small fragmented nuclei. When selective factors (such as ITSFn) were omitted in Protocol 5, the number of such potential apoptotic cells could be reduced further (see Fig. 1, Protocol 5).

We conclude that the generation of ES-derived functional insulin-producing cells without applying lineage-selection (see Soria *et al.*, 2000; Leon-Quinto *et al.*, 2004) is dependent on suitable differentiation protocols and differentiation factors. We therefore propose that preselection of ES-derived undefined progenitor cells by ITSFn and FGF-2 before induction of pancreatic differentiation should be omitted.

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