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Biochemical and Biophysical Research Communications 352 (2007) 84–90
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Neonatal pancreatic cells redifferentiate into both neural and pancreatic lineages

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Received 25 October 2006
Available online 10 November 2006

Abstract

Studies of islet neogenesis have suggested that the regeneration of islet cells can be achieved through redifferentiation of pre-existing islet cells. However, this hypothesis is largely unproven and fails to account for the diversity of observed islet neogenesis. Here we show that cultured neonatal pancreatic cells dedifferentiate into βIII tubulin-expressing precursors, which then expand and redifferentiate into both neural and pancreatic lineage progenies. Redifferentiation happens not only in the islet cells, but also in the ductal cells that may represent what are called ductal origin "pancreatic stem cells". The *in vitro* redifferentiation of neonatal pancreatic cells recapitulates the embryonic development by sequential endocrine differentiation accompanied by the coexpression of neuronal marker βIII tubulin with endocrine hormones until terminal differentiation. The neuronal differentiation of pancreatic cells, however, occurs prior to endocrine differentiation and gradually becomes dominant, thus implying a default neuronal lineage specification for cultured pancreatic cells.

Keywords: Neonatal pancreatic cell; Dedifferentiation; Redifferentiation; βIII Tubulin; Neural lineage; MIP-GFP transgenic mouse; Fluorescence-activated cell sorting; *In vitro* clonal assay

Various mechanisms have been suggested for pancreatic β cell homeostasis and regenerative repair, including differentiation from putative pancreatic stem cells [1,2] and self-duplication of pre-existing β cells [3]. Recently it was discovered that islet cells expressed ductal cell marker CK19 in culture and were supposed to be reversed to ductal phenotypic cells [4]. In addition, cultured human islet cells generate islet precursors by reversible epithelial–mesenchymal transition [5]. Subsequently, it has been proposed that mature islet cells including insulin-producing β cells can redifferentiate into multiple pancreatic lineages *in vitro* [4,6]. However, this hypothesis is largely unproven and fails to account for the diversity of the observed islet neogenesis.

Fluorescence-activated cell sorting (FACS) combined with a low density cell culture system was once used to identify the speculated pancreatic stem/progenitor cells, where single neonatal pancreatic cells proliferated clonally and formed pancreatic multilineage colonies with epithelial-like cells (EC) [7]. Here we show that under the same culture conditions, neonatal pancreatic cells dedifferentiate into βIII tubulin-expressing precursor cells, which then expand and redifferentiate into both neural and pancreatic lineage progenies. Our study provides the first direct evidence for the *in vitro* redifferentiation of pancreatic cells. This redifferentiation finding could help to develop new strategies to cure diabetes; in addition, it might make pancreatic cells a promising cell source for the cell therapy of neural diseases.

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doi:10.1016/j.bbrc.2006.10.179

Materials and methods

Animals, cell isolation, and primary culture. The animals used in this study were wild-type C57BL/6J mice (Clea, Tokyo, Japan) and MIP-GFP transgenic mice which express GFP under the control of mouse insulin promoter (a kind gift from Manami Hara of the University of Chicago) [8]. Neonatal pancreatic cells were isolated as previously described [7] and were filtrated with a 40 μm cell strainer (BD Falcon) prior to culture or flow cytometric analysis.

Freshly isolated wild-type pancreatic cells were cultured at 200 cells/cm^2 in a defined culture medium [7] in 35 mm culture dishes (BD Falcon). Hepatic growth factor (HGF, 50 ng/ml; Sigma) and epidermal growth factor (EGF, 20 ng/ml; Sigma) were added 24 h after culture initiation (Day 2). For the dedifferentiation experiments, dissociated pancreatic cells were plated at 2×10^4 cells per cm^2 and cultured briefly (~40 min) to allow partial cell attachment.

Flow cytometric analysis and cell sorting. Dissociated single neonatal pancreatic cells from MIP-GFP transgenic mice were analyzed with a MoFlo cell sorter (Dako Cytomation). Cells from the pancreas of wild-type littermates were used as the negative control. Pancreatic cells were profiled by the side scatter character (SSC) and GFP. Dead cells were excluded by propidium iodide (PI) staining. Different cell fractions were gated based on the intensity of GFP and sorted cells were cultured at 500 cells/cm^2. Floating dead cells were removed 24 h later by changing the growth medium along with the addition of growth factors. The numbers of EC colonies formed by each cell fraction were counted and the growth medium was further changed on day 6.

Immunocytochemistry. Frozen sections (6 μm) from wild-type neonatal pancreas and brain were fixed with cold acetone. Cultured cells were fixed with 4% paraformaldehyde (for CK7 staining the cells were fixed with cold methanol) and immunocytochemistry was carried out as a routine procedure. The primary and secondary antibodies can be found in supplementary methods online. Images were captured with a Zeiss AxioCam MRm or MRc 5 and processed with Photoshop. For quantitative analysis, at least 6 culture dishes were included. The absolute number counted per cell type was 500–700 cells for the acutely plated cells, and 100–300 cells for the day 6 EC colonies. To investigate cell proliferation, day 6 cultures were pulse-labeled with BrdU (Amersham) for 1 h for βIII tubulin-positive cells or for 24 h for insulin-producing cells. Cells were fixed with 4% PFA for 20 min followed by cold methanol for 10 min. Epitope retrieval was done with 2 N HCl. Immunocytochemistry was carried out using standard protocols.

RT-PCR analysis. Total RNA was extracted form single epithelial colonies on day 8 as described previously [7]. For investigating the expression of neural markers, RNA was extracted from five pooled EC colonies. Total RNA from neonatal brain and pancreas was used as the positive control. Samples were treated with DNase I (Invitrogen) prior to RT-PCR amplification when neural markers were detected. RT-PCR was carried out using a OneStep RT-PCR (Qiagen) kit. See the supplementary methods online for a list of primers used.

Results

Neonatal pancreatic cells generate both pancreatic and neural lineages

To obtain single cell-derived colonies, freshly isolated neonatal pancreatic cells were cultured at 200 cells/cm^2. This low density culture ensures that single cells proliferate clonally, and morphologically recognized colonies with epithelial-like cells (EC) were observed during culture [7]. Approximately 1.5% of neonatal pancreatic cells had formed EC colonies by day 6. Multiple pancreatic lineage progenies were identified in EC colonies (Fig. 1A–C). Endocrine hormones were found to be coexpressed (Fig. 1A and B and Table 1) and exclusively distributed (Fig. 1A) in single cells, similar to the hormone coexpression observed in the developing pancreas [9–11] and regenerative models of diabetes [12]. Insulin-producing cells identified here showed pro-β cell characters as they also expressed c-peptide (Supplementary Fig. 1A) and pancreas duodenal homeobox 1 (PDX-1) (Supplementary Fig. 1B). The pancreatic multilineage of the EC colonies was also confirmed by detecting the mRNA expression of various pancreatic lineage markers on day 8 (Supplementary Table 1) [7].

We then stained day 6 cultures with monoclonal antibody TUJ1 to detect βIII tubulin-expressing neuronal cells [2,13]. Various types of TUJ1-positive cells were observed (Fig. 1D–G). Their morphology ranged from oval-shaped cells located at the center of EC colonies (Fig. 1D and E) to neuron-like cells dispersed among the EC colonies (Fig. 1G). BrdU labeling revealed that these TUJ1-positive cells can proliferate and thus may be neuronal precursor cells (Fig. 1H) [14]. Collectively, these images suggested a process of neuronal maturation: TUJ1-positive immature oval-shaped cells were generated from the center of the EC colonies (Fig. 1D). These cells proliferated while extending cell processes (Fig. 1E), migrated outward (Fig. 1F), and finally were distributed among the EC colonies where they differentiated into mature neurons (Fig. 1G). As cell culture proceeded, TUJ1-positive cells gradually became dominant. On day 10 approximately 40% of the cells expressed βIII tubulin (Fig. 1I). In addition, pancreatic cells also generated other neural lineages. RT-PCR detected the mRNA expression of the astrocyte marker GFAP along with the neuronal markers βIII tubulin and MAP2 (Fig. 1J).

Neonatal pancreatic cell-derived progenies express both neural and pancreatic lineage markers

To understand the nature of the cells which expressed pancreatic lineage or neural lineage markers, day 6 culture cells were doubly stained with these two lineage markers. Almost all of the c-peptide (insulin)-positive cells expressed βIII tubulin (Fig. 2A and Table 1). Oval-shaped double positive cells expressed higher levels of c-peptide, while fibroblast-shaped double positive cells were immuno-positive for c-peptide through the cell body and proximal but not distal cell processes where βIII tubulin staining was present (Fig. 2A), suggesting an on-going neuronal lineage specification. In rare cases, TUJ1-positive neuron-like cells with longer cell processes budded from islet-like double positive cell clusters (Supplementary Fig. 2A). In contrast, cells expressing exclusively c-peptide and thus likely to be of pancreatic β cell lineage were rare (Supplementary fig. 2B). C-peptide (insulin) and βIII tubulin double positive cells incorporated BrdU (Fig. 2B), consistent with these cells being bipotent precursor cells.

In addition, glucagon (Fig. 2C), somatostatin (Fig. 2D), and CK7 (Fig. 2E) were coexpressed with βIII tubulin in EC colonies. Just as with the coexpression of insulin and glucagon, βIII tubulin and glucagon were also distributed

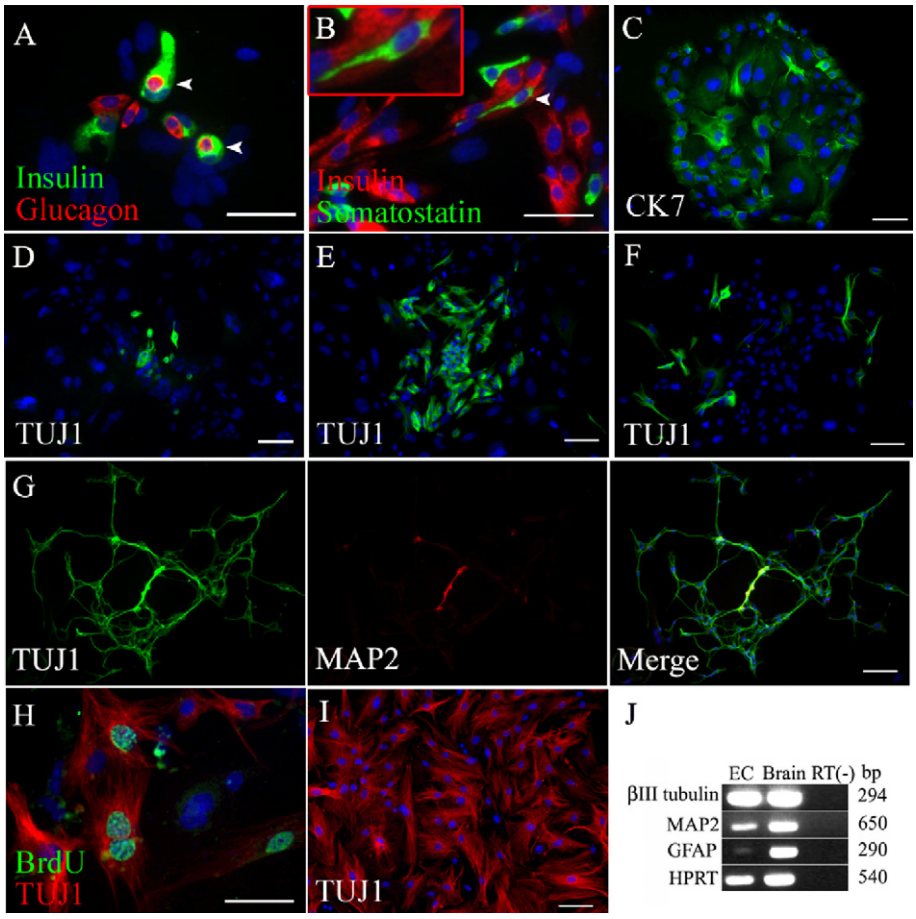

Fig. 1. Single neonatal pancreatic cells generated both pancreatic and neural lineages in a low density culture. (A–C) Single cell-derived EC colonies contain endocrine lineage insulin-positive cells (A,B), glucagon-positive cells (A), somatostatin-positive cells (B), and ductal lineage CK7-positive cells (C). Insulin and glucagon are coexpressed exclusively inside cells (A, arrowheads); somatostatin is also coexpressed with insulin (B, enlarged in the inset). Scale bar in (A,B) 50 μm; (C) 100 μm. (D–F) βIII Tubulin-positive cells that display oval or fibroblast morphologies are located at the center (D,E) or the periphery (F) of EC colonies, respectively. Scale bars: 100 μm. (G) In rare cases, βIII tubulin-expressing cells form extensive networks through contacts between cell processes. These cells express higher βIII tubulin and a few of them also express the mature neuron marker MAP2. Scale bar: 100 μm. (H) BrdU labeling demonstrates that βIII tubulin-positive cells can proliferate. Scale bar: 50 μm. (I) βIII Tubulin-positive cells are abundant on day 10. Scale bar: 100 μm. (J) RT-PCR detection of the mRNA expression of neural lineages in EC colonies.

Table 1
Coexpression of βIII tubulin and endocrine hormones in acutely plated pancreatic cells and in day 6 EC colonies (mean% ± SEM)

	βIII Tubulin+ in insulin+	βIII Tubulin+ in glucagon+	βIII Tubulin+ in somatostatin+	Insulin+ in glucagon+	Insulin+ in somatostatin+
Plated cells	100	46.6 ± 2.1	100	43.9 ± 1.9	41.2 ± 2.7
EC colonies	100	20.2 ± 3.9	22.6 ± 2.1	15.7 ± 4.0	8.2 ± 2.2

exclusively inside cells (Fig. 2C). Unlike the precursor state of insulin-producing cells, most glucagon- and somatostatin-positive cells did not express βIII tubulin (Fig. 2C and D, and Table 1), suggesting an earlier differentiation of the non-insulin-producing islet cells.

Dedifferentiation of acutely plated neonatal pancreatic cells

The above data demonstrate that single pancreatic cell-derived progenies coexpress pancreatic markers and βIII tubulin, a neuronal marker that was recently discovered to be expressed in the developing chicken pancreas [15]. As βIII tubulin is not detected in mouse pancreatic tissue [14], it is possible that pancreatic cells can express βIII tubulin before proliferation after cell plating in culture, a process which may correspond to the 'dedifferentiation' known in lower vertebrates [16]. To test this hypothesis we checked the βIII tubulin expression in pancreatic cells shortly after cell plating. All insulin-producing β cells (Fig. 3A and Table 1) and all somatostatin-producing δ cells (Fig. 3C and Table 1) expressed βIII tubulin, consistent with the hypothesis that they are descended from common precursors during development [17]. Glucagon-positive α cells (Fig. 3B and Table 1) and CK7-positive

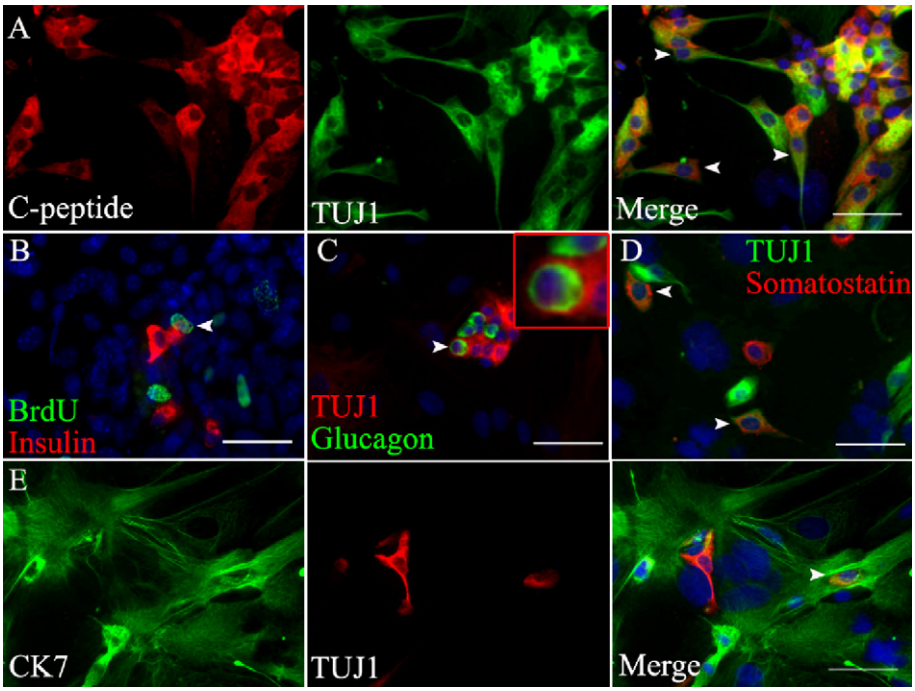

Fig. 2. Neonatal pancreatic cell-derived progenies express both neural and pancreatic lineage markers. (A) Coexpression of c-peptide and βIII tubulin. C-peptide staining is observed in oval-shaped cells and in fibroblast-like cells where the cell body and proximal but not distal cell processes are immunopositive (arrowheads). (B) BrdU labeling demonstrates that insulin-positive cells can proliferate (arrowhead). (C–E) Co-expression of βIII tubulin with pancreatic lineage markers glucagon (C), somatostatin (D), and CK7 (E). Note the exclusive distribution of βIII tubulin and glucagons in (C) (arrowhead, enlarged in the inset). Scale bars: 50 μm.

ductal cells (Fig. 3D) also expressed βIII tubulin. Since non-neuronal βIII tubulin expression is always found in embryonic stages or in neural progenitors *in vivo* [14], these results suggest that the mature pancreatic endocrine and ductal cells can revert to an immature state upon *in vitro* culture via 'dedifferentiation'.

Within the same plating period, many TUJ1-positive cells were found to express multiple endocrine hormones (Fig. 3E and F). We observed that 43.9% ($n = 651$) of glucagons-positive cells and 41.2% ($n = 512$) of somatostatin-positive cells expressed insulin (Table 1), but *in vivo* the coexpression of these endocrine hormones can only be found in the early mouse embryonic stages [11]. These observations suggest that the dedifferentiated cells which expressed βIII tubulin and single hormones (Fig. 3A–D) further dedifferentiated into more primitive cells expressing βIII tubulin and multiple endocrine hormones (Fig. 3E and F), a process which is just opposite to the *in vivo* endocrine differentiation in developing pancreas [10,11].

Pancreatic cells with insulin promoter potency redifferentiate into both neural and pancreatic lineages

To determine if lineage-specified neonatal pancreatic cells can differentiate into both pancreatic and neural lineages as observed in primary culture, we separated the insulin-producing cells and assessed their capability to form *in vitro* multilineage EC colonies. For this purpose we used MIP-GFP-transgenic mice that express GFP under the control of the mouse insulin promoter [8]. Neonatal pancreatic cells were analyzed and several cell populations were collected by fluorescence-activated cell sorting (FACS) according to the insulin promoter potency represented by the fluorescence intensity. Flow cytometric analysis revealed that the cell number decreased along with the increase in insulin promoter potency (Fig. 4A and B). When the sorted cells were used for *in vitro* clonal assay, cell populations of lower promoter potency displayed higher capability to form EC colonies (Fig. 4C).

To confirm the multilineage of EC colonies formed by sorted cells with insulin promoter potency, we stained for both neural and pancreatic lineage markers. βIII Tubulin was detectable around day 8, prior to the expression of endocrine hormones. On day 12, various neural and pancreatic progenies can be identified (Fig. 4D–F). Just as in primary culture, more βIII tubulin-positive neuronal progenies were generated and they gradually became dominant as the culture proceeded.

In light of the observed dedifferentiation of acutely plated pancreatic cells, these data indicate lineage-specified neonatal pancreatic cells redifferentiate into both neural and pancreatic lineages in culture condition.

Discussion

We describe here that the neonatal pancreatic cells including insulin-producing cells dedifferentiate into multipotent precursors, which expand and then redifferentiate into both pancreatic and neural lineages, including pancreatic endocrine hormone-expressing cells, exocrine acinar

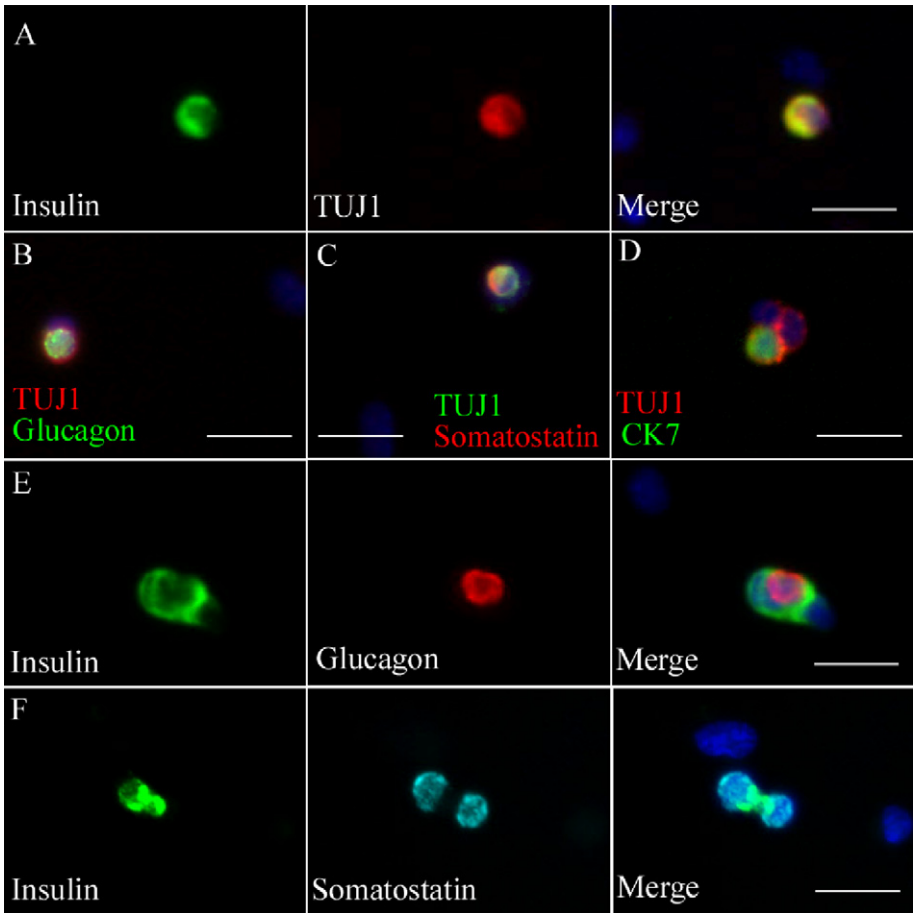

Fig. 3. Dedifferentiation of pancreatic endocrine cells and ductal cells. Freshly isolated pancreatic cells were cultured briefly to allow partial cell attachment and then were immunostained. (A–D) Dedifferentiation of pancreatic cells. All insulin-positive β cells express βIII tubulin (A). All somatostatin-positive δ cells (C), some glucagon-positive α cells (B), and some CK7-positive ductal cells (D) express βIII tubulin. (E,F) Dedifferentiation of dedifferentiated pancreatic cells. Dedifferentiated cells express additional endocrine hormones; the hormone coexpression could be insulin and glucagon (E) or insulin and somatostatin (F). Insulin and glucagon are distributed exclusively inside cells (E). Scale bars: 20 μm.

cells, and ductal cells, and neural progenies such as βIII tubulin-positive neurons and GFAP-positive astrocytes.

Dedifferentiation of neonatal pancreatic cells is characterized by the expression of βIII tubulin shortly after cell plating in endocrine cells and ductal cells. βIII Tubulin is generally recognized as a specific neuronal marker, but it is also expressed in non-neuronal tissue in embryonic stages [14]. It has been reported recently that βIII tubulin is also expressed in the developing chick pancreas and is partially colocalized with endocrine hormones [15]. Aberrant expression of βIII tubulin has been reported in many types of neuronal or non-neuronal tumors, including pancreatic neuroendocrine cancer [14,18]. Furthermore, the coexpression of βIII tubulin and endocrine hormones described here is reminiscent of the developing pancreas where all endocrine hormones first appear along with another neuronal marker, tyrosine hydroxylase (TH) [10]. All these suggest that the *in vitro* expression of βIII tubulin in mouse pancreatic cells is indicative of the emergence of an immature cellular state, a process known as 'dedifferentiation' [16].

Consistent with this hypothesis, all β cells and all δ cells, but only a part of α cells expressed βIII tubulin after acute plating. This is in accord with the proposal that β cells and δ cells, but not α cells, are thought to be derived from common precursors during development [17]. In addition, dedifferentiated βIII tubulin-expressing cells further dedifferentiate into cells expressing βIII tubulin and multiple endocrine hormones. This sequential dedifferentiation process is the reverse of the sequential endocrine differentiation process observed in the developing pancreas, except that the neuronal marker TH in embryonic development is substituted by the neuronal marker βIII tubulin [10,11]. Moreover, EC colonies formed by single cells with insulin promoter potency expressed βIII tubulin prior to endocrine hormones, consistent with the dedifferentiation of pancreatic cells *in vitro*.

Dedifferentiated pancreatic cells then proliferate and redifferentiate into both pancreatic and neural progenies. We have demonstrated that in primary culture, single neonatal pancreatic cells can generate epithelial-like colonies that contain terminally differentiated pancreatic and neural progenies, and we have confirmed this observation by using sorted pancreatic cells with insulin promoter potency. It has been suggested that cells with insulin promoter potency

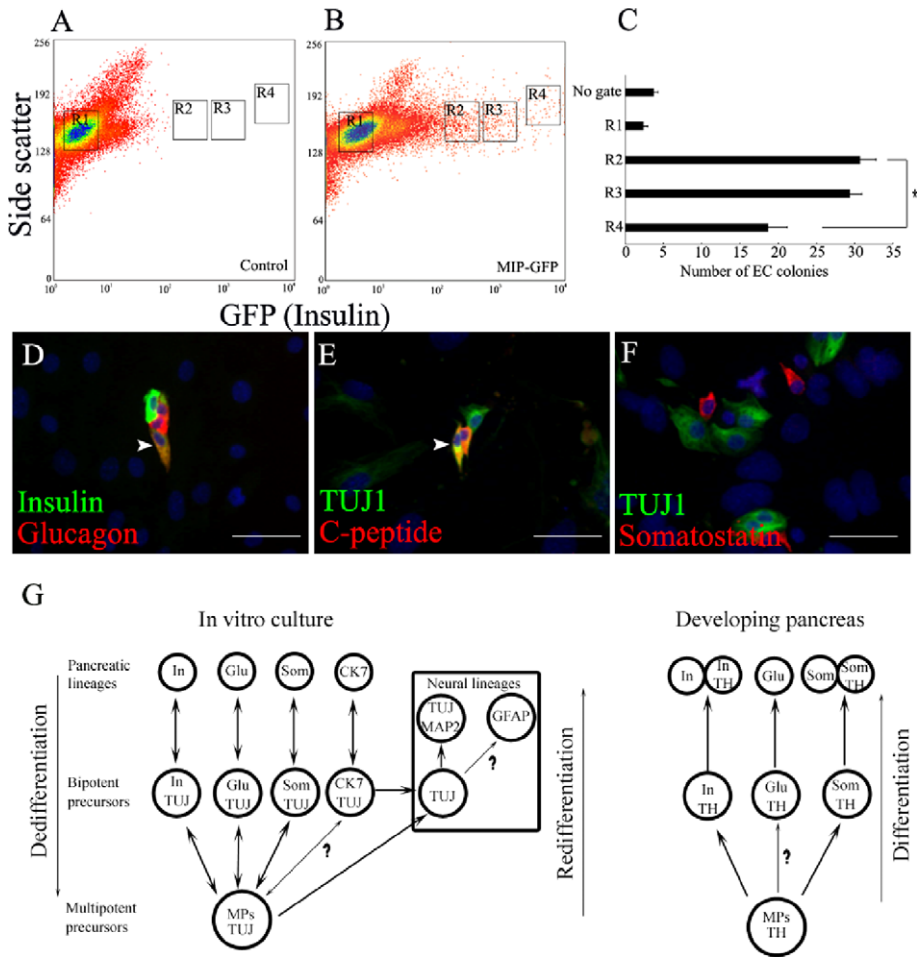

Fig. 4. Pancreatic cells with insulin promoter potency redifferentiate into both neural and pancreatic lineages. (A–C) Flow cytometric analysis and *in vitro* clonal assay of neonatal pancreatic cells with insulin promoter potency. Pancreatic cells display a spectrum of insulin promoter activities (A, wild control; B, MIP-GFP). The gates R1 to R4 represent Ins^{-}, $Ins^{+/low}$, $Ins^{+/mid}$, $Ins^{+/high}$ respectively. *In vitro* clonal assay demonstrates that the cells with lower insulin promoter potency have greater ability to form EC colonies (C). *, Mann–Whitney *U* test: $P < 0.05$. (D–F) Epithelial-like colonies formed by single sorted cells with insulin promoter potency contain progenies of both pancreatic and neural lineages. Progenies may coexpress insulin and glucagon (D, arrowhead). C-peptide-positive cells consistently express βIII tubulin (E, arrowhead). Somatostatin-positive cells are also generated (F). Scale bars: 50 μm. (G) Proposed model of *in vitro* redifferentiation of pancreatic cells. MPs, multiple pancreatic markers. Right panel shows the endocrine lineage differentiation in developing pancreas [10].

in postnatal mouse pancreas are lineage-specified [3]. In particular, cells with high insulin promoter potency, which indicates mature β cells, also form multilineage colonies. This result strongly supports the redifferentiation of pancreatic cells *in vitro*.

Our results indicate that the redifferentiation of pancreatic cells recapitulates the embryonic development of the pancreas. The coexistence of precursor cells and terminally differentiated cells within the single pancreatic cell-derived colonies suggests that the dedifferentiated βIII tubulin-expressing precursors generate both neural and pancreatic lineages. It is likely that the dedifferentiated cells expressing multiple endocrine hormones and the neuronal marker βIII tubulin redifferentiate into bipotent precursors that express single endocrine hormones and βIII tubulin and then give rise to both neural and endocrinal progenies. As an example, the endocrine hormone somatostatin was coexpressed with βIII tubulin in acutely plated cells but was progressively expressed alone during primary culture. This sequential *in vitro* endocrine redifferentiation process recapitulates the *in vivo* endocrine differentiation [10,11]. Although no neuronal lineage progenies are generated *in vivo*, it is predictable that similar precursors will generate neuronal progenies *in vitro*. The coexpressed endocrine hormones are distributed exclusively inside the precursor cells, which again recapitulate the embryonic development [9]. A proposed model of *in vitro* redifferentiation of pancreatic cells is shown in Fig. 4G.

Our data suggest that cell redifferentiation occurs not only in islet cells, but also in ductal cells. Like islet cells, acutely plated ductal cells dedifferentiate into βIII tubulin-expressing cells, and ductal cell marker CK7 is coexpressed with βIII tubulin during culture. Moreover, we show that insulin promoter active cells are almost exclusively responsible for *in vitro* multilineage colony formation. This is consistent with *in vivo* findings that pre-existing insulin-producing cells are the major source for new insulin-producing cells during normal cell turnover

and regeneration following 70% pancreatectomy in mice [3]. These results indicate that the proposed ductal origin ''pancreatic stem cells'' have weak insulin promoter potency. In accordance with this assumption, we found that cells with lower insulin promoter potency show higher cell plasticity, suggesting that these cells may represent the putative ductal origin ''pancreatic stem cells''. Ductal cells with lower insulin promoter potency redifferentiate into both neural and pancreatic lineages upon *in vitro* culture, the same as islet cells with higher insulin promoter potency (Fig. 4G).

In vitro culture of cells obtained from pancreas has been reported to give rise to neural lineages [2,13]. Our data differ from the previous reports in that we identified the neuronal marker βIII tubulin as the marker of dedifferentiation of pancreatic cells, and we showed that βIII tubulin is coexpressed with pancreatic markers during culture. Furthermore, we demonstrated that sorted pancreatic cells with insulin promoter potency also generate neural lineage progenies. All these observations unequivocally demonstrate that cultured pancreatic cells can generate neural lineages. In addition, progenies from sorted insulin-positive cells express βIII tubulin before expressing endocrine hormones. More neuronal progenies are generated during culture, and they gradually become dominant as in a previous report [2]. These findings reveal a close relationship between neurons and pancreatic cells and may suggest a default neuronal lineage specification for pancreatic cells *in vitro*.

Acknowledgments

We thank Hideaki Sato for cell sorting, Manami Hara for the gift of MIP-GFP transgenic mice, and all members of Taniguchi's laboratory for their expert technical help. This work was supported by the National Leading Project for realization of 'Regenerative Medicine' and Grants-in-Aid (14207046, 14081201, 16659340, and 17209043) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and grants from the MITSUBISHI Foundation and the Sankyo Foundation of Life Science.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.179.

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