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<td>Author(s)</td>
<td>Hori, Yuichi / Fukumoto, Miki / Kuroda, Yoshikazu</td>
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<tr>
<td>Citation</td>
<td>STEM CELLS, 26(11):2912–2920</td>
</tr>
<tr>
<td>Issue date</td>
<td>2008-11</td>
</tr>
<tr>
<td>Resource Type</td>
<td>Journal Article / 学術雑誌論文</td>
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<td>Resource Version</td>
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<td>Rights</td>
<td>definitive version is available at <a href="http://www.blackwell-synergy.com">www.blackwell-synergy.com</a>.</td>
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<tr>
<td>DOI</td>
<td>10.1634/stemcells.2008-0192</td>
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<td>URL</td>
<td><a href="http://www.lib.kobe-u.ac.jp/handle_kernel/90001573">http://www.lib.kobe-u.ac.jp/handle_kernel/90001573</a></td>
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PDF issue: 2018-11-02
Running head: pancreatic epithelial progenitor cells

**Enrichment of putative pancreatic progenitor cells from mice by sorting for prominin1 (CD133) and PDGFRβ**

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**Key Words.** Pancreas, Tissue-specific stem cells, diabetes, insulin-producing cells, prominin1, CD133
Abstract

Success in islet transplantation-based therapies for type 1 diabetes mellitus and an extreme shortage of pancreatic islets has motivated recent efforts to develop renewable sources of islet-replacement tissue. Although pancreatic progenitor cells hold a promising potential, only a few attempts have been made at the prospective isolation of pancreatic stem/progenitor cells, due to the lack of specific markers and the development of effective cell culture methods. We found that prominin1 (also known as CD133) recognized the undifferentiated epithelial cells, while PDGFRβ was expressed on the mesenchymal cells in the mouse embryonic pancreas. We then developed an isolation method for putative stem/progenitor cells by flow cytometric cell sorting and characterized their differentiation potential to pancreatic tissue using both an in vitro and in vivo protocol. Flow cytometry and the subsequent RT-PCR and microarray analysis revealed pancreatic epithelial progenitor cells to be highly enriched in prominin1^{high}PDGFRβ^{-} cell population. During in vivo differentiation, these cell populations were able to differentiate into endocrine, exocrine, and ductal tissues, including the formation of insulin-producing cell cluster. We established the prospective
isolation of putative pancreatic epithelial progenitor cells by sorting for prominin1 and PDGFRβ. Since this strategy is based on the cell surface markers common to human and rodents, these findings may lead to the development of new strategies to derive transplantable islet-replacement tissues from human pancreatic stem/progenitor cells.
**Introduction**

Advances in cell-replacement strategy for type 1 diabetes mellitus and the shortage of transplantable pancreatic islets have focused on renewable sources of insulin-producing cell (IPC) (1). Although embryonic stem (ES) cells hold a promising potential as a source of IPC, IPC clusters from ES cells still have a high degree of cellular heterogeneity, tumor-forming potential, and low insulin levels compared with pancreatic islets (2-4).

Recent studies in regenerative medicine have also focused on the isolation and characterization of repopulating tissue-specific stem/progenitor cells. However, only a few attempts have been made at the prospective isolation of pancreatic stem/progenitor cells, due to the lack of specific markers and the failure to develop a cell culture strategy to determine their capacity for self-renewal and multi-lineage potential. Putative adult pancreatic stem/progenitor cells that clonally expand, while expressing a low level of insulin and other pancreatic markers have been found in the mouse pancreas (5,6). However, their capacity for self-renewal and the ability to differentiate into functional islets remains undetermined. In contrast, Dor et al. reported that new β-cells, after birth
could be generated by the replication of existing β-cells, rather than by putative pancreatic stem cells (7). Taken together, the existence of adult pancreatic stem/progenitor cells still remains controversial.

On the other hand, there is no doubt that pancreatic stem/progenitor cells exist in the developing pancreas. The pancreas develops from the posterior foregut, emerging as buds from the ventral and dorsal area of the gut tube. Although it is thought that Pdx1 (also known as Ipf1)-expressing epithelial progenitor cells give rise to endocrine, exocrine, and ductal cells (8), evidence that isolated clonogenic cells are pancreatic stem/progenitor cells with characteristics indicating the capability for self-renewal and pluripotency still remains to be achieved.

This study describes that prominin1 (also known as CD133) recognized the undifferentiated epithelial cells, while platelet-derived growth factor receptor β (PDGFRβ, also known as CD140b) expressed on the surrounding mesenchymal cells within the embryonic pancreas. With the combination of these markers, putative pancreatic epithelial stem/progenitor cells were enriched in prominin1<sup>high</sup> PDGFRβ<sup>−</sup> cell population by cell sorting. This was verified by RT-PCR and a microarray analysis. An
extensive phenotypic characterization of prominin1⁺ PDGFRβ⁻ cells showed that these cells include putative specialized progenitors capable of generating all pancreatic epithelial cell types and were expandable *in vivo* during a serial transplantation. It was also possible to differentiate the putative prominin1⁺ PDGFRβ⁻ progenitor cells into not only IPC, but other pancreatic epithelial cells *in vitro* under the specific condition.
Materials and Methods

The supplemental methods are available in the online “supplemental data”.

**Isolation of embryonic pancreatic cells.** All animal experiments were conducted according to the “Guidelines for Animal Experimentation at Kobe University.”

Embryonic day (E) 11.5 and E13.5 fetal pancreases were microdisected from Jcl:ICR mice (Clea, Japan). Fetal pancreases were digested in PBS containing 0.04 units Liberase Blenzyme 3 (Roche) at 37°C for 5 min. This routinely yielded $1.3 \times 10^4$ cells from E11.5 and $2 \times 10^4$ cells from E13.5 embryonic pancreas, respectively.

**Cell lines and culture conditions.** Mouse stromal cell line, PA6 (a gift from Dr. Nishikawa, RIKEN, Kobe) was maintained in α-minimum essential medium (α-MEM: Invitrogen) containing 10% fetal calf serum (FCS).

**In vitro differentiation assays.** For the differentiation of insulin-producing cells, sorted cells were cultured on PA6 cells (a gift from S.I. Nishikawa, Center for Developmental Biology, RIKEN, Kobe, Japan) for 7 days in the medium containing α-MEM, 10% FCS.

Alternatively, for the differentiation of glucagon-producing cells, sorted cells were cultured on PA6 cells for 7 days in the SF-O3 (Sanko Junyaku, Japan), serum free
medium.

**In vivo differentiation assays.** Pancreatic cells were isolated from enhanced green fluorescent protein (eGFP) transgenic mice (9) to allow verification of donor origin by staining for GFP. For purification experiments, pancreatic cell suspensions were magnetically separated on the basis of prominin1 and PDGFRβ expression. Purities ranged from 90 to 95% for the prominin1⁺PDGFRβ⁻ cells and from 88 to 92% for prominin1⁻PDGFRβ⁻ cells by FACS (data not shown). Under general anesthesia, 5- to 6-week-old male nude mice (BALB/cAJcl-nu/nu) were engrafted with 5 x 10⁴ prominin1⁺PDGFRβ⁻ or 5 x 10⁴ prominin1⁻PDGFRβ⁻ cells in the subcapsular renal space with e13.5 embryonic pancreas tissues or pancreatic mesenchymal tissues. For the preparation of the mesenchymal tissues, dorsal pancreatic rudiment was incubated in 0.04 units Liberase Blenzyme 3 in PBS at 37°C for 1 hr. The enzyme digestion was stopped by 30 min incubation in an RPMI medium containing 50% fetal calf serum. Thereafter, the pancreatic mesenchymal tissues can be separated from the epithelial tissue using tungsten needles. Either one week or one month after transplantation, the grafts were subjected to an immunohistochemical analysis (n = 5). In preliminary
studies, we compared prominin1+PDGFRβ- and GFP+prominin1+PDGFR- cells, including cell sorting, *in vitro* and *in vivo* differentiation, and obtained similar results.

**Serial transplantation.** Two weeks after transplantation, grafts were removed and dissected, then digested in Liberase Blenzyme as described previously. Cells were separated on the basis of prominin1 expression and $5 \times 10^4$ prominin1+/GFP+ cells were re-implanted into the secondary mice as described above ($n = 3$).

**RT-PCR.** Total RNA was prepared and cDNA was synthesized as described (3). A total of 35 cycles of PCR were performed. The primer sequences are summarized in supplemental online Table 1.

**Immunohistochemistry.** Immunohistochemistry was performed as described previously (10). Briefly, cultured cells were fixed in methanol at -20°C for 10 minutes, and washed three times with PBS. The grafts were fixed in 4% paraformaldehyde, and embedded in OCT compound. They were then sectioned to generate 10-µm-thick tissue frozen sections. Primary antibodies are listed in supplemental online Table 2.

Anti-neurogenin3 antibody is a kind gift from M German (Diabetes Center, UCSF). For BrdU labeling *in vivo*, 1 ml / 100g body weight BrdU solution (ZYMED, CA) was
administered intraperitoneally, two hours before the mice were sacrificed. Tissue sections and fixed cells were incubated with the secondary antibodies labeled with Alexa conjugated goat anti-rabbit, -mouse or rat IgG 1:200 (Molecular probes). The cells were observed by confocal immunofluorescence microscopy (Zeiss LSM510).

**Statistical analyses.** All values were expressed as the means ± SEM. Statistical significance was determined by using the two-tailed unpaired Student’s t test, and difference were considered to be statistically significant when $P < 0.05$.

**Intracellular C-peptide quantification in IPCs.** Pancreatic islets were isolated by intraductal collagenase perfusion using standard methods for 2-week-old and 2-month-old mice. After isolation, we separated two similar sized groups to determine the intracellular C-peptide content and the cell number, respectively. Ten mouse islets were handpicked and washed with PBS, homogenized by sonication, and then intracellular C-peptide content was measured with a C-peptide ELISA kit (Shibayagi, Japan). For P1 and P7 postnatal mice, the pancreas was removed, minced, and digested with 0.04 units Liberase Blenzyme 3 for 5 min at 37°C. The islets were then isolated by the standard method. To measure the cell number, handpicked islets were digested with
0.05% trypsin/EDTA for 5 min at 37°C and then the cell number was counted with a hemocytometer. For C-peptide content or the cell number of IPC \textit{in vitro}, 5,000 prominin1$^+$PDGFRβ$^-$ cells on PA6 cells were cultured for 7 days in the 12 well-plates and counted insulin$^+$ cells by immunohistochemistry. This routinely yielded 150 ± 30 IPC per well in the 12 well-plates. In addition, the harvested cells from different wells were homogenized, and assayed for intracellular C-peptide content. To determine C-peptide content in IPC clusters \textit{in vivo}, 50,000 prominin1$^+$PDGFRβ$^-$ cells derived from eGFP transgenic mice were transplanted with either e13.5 embryonic pancreas tissues (not triturated single cells) or pancreatic mesenchymal tissues derived from wild type mice as described above. One month after transplantation, graft including GFP$^+$IPC clusters was removed under a Leica fluorescence microscope (supplemental online Fig. 1). The graft was incubated in 0.04 units Liberase Blenzyme 3 in PBS at 37°C for 1 hr. The enzyme digestion was stopped by 30 min incubation in a RPMI medium containing 50% fetal calf serum. Round GFP$^+$IPC clusters can be separated from GFP$^-$ tissues using tungsten needles and confirmed with fluorescence microscope and fluorescence-activated cell sorting (FACS). As shown in Fig.6, GFP$^+$IPC clusters did
not include GFP IPC. The cell number and C-peptide content were determined as described above.

Results

**Cell surface markers expressed on embryonic pancreatic cells.** The expression of stem cell markers in pancreatic buds was examined at E11.5 and E13.5, when the majority of the epithelium consists of undifferentiated progenitor cells.

Prominin1 (CD133) is a cell surface marker to identify hematopoietic (11) and neural progenitor cells (12). Prominin1 is also expressed on the apical membrane of the human embryonic gut tube and the neural tube (13). Immunohistochemistry revealed prominin1 expression on the apical membrane of the Pdx1-expressing pancreatic epithelial cells (Figs. 1A and 1E). This was also confirmed by Pdx1 staining on post-sorted prominin1\textsuperscript{high}PDGFRβ\textsuperscript{−} cells (Fig. 2D). Previous efforts to further expand the number of β-cells and other pancreatic epithelial cells were limited by mesenchyme-derived fibroblast overgrowth in culture. To rule out any other tissue-derived progenitor cells, including hematopoietic and neural progenitor cells, and to further establish an in vitro culture system, it was necessary to distinguish the epithelial cells from the surrounding
mesenchymal cells. Several surface markers were tested and this revealed that PDGFRβ is expressed on the surrounding mesenchymal cells of the embryonic pancreas (Figs. 1B and 1F). Consistent with previous report, the only mature pancreatic marker detectable was glucagon, expressed by a few scattered cells in the E11.5 bud, while insulin-expressing β-cells were identified in the E13.5 bud (Figs. 1C, 1D, 1G, and 1H). Moreover, no double positive cells for both prominin1 and hormone like insulin or glucagon could be detected (data not shown), thus suggesting that prominin1 expression diminishes through the differentiation. Notably, both insulin and glucagon-expressing cells in the E13.5 pancreas were Ki67 negative (supplemental online Fig. 8).

**Isolation of pancreatic epithelial progenitor cells expressing prominin1 by FACS.**

To determine whether pancreatic stem/progenitor cells can be enriched using prominin1 and PDGFRβ, embryonic pancreas was dissociated and fractionated into four subpopulations by FACS as follows; 1) prominin1<sup>high</sup>PDGFRβ<sup>−</sup> cells (15.3 ± 2.8%), 2) prominin1<sup>dim</sup>PDGFRβ<sup>−</sup> cells (11.4 ± 2.2%), 3) prominin1<sup>neg</sup>PDGFRβ<sup>−</sup> cells (6.5 ± 1.2%), 4) PDGFRβ<sup>+</sup> cells (66.8 ± 9.8%), (Fig. 2A). The expression of other surface markers on prominin1<sup>high</sup>PDGFRβ<sup>−</sup> cells was next examined, including pancreas or liver
stem/progenitor cell (c-met or CD49f)(6,14-16), hematopoietic stem/progenitor cell
(c-kit and Sca-1) (17), pan-leukocyte marker (CD45) (Fig. 2B). Both
prominin1<sup>high</sup>PDGFR<sup>β</sup> cells and prominin1<sup>neg</sup>PDGFR<sup>β</sup> cells were c-met<sup>−</sup>, c-kit<sup>−</sup>, Sca-1<sup>−</sup>,
and CD45<sup>−</sup>, indicating that blood cells were excluded in these cell populations. On the
other hand, 60% of prominin1<sup>high</sup>PDGFR<sup>β</sup> and 30% of prominin1<sup>neg</sup>PDGFR<sup>β</sup>
coexpressed CD49f (Fig 2B and data not shown), which belongs to integrin family and
have established roles in cell-cell adhesion in the pancreas (18). The gene expression of
the sorted cells was next examined by RT-PCR and this demonstrated that putative
markers of pancreatic stem/progenitor cells, including Foxa2, HNF4a, HNF6, Pdx1,
Hlbx9, Ptf1a and neurogenin3 (ngn3) are clearly enriched in prominin1<sup>high</sup>PDGFR<sup>β</sup>
fraction, whereas mature markers including insulin1, preproinsulin1 (β-cell marker) and
glucagon, preproglucagon (α-cell marker) are enriched in prominin1<sup>neg</sup>PDGFR<sup>β</sup>
fraction (Fig. 2C). These results were confirmed by the Pdx1 and insulin staining on
post-sorted prominin1<sup>high</sup>PDGFR<sup>β</sup> cells (Fig. 2D). We also detected a few percentage
of neurogenin3 (ngn3) –expressing cells in prominin1<sup>high</sup>PDGFR<sup>β</sup> cells (supplemental
online Fig. 6). These reproducible results of RT-PCR prompted the use of DNA chip
analysis to explore other new molecules essential for not only pancreatic stem/progenitors, but also early pancreatic development. Hybridization of amplified RNA from each of these subsets to Agilent whole mouse genome array chips indicated that prominin1^{high} PDGFRβ^-cells contain higher levels of early definitive endoderm transcripts and pancreatic stem/progenitor transcripts (supplemental online Table 3). Conversely, transcripts for the differentiated cell were found to be present at higher levels in prominin1^{neg} PDGFRβ^-cells (supplemental online Table 4). These results confirm that prominin1 could be one of the putative epithelial stem/progenitor markers in the embryonic pancreas and its expression is diminished through differentiation into mature pancreatic cells. Wider gene characterization of the prominin1^{high} PDGFRβ^-cells demonstrated that Sox17 (early definitive endoderm marker) and surfactant associated protein C (SftpC) (lung marker), but not thyroid transcriptional factor-1 (TTF-1) (master gene of lung development), were enriched in prominin1^{high} PDGFRβ^-cells (supplemental online Table 5-8). The expression of other endoderm derivatives or mesoderm or ectoderm derivatives were not detected or not significantly enriched in this population (supplemental online Table 8). The microarray data indicating an early
definitive endoderm and pancreatic stem/progenitor cells include many genes expressed in adult differentiated pancreatic cells. However, most genes enriched in prominin1$$^{\text{high}}$$PDGFR$$^{\beta^-}$$ cells are also essential for developing pancreas such as E13.5. We thus classified these genes as putative pancreatic stem/progenitor cell markers at this embryonic stage.

**Differentiation potential of putative pancreatic stem/progenitor cells in vitro.** To evaluate the differentiation potential of prominin1$$^{+}$$PDGFR$$^{\beta^-}$$ cells in vitro, several conditions were investigated. After plating these cells on PA6 mouse stromal cells in serum-containing medium for 7 days, we routinely yielded 150 ± 30 insulin-producing cells (IPCs) from 5,000 prominin1$$^{+}$$PDGFR$$^{\beta^-}$$ cells, but not from PDGFR$$^{\beta^-}$$ or prominin1$$^{+}$$PDGFR$$^{\beta^-}$$ cells (Figs. 3A and D). The growth rate of prominin1$$^{+}$$PDGFR$$^{\beta^-}$$ cells on PA6 cells in culture during 7 days was shown in supplemental online Fig. 7. IPC expressed Pdx1 in the nucleus and form clusters and each cluster contained 3-20 IPC (Fig. 3A). IPC also expressed C-peptide, indicating *de novo* insulin synthesis (Fig. 3B). To quantify the insulin expression in IPC more accurately, an insulin C-peptide specific ELISA was used. This demonstrated that the
intracellular C-peptide level in IPC in vitro was $2.2 \pm 0.1 \text{ pg / cell}$, whereas that in the islets of a 2 month-old mouse was $91.8 \pm 3.5 \text{ pg / cell}$ (Fig. 3E). This indicates that the insulin C-peptide content in one IPC in vitro is approximately 2.4% of the level in isolated β-cells. Consistent with these findings, 1,000 transplanted IPCs did not rescue streptozotocin-induced diabetic phenotype. However, IPCs could survive for one month and continue to express C-peptide and insulin (supplemental online Fig. 2). Transcripts for glucagon, somatostatin, and pancreatic polypeptide were consistently detected by RT-PCR in in vitro cell clusters in the presence of serum. In addition, transcripts for carb A and amylase were also found (data not shown). However, no gene product except for insulin could be detected by immunohistochemistry during in vitro differentiation, suggesting that the gene expression level is still very low. On the other hand, when prominin1^+^PDGFRβ^−^ cells were plated on PA6 cells and cultured in the serum-free SF-O3 medium, a glucagon-producing cell cluster was detected (Fig. 3C). However, no IPC were obtained under such serum-free conditions. In addition, exocrine cells could not be induced under any conditions examined.

**Putative pancreatic progenitor cells have the ability to differentiate into pancreatic**
tissue in mice. In preliminary experiments, cell growth could be routinely detected in PDGFRβ- epithelial cell suspensions, prepared by enzymatic digestion of eGFP transgenic E13.5 pancreas. These units regenerated microscopic outgrowth within 4 weeks after engraftment with either the embryonic pancreas tissues or pancreatic mesenchymal tissues from wild type mice into the subcapsular renal space of nude mice. One week after engraftment of prominin1+PDGFRβ- cells, but not prominin1−PDGFRβ- cells, tubular structure was identified in the graft, which was similar to that of the embryonic pancreatic epithelium. This was confirmed by the observation that these cells forming the tubular structure expressed Pdx1 in the nucleus and prominin1 in the apical membrane (Fig. 4A).

To investigate whether prominin1+PDGFRβ- cell can undergo self-renewal, GFP+ cells in the graft derived from primary transplants of 15,000 prominin1+PDGFRβ- cells were analyzed by flow cytometry and performed a serial transplantation as described previously (Fig. 4B) (19). The prominin1+GFP+ profile in the primary graft (4.1%) was comparable to that in donor pancreas as described above (3.44 ± 0.64%). For secondary transplantation, 12,000 prominin1+GFP+ cells in the primary graft were
used and retrieved 10,000 prominin1^GFP^ cells and yielded the same prominin1^GFP^ profile in the secondary graft (3.7%) as that in the primary graft.

The differentiation potential of the transplanted cell population was further investigated. One week after engraftment, gene products for insulin and glucagon were detected in the same cell cluster, but not in the same cell (Fig. 5A). Moreover, the IPC coexpressed Pdx1 in the nucleus and proinsulin C-peptide in the cytoplasm (Figs. 5B and C), suggesting that IPCs in vivo has features of pancreatic β-cells. In addition, the different clusters with features of pancreatic exocrine (amylase^+ or carboxypeptidase A^+) or ductal (Dolichos biflorous agglutinin^+) cells were observed, respectively (Figs. 5D-F).

**Putative pancreatic progenitor cells differentiate into mature endocrine islet-like cell clusters in vivo.** Prominin1^PDGFRβ^- cells differentiated into IPCs and glucagon-producing cells one week after transplantation. However, islet-like cell clusters in vivo are different from pancreatic islets in several ways. No other endocrine marker like somatostatin or pancreatic polypeptide was detected. Moreover, β-cells in pancreatic islets are characteristically postmitotic (20), but most of the IPC clusters
derived from prominin1⁺PDGFRβ⁻ cells include BrdU⁺ cells, thus suggesting that they were proliferating. On the other hand, prominin1⁻PDGFRβ⁻ cells also formed IPC clusters, which did not include BrdU⁺ cells (supplemental online Fig. 3A). This suggests that a prolonged culture in vivo might promote the maturation of endocrine islet-like cell clusters. Based on the observations of the intracellular C-peptide content, it takes from one to two months for pancreatic β-cells to differentiate and achieve maturation (Fig. 3E). Therefore, the graft with prominin1⁻PDGFRβ⁻ cells was analyzed one month after transplantation. Most islet-like cell clusters consisted of IPCs in the center and other pancreatic hormones positive cells in the periphery, including glucagon, somatostatin, and pancreatic polypeptide, which is characteristic of pancreatic islets (Figs. 6A-E). However, no somatostatin or pancreatic polypeptide was detected in some islet-like cell clusters. Furthermore, the prominin1 expression disappeared as islet-like cell clusters formed (supplemental online Fig. 3B). In addition, the intracellular C-peptide content in islet-like cell clusters was comparable to that of 2-month-old pancreatic islets (Fig. 3E). Further examinations need to be performed to show the maturation of islet-like cell clusters including glucose stimulation. On the other hand, we were not able to find any
islet-like cell clusters in the graft with prominin1 'PDGFRβ' cells one month after transplantation.
Discussion

Widespread interest in developing islet replacement strategies has emerged for treatment of human diabetes mellitus. Although a consensus of findings point to the existence of pancreatic stem/progenitor cells in the embryonic pancreas, it remains unclear what kind of cells show the characteristics of stem/progenitor cells, including self-renewal and multi-lineage potential.

Immunohistochemistry demonstrated that prominin1 is expressed on the apical membrane of the Pdx1-expressing pancreatic epithelial cells at E11.5 and E13.5. Prominin1 expression has been previously shown in putative stem/progenitor cells in brain (12), kidney (21), prostate (22), and ES cell-derived progenitors (23). More recently, prominin1 expression has also been reported in neonatal, adult pancreatic ductal progenitor cells (14) and fetal islet progenitor cells (16). In addition, PDGFRβ expression was observed on the surrounding mesenchymal cells at each stage of developing pancreas. Moreover, prominin1high PDGFRβ+ cells were observed in the embryonic pancreas (see Fig. 2A). Prominin1 (CD133) is also used to isolate endothelial progenitor cells (24) or a rare subset of hematopoietic stem cells (HSC) (11),
both of which are derived from mesoderm. To rule out the possibility of transdifferentiation of these cells into pancreatic epithelial cells, for the first time, PDGFRβ was employed as a mesenchymal marker. These results allowed us the prospective isolation of the putative pancreatic stem/progenitor cells.

This study demonstrated that prominin1⁻PDGFRβ⁻ cells include putative stem/progenitor cells of the embryonic pancreas. Several lines of evidence support to this conclusion. Transcriptional markers essential for putative markers of pancreatic stem/progenitor cells are clearly enriched in the prominin1<sup>high</sup>PDGFRβ⁻ cell population, whereas mature markers are enriched in the prominin1<sup>neg</sup>PDGFRβ⁻ cell population, suggesting that prominin1 expression might be specific to putative stem/progenitor cells and diminished through differentiation. Based on histochemistry and RT-PCR, prominin1 expression persists in the apical membrane of the peripheral exocrine acini, but not in islet in neonatal and adult pancreas (data not shown). Since the existence of adult pancreatic stem/progenitor cells is still controversial, further studies are required to determine whether prominin1 is also a putative cell surface antigen of stem/progenitor cells in postnatal and adult pancreas as well. An alternative protocol,
using the receptor for the hepatocyte growth factor (HGF), c-met, was reported to isolate the putative pancreatic progenitors from the neonatal and adult pancreas (6, 14). We performed immunohistochemistry for c-met in the embryonic pancreas and found that c-met is not expressed until E15.5. Thereafter c-met is expressed in the epithelial cells in the pancreas (supplemental online Figs. 4A-5E). These results are consistent with the FACS data (Fig. 2B). In the normal human pancreas, c-met is expressed at high levels only in β-cells (25). In the E18.5 pancreas, we detected prominin1⁺c-met⁺ cells (supplemental online Fig. 4F). In addition, we obtained the insulin⁺c-met⁺ or glucagon⁺c-met⁺ cells, but not DBA⁺c-met⁺ cells in the E18.5 pancreas (supplemental online Fig. 4G-I). Moreover, prominin1-expressing cells expressed DBA (data not shown). Taken together, we assume that c-met is not essential for the isolation of pancreatic progenitor cells at this stage. The exocrine tissue differentiation are also not well presented in the previous report (14). On the other hand, previous report has shown a CD49f⁺high prominin1⁺ population at E15.5 embryonic pancreas to be composed of differentiated exocrine cells and pancreatic progenitor cells (16). However, as we mentioned before, prominin1 is expressed on the cell surface of endothelial progenitor
cells (24) or a rare subset of hematopoietic stem cells (11). In addition, CD49f is also expressed in primitive hematopoietic cells and endothelial progenitor cells (26-29), both of which are derived from mesoderm. Since PDGFRβ expression was used to eliminate the mesenchymal cells and a microarray analysis strongly supported prominin1^high^PDGFRβ^-^cell population excluded the mesoderm derivatives, we assume prominin1^high^PDGFRβ^-^cells are different from prominin1^CD49f^high^cells. Moreover, we examined further cell sorting of prominin1^high^PDGFRβ^- and prominin1^neg^PDGFRβ^- cell population with CD49f, and obtained CD49f^high^ and CD49f^low^ cell population from prominin1^high^PDGFRβ^- cells, CD49f^high^ and CD49f^neg^ cell population from prominin1^neg^PDGFRβ^- cells, respectively (supplemental online Fig. 5A). This fractionation result was different from that previously described (16). Moreover, real-time quantitative RT-PCR for amylase showed both prominin1^high^PDGFRβ^-CD49f^high^ and prominin1^neg^PDGFRβ^-CD49f^high^ to include amylase gene expression (supplemental online Fig. 5B), thus suggesting that CD49f is available to stain the exocrine cells also in our protocol on E13.5 pancreas.
Stem cells are generally considered to exhibit the following properties: 1) differentiation into multilineage cells, 2) self-renewal (30). In this study, the expansion capacity of cultivated stem/progenitor cells is limited as well as it is in HSC. An arrest of the cell cycle or a slow rate of proliferation is characteristic of HSC and other stem cells. Moreover, most cells from tissues do not self-renew *in vitro* and eventually stop proliferating. In the steady state, only 8% of long term self-renewing HSC (LT-HSC) will enter the cell cycle per day in a regulated fashion (31). *In vitro* differentiation yielded only 150 IPCs from 5,000 prominin1⁺PDGFRβ⁻ cells in the presence of serum. One cluster includes 3-10 IPCs, so that the frequency of the insulin-producing progenitors was less than 1% of prominin1⁺PDGFRβ⁻ cells. This is consistent with a recent report (16). Under these conditions, no glucagon positive cells were detected. Previous studies reported that low blood glucose level allowed the fetal sheep pancreas to differentiate into glucagon-producing α cells rather than β cells (32). Based on this, the cells were switched to low glucose medium, and this yielded glucagon-positive cells in the serum-free condition. However, no other endocrine-positive cells like
somatostatin and pancreatic polypeptide, and exocrine cells were detected by immunohistochemistry.

To evaluate whether prominin1⁺PDGFRβ⁻ cells have a potential to differentiate into pancreatic cells in vivo, these cells were engrafted. This resulted in the differentiation of endocrine, ductal, and exocrine cells with characteristics of the pancreas tissue. Notably, one month after engraftment, intracellular C-peptide content in IPC increased and showed comparable level to that in pancreatic β-cells. However, since the issue of heterogeneity of the prominin1⁺PDGFRβ⁻ cell population still remains to be elucidated, we were not able to rule out the possibility that the different cell types detected in the graft arose from different progenitor cells. Even in the in vitro experiment, we did not detect the colonies with evidence of progeny with mixed lineage derived from a single cell. We therefore should note that it is difficult to conclude that prominin1⁺PDGFRβ⁻ cells have multi-lineage potential in this study. In addition, we employed a serial transplantation experiment to examine self-renewal. We detected similar population of prominin1⁺GFP⁺ profile in the graft and retrieved similar number of prominin1⁺GFP⁺ cells during a serial transplantation. However, we should note that
further experiments need to be performed to answer the following questions: 1) what cell type is self-renewing? 2) Do prominin1\(^+\)GFP\(^+\) cells proliferate and/or differentiate asymmetrically?

Although the concept of stem cells has been extended from HSC to many other tissues, only rarely have stem cells been identified as clonogenic precursors that include in their progeny both self-renewing stem cells and differentiated progeny. Based strictly on this definition, stem cells reported in other tissues are not clonogenic. To prove definitively that prominin1\(^+\)PDGFR\(\beta\)\(^-\) cells have stem cell characteristics, it is necessary to transplant individual prominin1\(^+\)PDGFR\(\beta\)\(^-\) cells and reconstitute the pancreas as well as the hematopoietic or mammary gland stem cells (19, 33). Alternatively, we need to employ genetic lineage tracing mice, which were established by Dor et al. (7) in this study to demonstrate that the prominin1\(^+\)PDGFR\(\beta\)\(^-\) population has a multilineage potential, but not including progenitors for different lineages.

**Conclusion**

We herein demonstrated that sorting for prominin1 and PDGFR\(\beta\) makes it possible to enrich putative fetal pancreatic progenitor cells and these cells are able to differentiate
into pancreatic tissues, including endocrine, exocrine, and duct tissues *in vivo*. 
Acknowledgments

We would like to thank Drs. Masato Kasuga and Susumu Seino for valuable advice and comments on the manuscript. We are grateful to Mitsuko Katsukawa for technical assistance of histology, Muneaki Miyata for FACS, Kenichi Sasamoto for MACS, Motohiko Tanino for DNA Chip analysis, and Dr. Masaru Okabe for the gift of eGFP transgenic mice. This research was supported by grants for the 21st Century COE Program, “Center of Excellence for Signal Transduction Disease: Diabetes mellitus as Model” (to Y.H.), other grants-in aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology of Japan (C) to Y.H. and (A) to Y.K., Kurozumi Medical Foundation (to Y.H.). YH is a Juvenile Diabetes Research Foundation (JDRF) advanced postdoctoral fellow. The authors indicate no potential conflicts of interest.
References


8  Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 2002;129:2447-2457.


Figure legends

Figure 1. Prominin1 (CD133) is expressed on the pancreatic epithelium whereas PDGFRβ is expressed on the surrounding mesenchyme of an embryonic pancreas. Prominin1 is expressed on the apical membrane of the Pdx1-expressing pancreatic epithelial cells at embryonic day (E) 11.5 and E13.5, when the majority of the epithelium consists of undifferentiated progenitor cells (A and E), whereas PDGFRβ (CD140b) is expressed on the surrounding pancreatic mesenchymal cells (B and F). Mature pancreatic marker, glucagon is expressed on a few scattered cells in the E11.5 pancreatic bud (D and H), while insulin-expressing β-cells are detected in the E13.5 pancreas (C and G). Scale bar = 50µm.

Figure 2. Prominin1 expression enriches embryonic pancreatic stem/progenitor cells. (A) Flow cytometric isolation of embryonic pancreatic cells with prominin1 and PDGFRβ monoclonal antibodies. The percentage of the gated cells is as follows (n = 15; data are represented as mean ± SEM); 1) prominin1\textsuperscript{high}PDGFRβ\textsuperscript{−} cells (15.3 ± 2.8%), 2) prominin1\textsuperscript{dim}PDGFRβ\textsuperscript{−} cells (11.4 ± 2.2%), 3) prominin1\textsuperscript{neg}PDGFRβ\textsuperscript{−} cells
(6.5 ± 1.2%), 4) PDGFRβ⁺ cells (66.8 ± 9.8%). (B) prominin₁^{high}PDGFRβ⁻ cells were characterized with other stem/progenitor cell surface markers including c-met, c-kit, Sca-1, CD45, and CD49f. (C) Gene expression analysis of each cell population by RT-PCR. (D) Immunofluorescent images of post-sorted prominin₁^{neg}PDGFRβ⁻ cells and prominin₁^{high}PDGFRβ⁻ cells. Pdx-1 expressing stem/progenitor cells are enriched in prominin₁^{high}PDGFRβ⁻ cells by cell sorting. Scale bar = 50μm.

Figure 3. In vitro differentiation potential of pancreatic stem/progenitor cells.

(A-C) Immunofluorescent images of differentiated cells derived from prominin₁⁻PDGFRβ⁻ cells were obtained by confocal microscopy. Pdx1 and insulin (A), C-peptide and insulin (B), and glucagon (C). Scale bar = 50μm. (D) 5,000 cells from each population were sorted onto PA6 cells in 12-well plate. The number of IPCs was counted after 7 days (n = 5; mean ± SEM; *P < 0.01). (E) Intracellular C-peptide content in IPC in vitro after 7 days culture, IPC in vivo after one-month transplantation, and islet at each stage (post natal day 1, 7, 2 weeks, and 2 months).
Figure 4. Serial transplantation of pancreatic stem/progenitor cells.

(A) Immunofluorescent images of prominin1 and Pdx1 double-positive cells after 7-days transplantation. The original magnification was 400X. (B) Pancreatic stem/progenitor cells (prominin1⁺PDGFRβ⁻ cells derived from eGFP transgenic mice) can be detected and characterized by their ability to differentiate into pancreatic tissue when transplanted into recipient nude mice and by their properties as assessed by transplantation into secondary recipients.

Figure 5. Multi-lineage potential of pancreatic stem/progenitor cells.

5 x 10⁴ pancreatic stem/progenitor cells (prominin1⁺PDGFRβ⁻ phenotype) or 5 x 10⁴ prominin1⁺PDGFRβ⁻ were transplanted in the subcapular renal space.

Immunofluorescent images of engrafted prominin1⁺PDGFRβ⁻ cells after 7-days transplantation. (A) insulin and glucagon, (B) insulin and Pdx1, (C) C-peptide and insulin. The original magnification was 400X. (D) amylase and (E) carboxypeptidase A are exocrine markers, (F) DBA is ductal markers. The original magnification was 1000X.
Figure 6. Endocrine differentiation and maturation of pancreatic stem/progenitor cells with the characteristics of a mature endocrine islet.

$5 \times 10^4$ pancreatic stem/progenitor cells (prominin1$^+$PDGFR$^-$ phenotype) or $5 \times 10^4$ prominin1$^-$PDGFR$^-$ were transplanted in the subcapular renal space.

Immunofluorescent images of engrafted prominin1$^+$PDGFR$^-$ cells after one-month transplantation. (A) insulin and Pdx1, (B) C-peptide and insulin, (C) insulin and glucagon, (D) insulin and somatostatin, and (E) insulin and pancreatic polypeptide (PP).

Scale bar = 50µm.
A) 

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<th>GFP</th>
<th>CD133</th>
<th>Pdx1</th>
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B) 

Transplant cells with pancreatic mesenchyme or pancreas from e13.5 WT mice

embryonic pancreas from e13.5 GFP transgenic mice

CD133+/PDGFRβ-

2 weeks graft analysis

CD133

GFP

2 weeks graft analysis

CD133

GFP