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The Adult Mouse and Human Pancreas Contain Rare Multipotent Stem Cells that Express Insulin

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SUMMARY

The search for putative precursor cells within the pancreas has been the focus of extensive research. Previously, we identified rare pancreas-derived multipotent precursor (PMP) cells in the mouse with the intriguing capacity to generate progeny in the pancreatic and neural lineages. Here, we establish the embryonic pancreas as the developmental source of PMPs through lineage-labeling experiments. We also show that PMPs express insulin and can contribute to multiple pancreatic and neural cell types in vivo. In addition, we have isolated PMPs from adult human islet tissue that are also capable of extensive proliferation, self-renewal, and generation of multiple differentiated pancreatic and neural cell types. Finally, both mouse and human PMP-derived cells ameliorated diabetes in transplanted mice. These findings demonstrate that the adult mammalian pancreas contains a population of insulin$^+$ multipotent stem cells and suggest that these cells may provide a promising line of investigation toward potential therapeutic benefit.

INTRODUCTION

The search for putative precursor cells within the pancreas has been the focus of extensive research, largely because of their envisioned use in the generation of new β cells for therapeutic cell replacement strategies in the treatment of diabetes. Previous work identified a novel precursor population, termed pancreas-derived multipotent precursors (PMPs), resident within the adult murine pancreas (Seaberg et al., 2004). A single PMP cell was capable of in vitro proliferation to form a clonally derived sphere colony, which expressed both neural and pancreatic precursor genes. Upon differentiation, clonal PMP spheres generated a cellular output containing multiple differentiated cell types of both lineages. Further, the β cells produced de novo were found to display functional glucose-stimulated Ca^{2+} responses and insulin secretion.

The intriguing capacity of these cells to generate progeny of distinct germ layers—endodermal pancreatic and ectodermal neural—raises questions as to their in vivo identity and ontogenic developmental compartment. Use of an in vitro sphere formation assay, while facilitating study of these cells, does not yield any prospective information regarding the in vivo characteristics of the PMP cells. We found that the mouse PMP cells do not express nestin as a specific marker, nor do they have an ESC or mesodermal precursor character (Seaberg et al., 2004). The neural crest (NC) was considered as the ontogenic origin of PMP cells, because it is a developmental structure that has a broad contribution to various lineages and has been proposed to be the source of most, if not all, highly plastic adult stem cells (Pierret et al., 2006). Though PMP sphere colonies were found not to express genes associated with the NC, such as *P75*, *Pax3*, *Twist*, *Sox10*, and *Wnt1* (Seaberg et al., 2004), their absence does not rule out a NC origin. The current study resolves this issue by including NC lineage-labeling and *PDX-1* lineage-labeling experiments, which exclude the NC, and establishes the embryonic *PDX-1* lineage as the source of PMP cells in the adult.

Although it has been known that β cells retain a limited capacity for replication (Messier and Leblond, 1960; Kassem et al., 2000), a surprising study by Dor et al. (2004) argued that new β cells in the adult were formed exclusively by self-duplication without any contribution from stem cell differentiation. This study generated controversy within the field, because it challenged the significance of work from many groups investigating the existence and role of putative adult pancreatic stem cells. Another recent study from the same group furthered these findings to suggest that there are no pancreatic stem cells in the developing embryo nor the adult and that the pancreas is formed by progenitors that are autonomously restricted, capable of producing only a fixed amount of tissue (Stanger et al., 2007). In the Dor et al. (2004) study, the authors utilized an elegant lineage-tracing technique by using mice with a tamoxifen-inducible transgene driving expression of the Cre recombinase enzyme under control of the rat insulin promoter (*RIP-Cre-ER* mice). After breeding with a reporter mouse strain, tamoxifen injections induced indelible labeling of insulin-expressing cells and their progeny. Dor et al. (2004) provided evidence that new β cells were generated entirely from such lineage-labeled cells, and thus concluded that old β cells were the source of new. However, such a conclusion is based on the assumption that

all insulin-expressing cells are true mature β cells and the corollary point that any putative stem cells would not express insulin. This may not necessarily be true, because stem cells may express ostensible differentiation markers in vivo, as has been observed with the expression of GFAP, a differentiated astrocyte marker, in neural stem cell populations within the brain (Morshead et al., 2003). As such, it is possible that any putative pancreatic stem cell population actually may express insulin in vivo. To investigate whether our PMP cell population expressed insulin in vivo, we used transgenic mice constitutively expressing GFP under control of the mouse insulin promoter (*MIP-GFP* mice) (Hara et al., 2003). In addition, the same *RIP-Cre-ER* mice used by Dor et al. (2004) were employed to verify the findings obtained with the *MIP-GFP* mice and to explore the lineage potential of insulin$^+$ cells in vivo. These experiments demonstrate conclusively that the PMP cells do indeed express insulin in vivo and that insulin$^+$ cells have the capacity to generate multiple types of pancreatic and neural cells in vivo in the normal adult pancreas.

We also sought to determine whether the human pancreas contained cells analogous to the PMP cells that we have identified in mice. It was found that adult human islet tissue contained cells capable of clonal proliferation to form colonies that were self-renewing and multipotent in the neural and pancreatic lineages, which produced functional β cells. The therapeutic potential of PMPs was then established by transplantation of mouse or human PMP-derived cells into diabetic mice. Thus, PMP cells represent an insulin-expressing multipotent stem cell population resident within the adult mammalian pancreas.

RESULTS

Pancreas, and Not Neural Crest, Is the Embryonic Source of PMP Cells

We have demonstrated by RT-PCR analysis that clonal spheres produced by proliferating PMP cells do not express genes associated with the NC (Seaberg et al., 2004); however, the transient nature of these markers is such that their absence in adult cell-derived colonies does not preclude a NC origin for PMP cells during development. To address this, we employed transgenic mice expressing the Cre enzyme under the control of the *Wnt1* promoter (*Wnt1-Cre* mice) (Danielian et al., 1998). *Wnt1* expression is restricted to the NC in the periphery (Echelard et al., 1994). Crossing the *Wnt1-Cre* mice with the *Z/EG* reporter strain (Novak et al., 2000) yielded mice in which the NC lineage remained fluorescently labeled with GFP. Confocal visualization of pancreatic sections from these *Wnt1-Cre* × *Z/EG* mice revealed the presence of GFP$^+$ cells displaying process-bearing morphologies, not typically observed in pancreatic exocrine and endocrine cells, which represent peripheral neuronal or Schwann cells (not shown). Primary cells from both islet and ductal tissue compartments were subjected to FACS followed by plating of positive and negative fractions in the PMP sphere formation assay. FACS showed that 5.0% ± 0.2% of primary islet and 4.1% ± 0.3% of duct compartment cells were fluorescent and thus derived from the NC. Notably, 100% of the PMP spheres were generated by cells in the sorted negative fraction. At day 4 of the assay, the negative cells were already proliferating to form spheres, whereas the positive cells were largely adherent, developing spindle-like morphologies by day 8 (not shown). These data show that all PMP spheres came from unlabeled cells and thus verify that the sphere-initiating PMP cells are not NC derived. To determine whether PMPs arose from a pancreatic endoderm source developmentally, we utilized *PDX-1-Cre* (Gu et al., 2002) × *Z/EG* mice to GFP label the entire *PDX-1* lineage in adult mice. FACS of primary cells and plating of sorted fractions in the PMP assay revealed that the GFP$^+$ cell fraction had higher sphere-formation capacity (4.0 ± 1.2 spheres/10,000 cells) than did the GFP$^-$ fraction (0.9 ± 0.5 spheres/10,000 cells). As a result, the vast majority (95.2% ± 0.6%) of spheres came from the GFP$^+$ cell fraction, verifying that the PMP cell arose from a *PDX-1*$^+$ pancreatic lineage developmentally.

PMP Cells Express Insulin In Vivo

Published studies have suggested that any newly generated β cells in vivo arise from insulin-expressing cells (Dor et al., 2004). To determine whether PMP cells express insulin in vivo, transgenic *MIP-GFP* mice were used. β cells within islets fluoresced with GFP (Figure S1A available online). We verified the fidelity of the transgene by immunostaining of primary cells. The insulin$^+$ cells were GFP$^+$, the GFP$^+$ cells were C-peptide$^+$, and both glucagon$^+$ and somatostatin$^+$ cells were GFP$^-$ (Figures S1B–S1E). Primary cells from both islet and ductal tissue compartments were subjected to FACS followed by plating of GFP-positive and -negative fractions in the PMP sphere formation assay. The positive fractions contained sphere-initiating cells at a frequency ~50-fold greater than the negative fractions (4.59 ± 0.81 and 0.09 ± 0.04 spheres/10,000 cells, respectively). On average, 95.3% ± 2.1% of the resultant spheres were generated by cells within the positive fractions. Indeed, in two of four experiments, 100% of the islet-derived spheres arose from the positive fraction. In an effort to discriminate subpopulations of insulin$^+$ cells, the *MIP-GFP*-positive fraction was sorted based on antibody staining to the β cell surface glucose transporter, Glut-2 (Figure S1F), and 100% of the resultant spheres arose from the sorted Glut-2^{low} cell fraction. These data show that PMP cells do in fact express insulin in vivo but do not display a mature β cell phenotype, as evidenced by low Glut-2 expression.

The spheres produced (Figure 1A) from the single insulin-expressing GFP$^+$ PMP cell do not then express GFP uniformly in all cells (58.8% ± 3.8% of sphere cells were GFP$^+$), as we might expect if these multipotent sphere colonies were capable of producing other pancreatic and neuronal progeny in addition to β cells. To demonstrate the multipotentiality of the clone-initiating GFP$^+$ PMP cell, spheres from the positive fraction were differentiated and subsequently immunostained. The generation of insulin-expressing β cells was confirmed by staining for insulin, which colocalized with the GFP from the transgene (Figure 1B). Coexpression of PDX-1 and GFP was also observed (not shown). The *MIP-GFP* transgene further allowed for FACS sorting of β cells from the differentiated spheres, which were then subjected to electron microscopy analysis. Figure 1C shows a sample electron micrograph of an in vitro-generated β cell, displaying archetypical β cell cytoarchitecture, including many large dense-core insulin granules. Other endocrine cell types were also produced. Note that the expression of glucagon in α cells (Figure 1D) and somatostatin in δ cells (Figure 1E) did

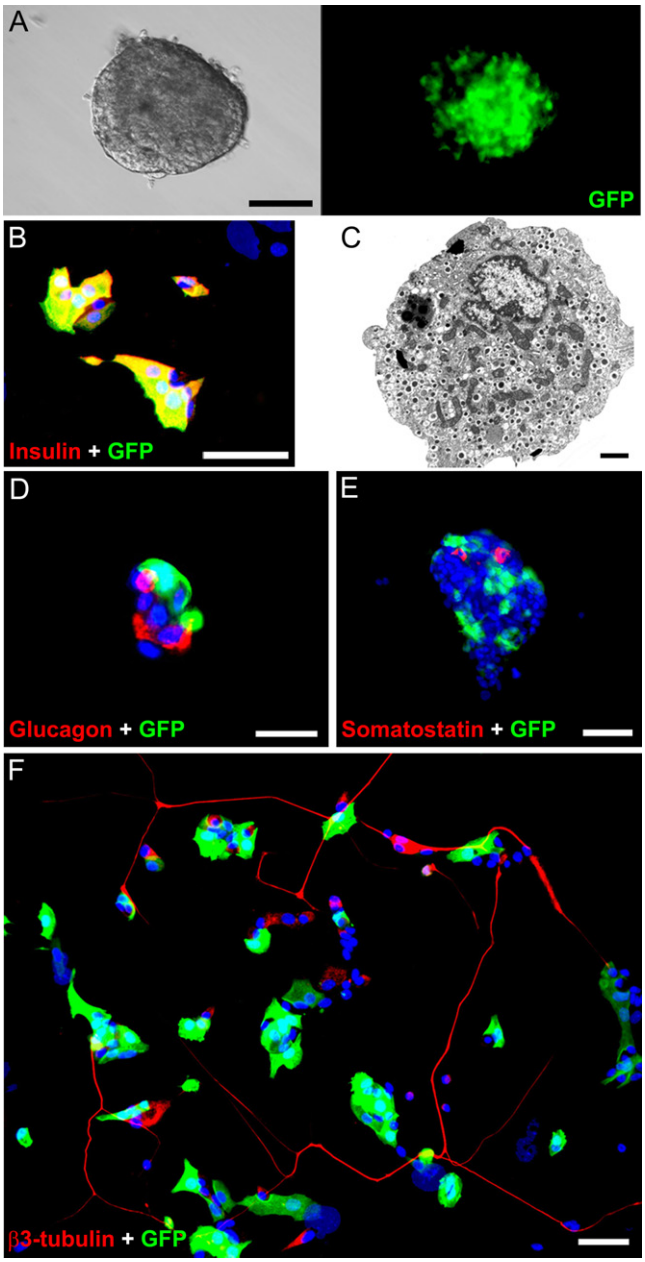

Figure 1. Insulin-Expressing Cells from *MIP-GFP* Mice Proliferate to Form PMP Spheres that Are Multipotent in the Pancreatic and Neural Lineages

(A) Spheres formed by proliferation of a single GFP$^+$ insulin-expressing cell do not display insulin expression in all cells.

(B–D) Such spheres could be differentiated to yield (B) insulin$^+$/GFP$^+$ β cells, one of which is shown in an electron micrograph (C), as well as (D) glucagon$^+$ α cells and (E) somatostatin$^+$ δ cells. Note that glucagon and somatostatin expression does not overlap with that of GFP.

(F) The same clonally derived sphere that generated GFP$^+$ insulin-expressing β cells was also able to produce β3-tubulin$^+$ neurons.

Cell nuclei are stained blue. Scale bars represent 50 μm in (A), (B), (E), and (F), 1 μm in (C), and 25 μm in (D). See also Figure S1.

not colocalize with the transgenic GFP. To verify their bilineage cross-germ layer potential, the differentiated colonies were stained for the neuronal marker β3-tubulin, which labeled distinct cells with obvious neuronal morphologies in the same clonally derived sphere that produced GFP$^+$ insulin-expressing β cells (Figure 1F).

To verify the findings obtained with the constitutive *MIP-GFP* mice, the inducible *RIP-Cre-ER* mice used in Dor et al. (2004) were crossed with the *Z/EG* strain. Adult *RIP-Cre-ER* × *Z/EG* mice were injected with 6 mg tamoxifen every 2 days (three injections total), and the mice were sacrificed 1 day after the final injection. This protocol GFP-labeled a percentage of the in vivo insulin-expressing cells, with inherited labeling of the progeny of these cells. Primary islets displayed GFP expression in a fraction of total cells (Figure 2A). As a control, tamoxifen was administered to *Z/EG* mice, and no GFP expression was observed. Cell quantification of immunostained pancreatic sections (fixed at time of sacrifice) revealed that 26.3% ± 1.4% of insulin$^+$ cells were GFP$^+$ (Figure 2B). Primary cells from both islet and ductal tissue compartments were subjected to FACS followed by plating of positive and negative fractions in the PMP sphere-formation assay. As expected, clonal spheres were generated from both fractions (Figures 2C and 2D), with 35.8% ± 6.6% arising from the positive fraction (at a frequency of 3.56 ± 0.52 spheres/10,000 cells) and 64.2% ± 6.6% from the negative fraction (at a frequency of 1.06 ± 0.20 spheres/10,000 cells). Spheres derived from the positive fraction were composed of 98.9% ± 0.3% GFP$^+$ cells (indicating very rare transgene shutoff), whereas sphere cells from the negative fraction were GFP$^-$. The presence of PMP cells was expected in both fractions, because tamoxifen induction of the transgene occurred within only a portion of the insulin-expressing cells (Dor et al., 2004), and thus there were insulin-expressing cells in both fractions. The multipotentiality of the clonal spheres derived from a single GFP$^+$ cell was confirmed by differentiation and immunostaining (Figure S2). The formation of PMP spheres from the positive fraction confirms that PMP cells arise from an insulin-expressing cell population in vivo.

Insulin-Expressing Cells within Undifferentiated PMP Spheres Are Distinct from Mature β Cells and Exhibit a Precursor Phenotype

The present data indicate that PMP cells represent a rare insulin$^+$ precursor population comprising a very small subset of the larger population of insulin-expressing cells in vivo. Given this finding, we sought to better characterize the insulin-expressing cells within the undifferentiated PMP spheres and to compare them to the insulin-expressing cell population within adult pancreatic islets (predominantly mature β cells). To do this, we applied FACS to primary pancreatic islet cells and PMP sphere cells from *MIP-GFP* mice (Figure S3), allowing us to obtain two pure populations of insulin-expressing cells from these distinct sources. Subsequently, RT-qPCR analysis was performed to assess the expression levels of key pancreatic genes (Figure 3A). Interestingly, the expression levels of both murine insulin genes were substantially lower in the PMP-derived cells as compared to the islet-derived cells. The low insulin mRNA expression revealed by qPCR could be validated at the protein level by the *MIP-GFP* transgene product's fluorescence intensity (measured during

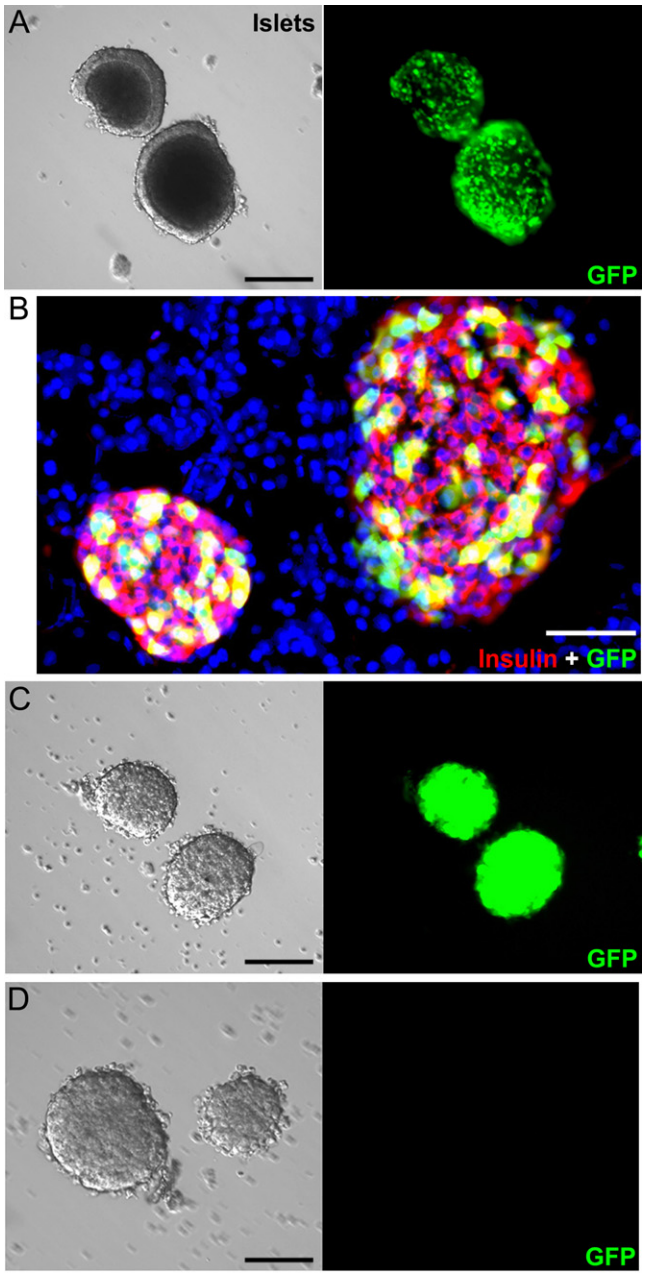

Figure 2. PMP Spheres Were Generated by Cells in the GFP-Positive and GFP-Negative Cell Fractions from *RIP-Cre-ER* × *Z/EG* Mice

Tamoxifen injections into *RIP-Cre-ER* × *Z/EG* mice labeled a fraction of insulin-expressing cells with GFP in (A) live primary islets and (B) in immunostained pancreatic sections. Cell nuclei are stained blue. After FACS and plating in the PMP sphere assay, single cells from both the (C) GFP$^+$ and (D) GFP$^-$ cell fractions were capable of producing PMP spheres. Scale bars represent 100 μm. See also Figure S2.

FACS), as shown by the fact that the median GFP intensity of the PMP-derived cells was 31.2% ± 13.2% of that observed for the islet-derived cells. On the contrary, *PDX-1* and *Nkx6.1* expression were observed in the PMP-derived cells at levels comparable to those of the islet-derived cells (Figure 3A). Moreover, it appeared that *Ngn-3* expression in the PMP-derived cells was actually higher than the levels observed in islet-derived cells, consistent with our detection of rare insulin$^+$/Ngn-3$^+$ cells in the normal adult pancreas (Figure S4). Notably, *Glut-2* was expressed at near-absent levels in the PMP-derived cells, whereas levels in the islet-derived cell population were much higher. The low level of *Glut-2* within insulin-expressing cells of undifferentiated PMP spheres is similar to the in vivo PMP cell phenotype (see preceding section) and was confirmed by immunostaining and confocal analysis of BalbC pancreatic islet and PMP sphere sections (Figure 3B). Additionally, we extracted another relevant parameter from the FACS analysis. Mature β cells exhibit a high level of granularity because of their content of many large dense-core insulin granules, which translates to increased FACS side-scatter. The FACS side-scatter profiles (Figure S3) revealed that the PMP-derived cells displayed a median side-scatter that was 44.7% ± 0.6% of that displayed by the islet-derived cells. Collectively, these data indicate that the insulin-expressing cells within a PMP sphere display characteristics distinguishing them from mature β cells and appear to exhibit a precursor phenotype. Additional RT-qPCR experiments demonstrated that the precursor phenotype of PMP spheres was dissimilar from that of mesenchymal stem cell (MSC) populations derived from the bone marrow (Figure S5), further supporting their unique identity.

Insulin-Expressing Cells Generate Multiple Pancreatic and Neural Cell Types In Vivo

The present data demonstrate that a subset of the insulin-expressing cells in vivo (i.e., PMPs) represent a population of cells that have been characterized to possess precursor properties in vitro; however, the question remains as to whether they function in vivo as precursor cells and whether they are relevant to normal adult pancreatic cell turnover. To address this, adult *RIP-Cre-ER* × *Z/EG* mice injected with tamoxifen were maintained for 10 weeks after the final injection before sacrifice. This was the "chase" group of mice, as opposed to the "pulse" group that was sacrificed 1 day after the final injection. Because these mice are insulin lineage labeled, any evident GFP$^+$ cells arose from an insulin-expressing cell in vivo. Tissue sections from the pulse and chase groups were immunostained and subjected to confocal microscopy analysis to carefully characterize the GFP$^+$ cell population. The percentage of insulin$^+$ cells labeled with GFP did not change significantly between the pulse and chase groups (Figure 4A). Notably, however, after the 10-week chase period, 14.2% ± 4.6% of the GFP$^+$ cells were insulin$^-$, whereas virtually all (~99%) GFP$^+$ cells in the pulse group were insulin$^+$ (Figures 4B and 4C), indicating that insulin$^+$ cells had given rise to non-β cells in vivo during the chase period. Additional immunostaining analysis revealed the presence in vivo of various GFP$^+$ pancreatic cell types, with a large increase observed comparing the chase to the pulse groups. These included endocrine glucagon$^+$ α cells, somatostatin$^+$ δ cells, and exocrine amylase$^+$ cells (Figures 4D–4F). Further, GFP$^+$ neural cell types were observed, with GFP coexpressed by GFAP$^+$ glial cells and by Neurofilament-M$^+$ and MAP2$^+$ neuronal cells (Figures 4G–4I). Quantification was performed to determine the percentage of some of the marker-positive cells relative to the entire GFP$^+$ cell population in both the pulse and chase groups (Figure 4J), illustrating the progressive production of non-β cells

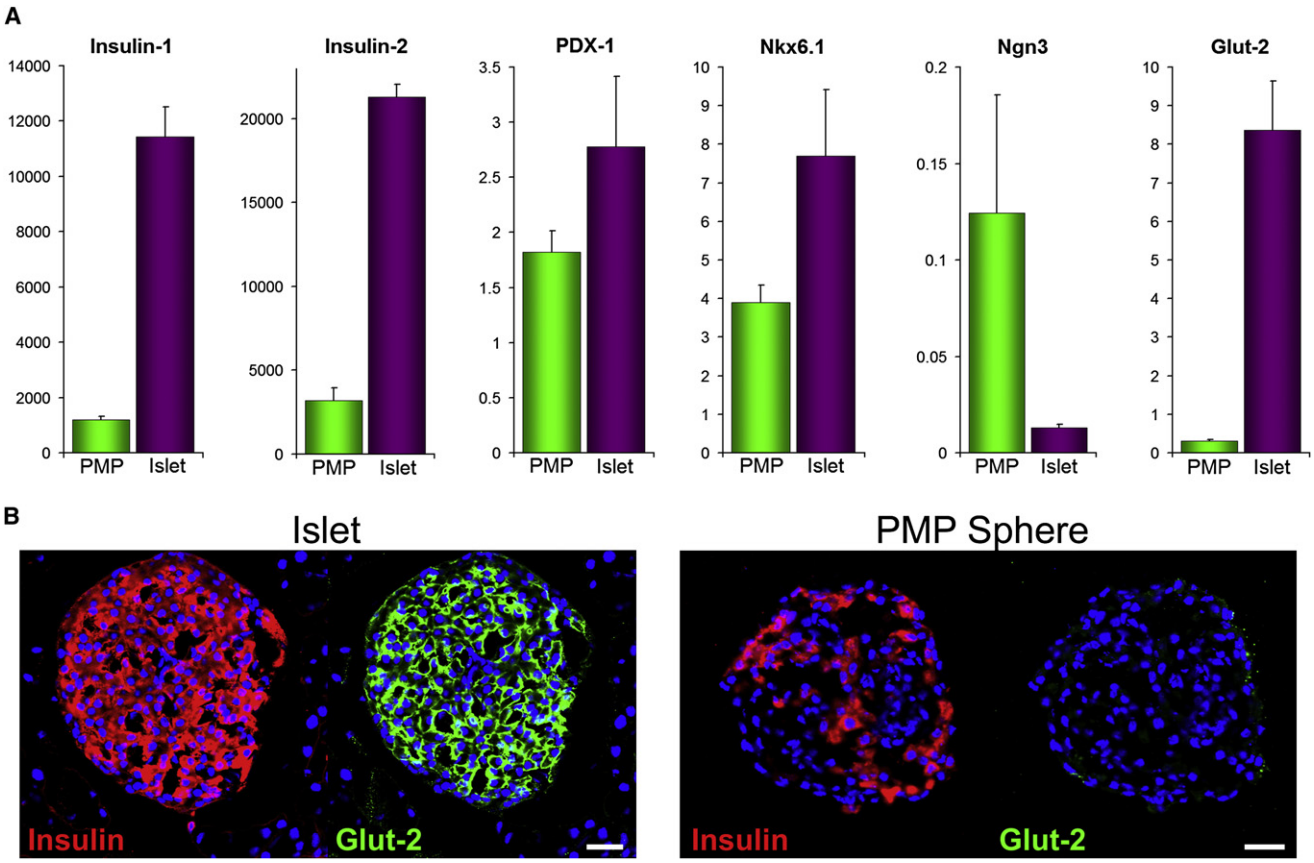

Figure 3. Insulin-Expressing Cells within Undifferentiated PMP Spheres Are Distinct from Mature β Cells

(A) RT-qPCR analysis of key genes expressed in FACS-purified populations of insulin-expressing cells from *MIP-GFP* undifferentiated PMP spheres and islets revealed that they displayed distinct profiles. Error bars are ±SEM.

(B) Confocal slice micrographs of BalbC pancreatic islet and undifferentiated PMP sphere sections, immunostained for insulin and Glut-2. Cell nuclei are stained blue. Scale bars represent 25 μm.

See also Figures S3–S5.

from the original labeled insulin$^+$ population over the 10-week chase period. The presence of rare GFP$^+$ cells expressing the above markers in the pulse group indicates that even during the 6 days from tamoxifen injection commencement, insulin$^+$ cells had given rise to these other cell types. Additional quantification was performed to assess the contribution of GFP$^+$ cells to each marker$^+$ population (Figure 4K), demonstrating significant participation of insulin$^+$ precursor cells in normal adult cell turnover within these non-β cell populations. For this interpretation to be accurate, we needed to verify that tamoxifen induction initiated GFP expression exclusively in insulin-expressing cells. This was performed by tracking GFP expression upon in vitro administration of tamoxifen, which confirmed both the insulin specificity of GFP initiation in the short term and the multipotentiality of insulin$^+$/GFP$^+$ cells over several days in vitro (Figure S6). Thus, these data recapitulate the in vitro demonstrated PMP cell multipotentiality, with multiple pancreatic and neural cell types being generated in vivo from insulin$^+$ precursor cells.

Human PMP Cells

The current and previous results (Seaberg et al., 2004) demonstrate the existence of PMPs and characterize some of their properties, but they are limited to the mouse species. To extend our studies to larger mammals, we obtained adult human pancreatic tissue (n = 17 individuals) and sought to determine whether it contained PMP cells analogous to those we identified in mice. Plating of dissociated human islet tissue in our PMP sphere assay conditions revealed that it contained cells capable of proliferation to form clonal spheres (Figure 5A, inset) at a frequency of 2.6 ± 0.1 spheres/10,000 cells (Figure 5A), a range similar to that seen in mouse (Seaberg et al., 2004). Further, these spheres could be dissociated and subcloned to yield secondary and tertiary sphere colonies (Figure 5A). As a verification of proliferation, primary spheres were grown in the presence of BrdU, and subsequent immunostaining revealed the BrdU positivity of the sphere cells, including cells expressing insulin (Figure S7). To determine the character of the human spheres, we performed RT-PCR analysis. Table S1 shows that the spheres expressed a panel of neural precursor genes (*nestin*, *Sox2*, *Hash-1*, *Ngn-1*, *Ngn-3*, *Olig-1*, and *Pax-6*), early endodermal markers (*HNF3b*, *HNF4*, and *Gata-4*), and the pancreatic transcription factor *PDX-1*. Some of these "neural" precursor genes are also normally expressed in the pancreatic lineage (e.g., *Ngn-3*, *Pax-6*) (Zhang and Sarvetnick, 2003). Early mesodermal markers (*brachyury*, *Gata-1*) were not

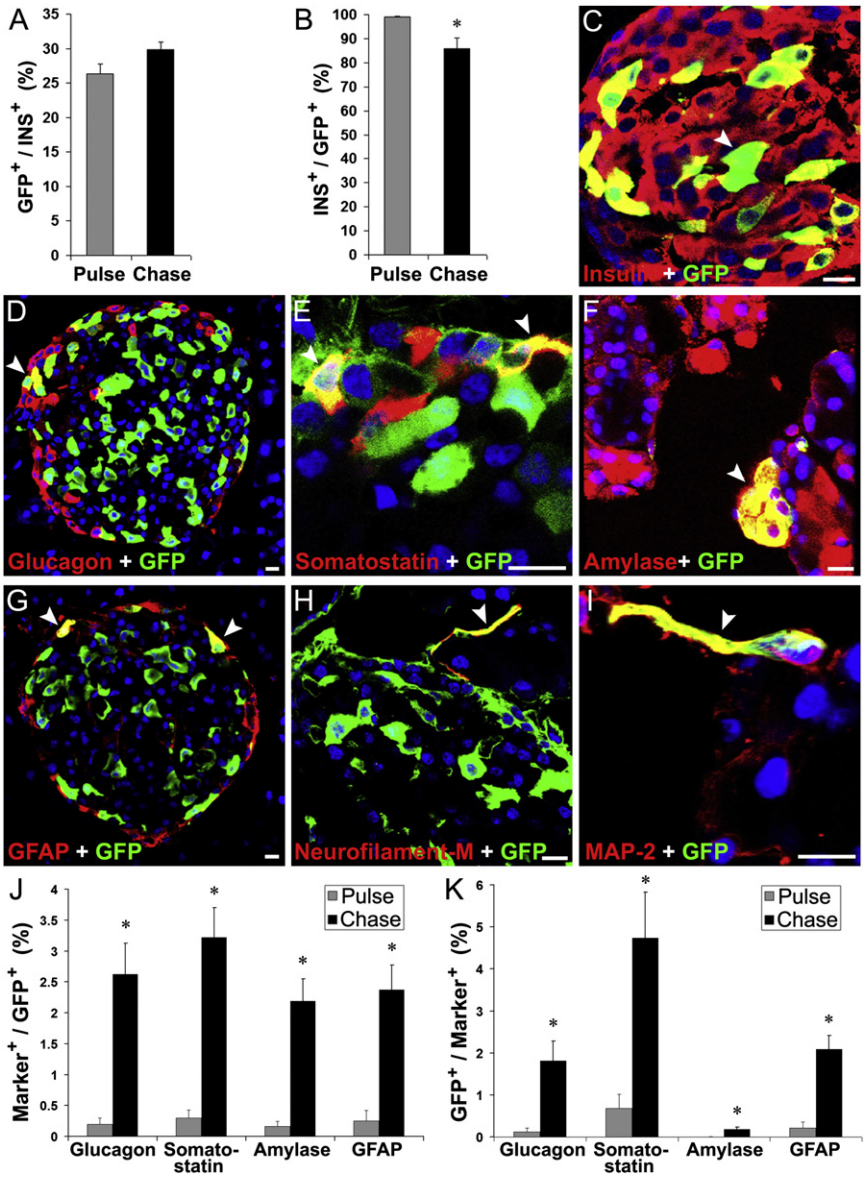

Figure 4. Insulin-Positive Cells Generate Multiple Pancreatic and Neural Cell Types In Vivo

(A–I) Confocal analysis of immunostained pancreatic sections from pulse and chase groups of *RIP-Cre-ER* × *Z/EG* mice showed (A) no increase in the percentage of insulin$^+$ cells labeled with GFP, (B) while the percentage of GFP$^+$ cells expressing insulin dropped during the chase period, as can be seen in (C), in which GFP$^+$/insulin$^-$ cells are indicated by an arrowhead. The insulin-lineage cells gave rise to multiple cell types coexpressing GFP (marked by arrowheads), including pancreatic (D) glucagon$^+$ α cells, (E) somatostatin$^+$ δ cells, (F) amylase$^+$ exocrine cells; neural (G) GFAP$^+$ glial cells; and (H) neurofilament-M$^+$ and (I) MAP-2$^+$ neuronal cells. All images are a single confocal slice, except for MAP-2, which is a z-stack (8 μm thick). Cell nuclei are stained blue. Scale bars represent 10 μm.

(J and K) Quantification of these marker$^+$/GFP$^+$ cells for both the pulse and chase groups is shown (J) as the percentage of GFP$^+$ cells expressing each marker, and (K) as the percentage of the total marker$^+$ cell population that was labeled with GFP. Error bars are ±SEM. *$p < 0.01$ versus pulse in (B), *$p < 0.001$ versus respective pulse in (K)–(J). See also Figure S6.

observed. These findings suggested that the human spheres had both a neural and pancreatic precursor character. To provide additional characterization, RT-qPCR analysis was performed for various key pancreatic genes, comparing the human PMP spheres to primary human islets (Figure 5B). Whereas the expression level of insulin was perhaps somewhat lower in the PMP spheres, it appeared that the human PMP spheres expressed substantially higher levels of *PDX-1*, *Nkx6.1*, *Ngn-3*, *Pax-6*, and *Pax-4* and substantially lower levels of *Glut-2* (similar to the mouse data), consistent with a precursor phenotype.

The human PMP spheres were then differentiated and immunostained to examine their differentiation potential. Generation of β cells was observed by the coexpression of insulin and C-peptide (not shown) and insulin and PDX-1 (Figure 6A), as well as coexpression of insulin with the β cell glucose transporter Glut-2 (Figure 6B). The β cells comprised 11.6% ± 1.1% of the differentiated cell population. In addition, other pancreatic glucagon$^+$ and somatostatin$^+$ endocrine (Figure 6C) and amylase$^+$ exocrine (Figure 6D) cell types were generated. Demonstrating the neural potential of the human spheres, the same clonally derived spheres that produced C-peptide$^+$ β cells also yielded β3-tubulin$^+$ neurons (Figure 6E). Mature neuron production was evidenced by the expression of MAP-2 (Figure 6F) and glial cell generation was demonstrated by the presence of GFAP$^+$ (Figure 6G) and O4$^+$ (Figure 6H) cells. Occasionally, somatostatin$^+$ cells with a neuronal morphology were observed (not shown), possibly suggesting the production of this neuroendocrine cell subtype. RT-PCR analysis of the differentiated spheres verified expression of the differentiated neural and pancreatic cell markers as well as an expanded array of mature β cell markers, whereas the muscle transcription factor *MyoD* was not observed (Table S2). To determine whether the human PMP spheres had an ESC-like character, undifferentiated spheres were immunostained for the ESC markers Oct-4, nanog, SSEA-4, and Tra-160, whose expression was found to be absent in PMP sphere cells (not shown).

Insulin content measurements were performed on differentiated human PMP spheres. They contained 24.8 ± 2.9 ng of insulin/μg of DNA whereas primary human islets contained 204.3 ± 27.9 ng of insulin/μg of DNA or 3.3 ± 0.5 ng of insulin/differentiated PMP sphere and 45.5 ± 8.8 ng of insulin/islet. To demonstrate the functionality of the newly generated β cells, insulin

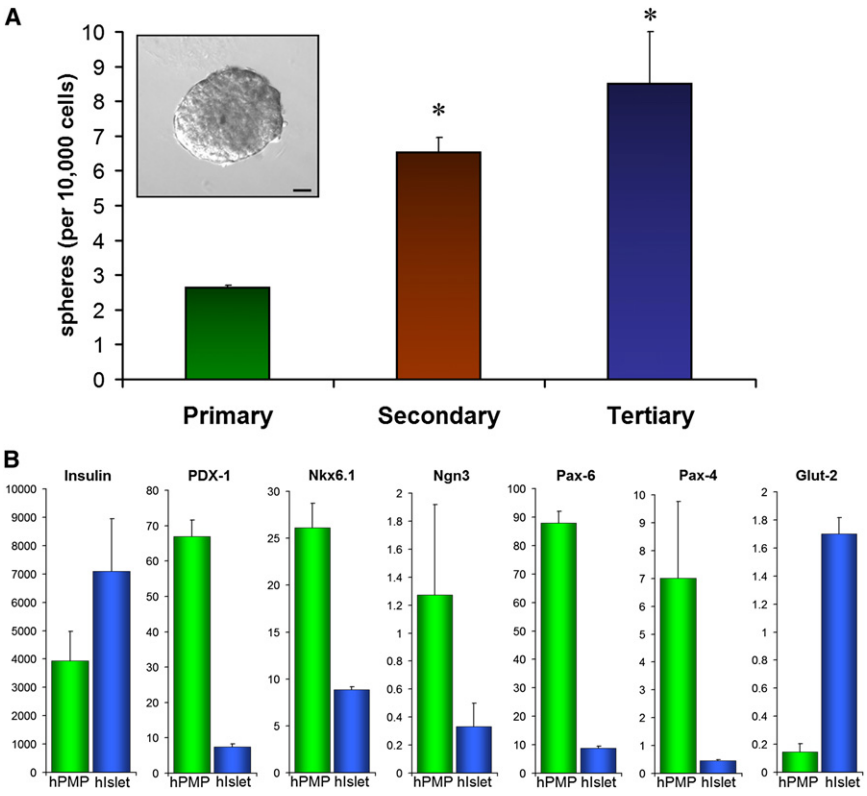

Figure 5. Human Islet Tissue Contains Cells Capable of Proliferation to Form Self-Renewing PMP Sphere Colonies, which Display a Precursor Gene Expression Profile

(A) Primary human islets were dissociated into single cells and cultured in the PMP sphere formation assay. Rare cells proliferated to form spheres (inset), which could be dissociated and subcloned to produce secondary and tertiary spheres. Scale bar represents 25 μm.

(B) RT-qPCR analysis of key genes comparing human PMP spheres (hPMP) to human islets (hIslet) suggested that the PMP spheres possessed a distinct precursor character.

Error bars are ±SEM. ANOVA $F(2) = 66.7$; *$p < 0.001$ versus primary in posttest in (A). See also Figure S7 and Table S1.

secretion measurements were performed. Basal insulin secretion over a 1 hr period under low-glucose (2.5 mM) conditions was 38.9 ± 8.4 pg insulin/differentiated PMP sphere. Insulin release stimulated by high glucose (20 mM) was 2.4- ± 0.5-fold of low glucose-stimulated insulin secretion (Figure 6I). The insulin release stimulated by high glucose could be moderately enhanced by GLP-1 (30 nM), a physiological enhancer of insulin secretion (MacDonald et al., 2002), or by tetraethylammonium (TEA; 10 mM), a compound that inhibits delayed rectifier K^+ currents, prolonging cellular depolarization, and thus potentiating the glucose-stimulated insulin response (Roe et al., 1996). The voltage-dependent Ca^{2+} channel blocker Verapamil (100 μM) abolished glucose-stimulated insulin release (Figure 6I). The lack of robust enhancement of insulin release by GLP-1 inclusion during the secretion assay may be due to the prolonged continuous exposure to GLP-1 during the differentiation conditions. One of the primary modes of GLP-1 action is via cAMP signaling (MacDonald et al., 2002). Therefore, a combination of the membrane-permeable cAMP analog pCPT-cAMP (100 μM) and the cAMP phosphodiesterase inhibitor IBMX (500 μM) was used to potently activate cAMP-induced insulin secretion (Figure 6I). This combination showed a much-increased enhancement of glucose-stimulated insulin release. These data demonstrate that human islet tissue contains a population of multipotent PMP cells with the capacity to generate functional β cells.

PMP Sphere Transplantation Improves the Diabetic State in Mice

Although we have demonstrated the generation of nascent functional β cells from PMPs in vitro, a critical first step to envisioned future therapeutic applications is to test their in vivo function in the glycemic improvement of diabetic mice after transplantation of PMP-derived cell populations. As such, we undertook experiments in which streptozotocin (STZ)-induced diabetic BalbC and NOD-Scid mice were used as recipients for transplantation of mouse and human PMP spheres, respectively. Given that we have not yet achieved high-efficiency β cell production in vitro from PMPs (~5% mouse and ~11% human β cells in differentiated PMP colonies; see above and Seaberg et al. [2004]), and in light of recent reports that the in vivo environment promotes differentiation/maturation of β cells from embryonic stem cell-derived endodermal pancreatic precursors (Kroon et al., 2008), we reasoned that we might be able to enhance mature β cell yield by using undifferentiated PMP spheres for transplantation. Diabetogenesis was initiated by single injection of STZ, and 7 days later mice with comparable levels of hyperglycemia were selected for transplant and control groups. Nonfasted blood sugar levels were then monitored twice a week for 50 days posttransplant. Transplantation of either mouse (Figures 7A and 7B) or human (Figures 7F and 7G) PMP spheres produced a significant amelioration of hyperglycemia, with the mouse PMPs having an effect comparable to that observed with transplantation of similar numbers of mouse islets (group averages are shown in Figures 7A and 7F and individual mouse data are shown in Figures 7B and 7G). The effectiveness of PMP sphere transplantation was also supported by body weight data (in which weight at 50 days posttransplant was compared to weight on STZ-injection date). STZ-diabetic BalbC mice that did not receive transplants lost ~1% of their body weight, whereas this weight loss was reversed in mouse islet and PMP sphere recipients (~3.2% and ~3.5% weight gain, respectively) (Figure 7C). A similar mitigation of weight loss was observed with human PMP sphere transplantation (Figure 7H), but it appeared that the nontransplanted diabetic NOD-Scid mice displayed more severe weight loss than did their BalbC counterparts (~12.3% versus ~1% loss, respectively), and the effect of the

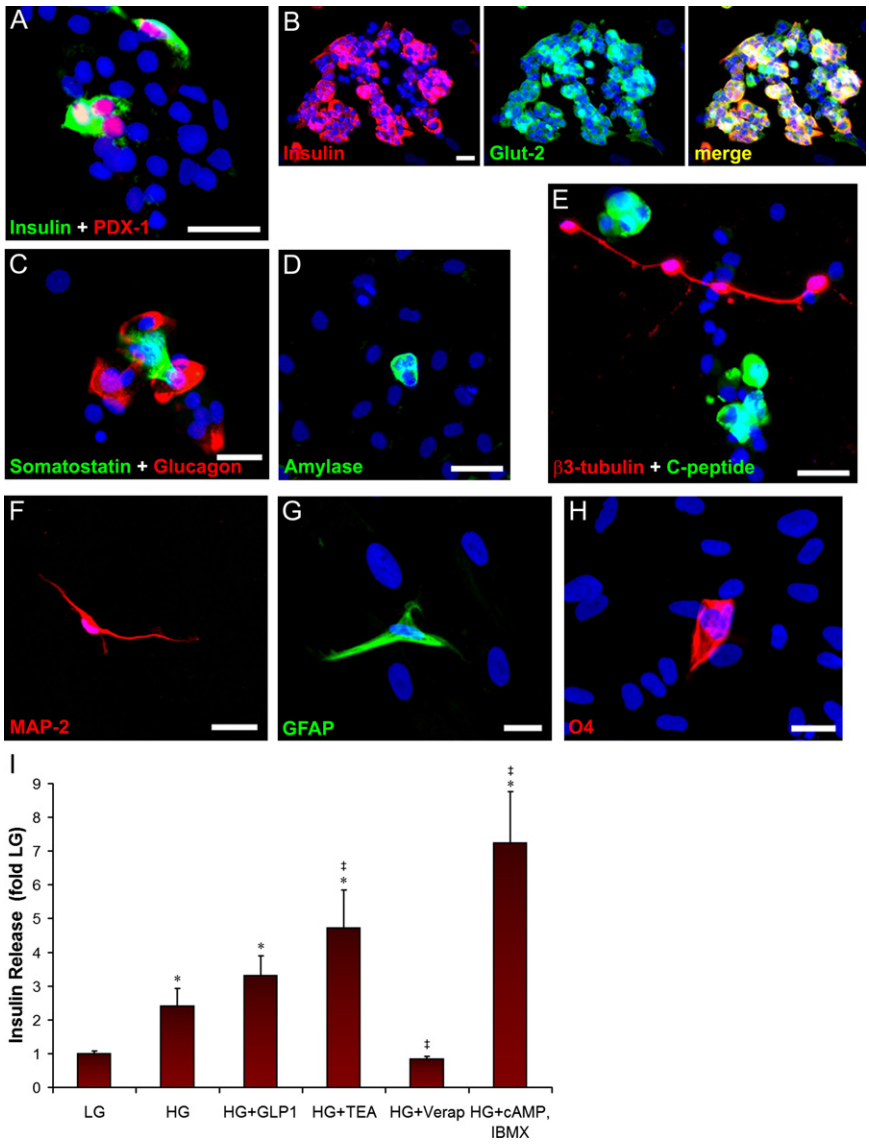

Figure 6. Human PMP Sphere Colonies Generate Multiple Pancreatic and Neural Cell Types upon Differentiation, and De Novo Generated β Cells Demonstrate Regulated Glucose-Stimulated Insulin Release

(A–D) Individual PMP spheres derived from single human islet cells could be differentiated to produce (A) insulin+/PDX-1+ cells, (B) insulin+/Glut-2+ β cells, (C) glucagon+ α cells and somatostatin+ δ cells, and (D) amylase+ exocrine cells.

(E) The same clonally derived sphere that produced C-peptide+ β cells was also capable of generating β3-tubulin+ neurons.

(F–H) MAP-2 expression demonstrated the production of mature neurons (F), and glial cell generation was demonstrated by the presence of (G) GFAP+ astrocytes and (H) O4+ oligodendrocytes. Cell nuclei are stained blue.

Scale bars represent 25 μm.

(I) Differentiated human PMP sphere colonies exhibited increased insulin secretion in response to high glucose (HG), an effect that could be enhanced by addition of GLP-1 or TEA. Inclusion of verapamil (Verap) abolished the glucose-stimulated insulin release, while the combination of pCPT-cAMP (cAMP in figure) with IBMX provided potent augmentation. Error bars are ±SEM. ANOVA $F(5) = 7.3$; *$p < 0.05$ versus low glucose (LG), ‡$p < 0.05$ versus HG in posttest.

See also Table S2.

human PMPs was to reduce this weight loss to ~2%. These data demonstrate a clear effect of PMP sphere transplantation in improving both glycemic control and body weight preservation in diabetic recipients.

The maintained glycemic improvement observed in PMP sphere recipients indicated that the grafted spheres differentiated to yield functional β cells which persevered throughout the analysis period. To confirm this, we sectioned and immunostained the kidneys of recipient mice to assess for the presence and fate of grafted cells. As shown in Figures 7D and 7E, clusters of insulin+ β and glucagon+ α cells were found in the renal subcapsular region of mouse PMP sphere-transplanted kidneys. As a control, the nontransplanted kidneys of recipient mice were analyzed, and no pancreatic hormone+ cells were evident (not shown), substantiating that the hormone+ cells observed in the transplanted kidneys were graft derived. Perdurance of human insulin+ β cells derived from the transplanted human PMP spheres was evident in the recipient kidneys (Figures 7I and 7J). To unambiguously verify the human graft origin of these cells, immunostaining was performed against human nuclear antigen, which displayed nuclear costaining in the insulin+ cells. Although the functional nature of these human PMP graft-derived insulin+ cells is indicated by the improved glycemia, their in vivo functionality was more directly assessed by ELISA measurement of plasma human C-peptide levels (Figure 7K). The plasma human C-peptide levels of the human PMP sphere recipients was 173 ± 30 pM, ~20% of that measured in nonfasted human plasma samples (838 ± 124 pM). These data establish the functional perseverance of both mouse and human PMP graft-derived β cells in diabetic recipients.

DISCUSSION

This study demonstrates that the adult mammalian pancreas contains a small population of insulin+ multipotent stem cells, capable of dividing and contributing to the pancreatic and neural lineages. The particular bilineage cross-germ layer potency exhibited by these adult PMP cells raises questions regarding their ontogenic origin and in vivo identity. PMPs do not appear to be generalized endodermal/ectodermal precursors, because they do not generate nonpancreatic endodermal progeny (e.g., hepatocytes) or nonneural ectodermal progeny (e.g., epidermal cells) (Seaberg et al., 2004). Rather, they appear to

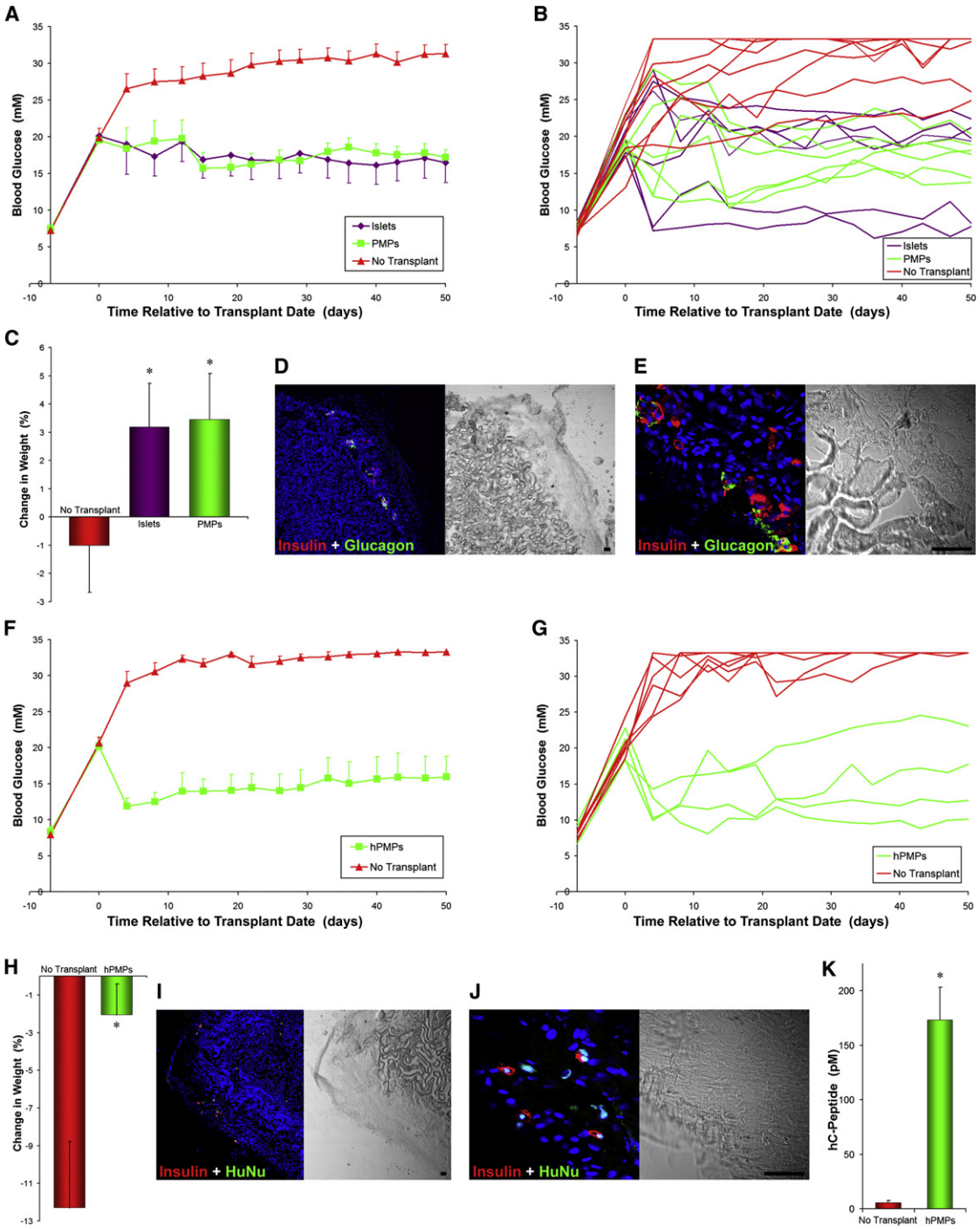

Figure 7. PMP Sphere Transplantation Ameliorates the Diabetic State in Recipient Mice

(A–J) Transplantation of (A–E) mouse islets or PMP spheres or (F–K) human PMP spheres under the kidney capsule of STZ-diabetic mice was performed. This resulted in both (A, B, F, G) a reduction of hyperglycemia (group averages are shown in A and F and individual mouse data are shown in B and G) and (C, H) weight loss in recipient mice. Confocal postanalysis of the transplanted kidneys from recipient mice revealed the perseverance of graft-derived cells.

(D and E) Low-power (10× obj.) and high-power (60× obj.) magnifications of graft-derived immunostained insulin$^+$ and glucagon$^+$ cells in mouse PMP sphere recipient kidneys.

(I and J) Low-power (10× obj.) and high-power (60× obj.) magnifications of graft-derived immunostained insulin$^+$ and human nuclear antigen (HuNu)$^+$ cells in human PMP sphere recipient kidneys.

Cell nuclei are stained blue. Scale bars represent 25 μm.

(K) An ELISA assay was performed, demonstrating substantial levels of circulating human C-peptide in the plasma of human PMP sphere recipients.

Error bars are ±SEM. *$p < 0.05$ versus No Transplant in (C) and (H); *$p < 0.001$ versus No Transplant in (K).

exhibit specification toward pancreatic and neural cellular output. This distinct potency appears unique to PMP cells, which have neither an ESC-like nor mesodermal character (Seaberg et al., 2004). The NC was considered as the ontogenic origin of PMP cells, because it is a developmental structure with broad contribution to various cell types and lineages (Dupin et al., 2006). The NC is thought to be the source of adult multipotent skin-derived precursors (SKPs) (Fernandes et al., 2004) and has been hypothesized to be the source of most adult stem cell populations (Pierret et al., 2006). The current study used genetic lineage-tracing techniques to exclude the NC and to establish the PDX-1$^+$ embryonic pancreatic endoderm as the PMP source. One of the rather interesting characteristics of PMPs is their ability to generate neural cells, even in vivo, a finding which is further supported by the observation that not all neural cells were GFP$^+$ in the *Wnt1-Cre* × *Z/EG* NC lineage-labeled mice (unpublished observations), indicating a non-NC origin for these intrapancreatic neural cells.

Some key findings, demonstrated through the use of two different transgenic mouse models, were that the PMP cell expresses insulin in vivo and that insulin$^+$ cells within PMP spheres have a precursor character distinct from mature β cells. Thus, PMP cells display gene expression properties generally thought to be restricted to differentiated β cells within the pancreas. The finding that PMP cells have low expression of Glut-2 suggests that they may not display functional properties of β cells (i.e., regulated insulin secretion) and may have a unique stem cell identity. The lack of *Glut-2* was maintained in the insulin$^+$ cells within undifferentiated PMP spheres, which also displayed much lower insulin gene expression than mature β cells. However, these cells evinced comparable or elevated levels of the transcription factors *PDX-1*, *Nkx6.1*, and *Ngn-3*, indicating that they had a precursor character or were immature proto-β cells derived from the PMP. These findings are also consistent with a mechanism in which the PMP-derived cells are reactivating portions of pancreatic developmental genetic programs in the generation of new pancreatic cells.

The finding that mouse PMP cells express insulin in vivo is of particular note, because it provides partial reconciliation with the work of Dor et al. (2004), which argued that new β cells were derived exclusively from pre-existing ones. Because the precursors express insulin, simple insulin expression-dependent lineage labeling of β cells cannot discriminate between self-replication of existing mature β cells and proliferation and differentiation from insulin$^+$ stem cells. Total reconciliation with Dor et al. (2004) has not been achieved, because they do not observe the production in vivo of non-β cells in their lineage-tracing experiments. To the contrary, we demonstrate that insulin$^+$ cells give rise in vivo to multiple pancreatic and neural cell types in the normal adult pancreas, thus corroborating this in vitro demonstrated bilineage multipotentiality of PMP cells and establishing that this potential was not a culture artifact. It is possible that this discrepancy is due to the increased sensitivity of the transgenic insulin-lineage marker used in the current study, because Dor et al. (2004) used alkaline phosphatase, whereas we used a GFP signal amplified by a secondary antibody fluorophore, which would be expected to yield enhanced detection. Indeed, the absence of evidence for in vivo non-β cell contribution in Dor et al. (2004) is not evidence of absence for this capacity. Similar to Dor et al. (2004), we do not observe an increase in the percentage of insulin$^+$ cells labeled with GFP over the chase period in vivo, suggesting that there is proportional contribution to new β cells from replication of labeled and unlabeled existing β cells and PMP cells. Occasionally, we would observe an entirely GFP$^+$/insulin$^+$ cell cluster (typically <10 cells), though no large islets that were entirely GFP$^+$, which would be the case if the islet was wholly derived from a labeled PMP cell.

Several other studies have utilized various experimental and modeling techniques to suggest that all β cells have equivalent, and very slow, replication rates and that precursors do not play a role in maintaining adult β cells (Teta et al., 2005, 2007; Brennand et al., 2007). Reasons for the discrepancies between these and the current study are unclear, but it is quite possible, given the rarity of PMPs, that they were simply missed. For example, in Brennand et al. (2007), the same *RIP-Cre-ER* mice used in the current study were utilized in combination with the mosaic analysis with double markers (MADM) system, a technique that allows low-frequency lineage labeling of insulin$^+$ cells. After a chase period, the clones from such labeled insulin$^+$ cells were analyzed, and they were found to all be of equivalent size, without any large clones being evident. The authors use these data to argue against the existence of any highly replicative insulin$^+$ precursors, but their technique labeled only 0.1%–.05% of insulin$^+$ cells, and thus PMPs would easily have been overlooked. Related to this, a pertinent point is that whereas PMPs display high replicative capacity within our in vitro sphere-formation assay, it is unlikely that they proliferate in vivo at the same rate and to the same extent. One must be cognizant that we are employing potent mitogens in the sphere-formation assay to reveal proliferative ability, which almost certainly manifests to a lesser degree in vivo, especially in the uninjured adult pancreas. It therefore remains likely, and consistent with our findings, that β cell replication remains a major mechanism for normal adult β cell homeostasis, although it is also likely that this mechanism cannot fully account for the extensive expansion occurring during early postnatal life, pregnancy, and in certain regenerative states. Indeed, recent modeling of the dynamics of rodent postnatal β cell mass expansion and maintenance revealed a large (~80%) dependence on nonreplicative sources (Manesso et al., 2009), supporting the existence of a precursor cell compartment.

In the current in vivo lineage-labeling studies, the notably increased appearance of GFP$^+$ non-β cells over the chase period clearly establishes the in vivo relevance of PMPs to normal non-β cell turnover. A recent study demonstrated that in a certain injury model inducing β cell regeneration (partial duct ligation), endogenous tissue-resident multipotent precursors were activated in a facultative and Ngn-3-dependant manner (Xu et al., 2008). The authors show the expansion of Ngn-3$^+$ cells in the tissue during regeneration and demonstrate the production of multiple islet cell types in vitro from Ngn-3$^+$ cells. It is quite possible that the originating precursor cells may be our PMP cell population, as we (Figure S4) and others (Gu et al., 2002; Dror et al., 2007) have demonstrated the presence of insulin$^+$/Ngn-3$^+$ cells in the normal adult pancreas.

Other studies have suggested that under specific culture conditions, β cells can undergo a process of epithelial-to-mesenchymal transition (EMT), a process by which epithelial

cells undergo "dedifferentiation" to lose many of their mature characteristics and acquire features associated with mesenchymal cell types (Gershengorn et al., 2004; Ouziel-Yahalom et al., 2006; Russ et al., 2008). After EMT of β cells, the resultant fibroblast-like cells can be extensively expanded, followed by "redifferentiation" into pancreatic endocrine cell types. Such EMT of β cells may also provide reconciliation with the findings of Dor et al. (2004), albeit through a different mechanistic interpretation than our model of resident insulin+ stem cells. However, most of these EMT studies do not utilize any lineage-labeling techniques to verify that the proliferative fibroblast-like cells are specifically derived from β cells. Indeed, several recent studies with lineage-tracing techniques found that such islet-derived fibroblast-like cells were not derived by EMT from either PDX-1- or insulin-expressing cells but rather were MSC-like cells, similar to those isolated from bone marrow (Chase et al., 2007; Atouf et al., 2007). This is in contrast to the work of Russ et al. (2008) in which lentivirus-based GFP insulin lineage labeling was performed to support human β cell EMT. However, with this technique, a substantial percentage (~30%) of the GFP-labeled cells were insulin negative (and many of these were positive for other islet hormones) when examined 4–5 days after viral infection, consistent with our interpretation of the presence of insulin+ multipotent precursor cells. Support for the dissimilarity between PMP-derived and true (bone marrow-derived) MSC populations was obtained by RT-qPCR analysis for key genes (Figure S5). Although the PMP spheres expressed substantially higher levels of *nestin* and comparable levels of *vimentin* to MSCs, the expression of pancreas-associated genes was absent from the MSC samples, verifying the nonequivalence of these populations.

A recent study (Stanger et al., 2007) suggested that the final size of the pancreas is determined by the number of embryonic progenitor cells, each with an autonomous restriction on the amount of tissue it was capable of generating (i.e., nonstem cells). The authors draw comparisons to the intrinsic "counting mechanism" that had been initially suggested to limit the number of times a central nervous system oligodendrocyte precursor cell can divide (Temple and Raff, 1986). However, such intrinsic limitations clearly do not exist with the PMP cell population described in the current study, because each PMP can generate many β and other differentiated cells when given the appropriate conditions. As well, PMPs demonstrate some capacity for self-renewal in vitro and can be isolated from both mouse and human throughout the entire lifespan, suggesting maintenance of this population in vivo. Thus, the limitation on PMP cell contribution in vivo appears to be cell extrinsic. This is also the case for the above-mentioned oligodendrocyte precursors, which were later found to be free of "counting mechanism" limitations when provided with specific growth factors in vitro, ones that are upregulated in the brain in response to injury in some cases (Wolswijk and Noble, 1992).

Though much of the present characterization of PMP cells relied on the use of mice and their genetic tractability, any envisaged application of pancreatic precursors in therapeutic β cell replacement strategies would obviously be facilitated by the use of human-derived cells. To that end, we have demonstrated the presence of a multipotent PMP cell population in the islets of adult human pancreatic tissue. A single differentiated human PMP colony was found to contain ~11% β cells and 3.3 ng of insulin, whereas a human islet contains ~55% β cells (Brissova et al., 2005) and contained 45.5 ng of insulin. Thus, the insulin content of PMP-generated β cells was 36% of primary human β cells. Further, the de novo generated human β cells were shown to display functional regulated insulin release. Under basal low-glucose conditions, differentiated PMP colonies released 39 pg insulin/colony/hr, a value ~18% of that typically obtained in our lab from individual human islets under similar conditions (unpublished observations). Taking into account the differing numbers of β cells (11%, PMP colony; 55%, islet), the basal insulin release from human PMP-derived β cells, on a per β cell basis, was ~90% of that released from human islet β cells.

These promising functional data led us to undertake the transplantation experiments, in which an amelioration of hyperglycemia and weight loss was observed in mouse and human PMP sphere recipients. The magnitude of the demonstrated glycemic improvement is actually an underestimation, because the glucometer range maximum was 33.3 mM glucose and many of the nontransplanted mice reached this measurement ceiling at many time points. Postanalysis of the grafted kidneys revealed the presence of transplant-derived insulin-expressing cells. The perseverance and evident functionality of these cells suggest that they developed access to the vasculature, enabling both nutrient supply and gas exchange as well as providing conduits for blood glucose sensing and insulin release. Given the insulin+ nature of PMPs and some of the precursors within PMP spheres, it is possible that some of these insulin-expressing cells derived from the grafts do not represent mature β cells (and maintain a precursor identity); however, the clear effect on metabolic parameters (blood glucose, weight) and the demonstration of substantial circulating human C-peptide indicate that many of them must have matured to functionality. Such findings provide the basis for further investigation of the therapeutic potential of these multipotent putative adult pancreatic stem cells.

EXPERIMENTAL PROCEDURES

Animals, Human Tissue, Cell Isolation, and Culture

All studies were approved by the Office of Research Ethics at the University of Toronto. Mice used in these studies included wild-type BalbC and CD1, NOD-Scid (Charles River), the *Z/EG* reporter strain, *Wnt1-Cre*, *PDX-1-Cre*, *MIP-GFP*, and *RIP-Cre-ER* mice. Pancreatic islet and ductal tissue was isolated and processed as previously described (Seaberg et al., 2004). MSC populations were derived from the femurs and tibias of CD1 mice as previously described (Itoh and Aubin, 2009). PMP spheres were successfully obtained from adult mice ranging in age from ~4 weeks to >18 months, though experiments were typically initiated with 5- to 10-week-old mice. Human islet tissue (postmortem) from 17 donors ranging in age from 19 to 71 years (9 male, 8 female) was isolated according to the Edmonton Protocol and generously provided via J. Shapiro and the Human Islet Distribution Program at the University of Alberta. Human islets were handpicked to ensure purity. All human samples yielded PMP spheres. The PMP sphere formation assay was performed as previously described (Seaberg et al., 2004); see Supplemental Information for details.

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Pancreatic cells were isolated as described, and cells were sorted with an EPICS Elite Cell Sorter (Beckman-Coulter) or a FACSAria System (BD Biosciences). All cells from the various transgenic mice were sorted into positive and negative cell fractions on the basis of GFP fluorescence. Glut-2 sorting was performed after immunostaining live cells with a primary anti-Glut2 rabbit

antibody followed by staining with an Alexa-647 goat anti-rabbit secondary antibody. Experiments involving sorting populations followed by their plating in the PMP sphere formation assay were performed 3–4 times. Quantification of spheres from the sorted fractions are reported both as the percent of total spheres derived from the positive and negative fractions, as well as average sphere formation frequency values for the two fractions, as indicated in the text.

Immunocytochemistry

Fixation and immunocytochemical analysis of cells was performed as described previously for neurospheres (Tropepe et al., 1999); see Supplemental Information for additional details.

Insulin Release and Content Studies

Insulin release and content experiments were generally performed as previously described (Seaberg et al., 2004); see Supplemental Information for details and modifications.

Transplantation Studies

Transplants were performed with BalbC PMP spheres into BalbC recipients and human PMP spheres into NOD-Scid recipients. Recipients were made diabetic with a single intraperitoneal injection of streptozotocin (170 mg/kg BalbC and 125 mg/kg NOD-Scid) in a pH 4.5 citrate buffer 7 days prior to transplant. Blood glucose was measured from tail vein samples with a OneTouch Ultra 2 glucometer (LifeScan). Recipient and control group mice were selected based on nonfasted hyperglycemic (>16 mM) measurements for two consecutive days (day 2 being the transplant date). ~500 mouse islets, ~400–500 mouse PMP spheres, or ~350–400 human PMP spheres were transplanted underneath the kidney capsule of recipient mice according to Szot et al. (2007). Nonfasted blood glucose was measured twice weekly for 50 days posttransplant, after which final weights and plasma samples were collected, mice were fixative perfused, and kidneys were harvested. Human C-peptide was measured in plasma samples with an ALPCO ELISA 80-CPTHU-E01.1 Kit according to manufacturer's instructions.

Statistics

Data are expressed as means ± SEM unless specified otherwise. Statistical comparisons between two groups was performed with a t test where appropriate or by one-way analysis of variance (ANOVA) with Dunnett's posttest for comparing groups to control or the Bonferroni posttest for comparisons between groups where an acceptable level of significance was considered at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.stem.2011.01.015.

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