Evaluating Protocols for Embryonic Stem Cell Differentiation into Insulin-Secreting β -Cells Using Insulin II-GFP as a Specific and Noninvasive Reporter

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Abstract

Stable and full differentiation of pluripotent stem cells into functional β -cells offers the potential to treat type I diabetes with a theoretically inexhaustible source of replacement cells. In addition to the difficulties in directed differentiation, progress toward an optimized and reliable protocol has been hampered by the complication that cultured cells will concentrate insulin from the media, thus making it difficult to tell which, if any, cells are producing insulin. To address this, we utilized a novel murine embryonic stem cell (mESC) research model, in which the green fluorescent protein (GFP) has been inserted within the C-peptide of the mouse *insulinII* gene (InsulinII-GFP). Using this method, cells producing insulin are easily identified. We then compared four published protocols for differentiating mESCs into β -cells to evaluate their relative efficiency by assaying intrinsic insulin production. Cells differentiated using each protocol were easily distinguished based on culture conditions and morphology. This comparison is strengthened because all testing is performed within the same laboratory by the same researchers, thereby removing interlaboratory variability in culture, cells, or analysis. Differentiated cells were analyzed and sorted based on GFP fluorescence as compared to wild type cells. Each differentiation protocol increased GFP fluorescence but only modestly. None of these protocols yielded more than 3% of cells capable of insulin biosynthesis indicating the relative inefficiency of all analyzed protocols. Therefore, improved β -cells differentiation protocols are needed, and these insulin II GFP cells may prove to be an important tool to accelerate this process.

Introduction

DIABETES MELLITUS is characterized by β -cell loss and/or dysfunction leading to chronic hyperglycemia and concomitant metabolic consequences. The autoimmune destruction of β -cells culminates in insulin deficiency in patients with type 1 diabetes mellitus (T1DM). In type 2 diabetes mellitus (T2DM), β -cell dysfunction results in a relative insulin deficiency. As the prevalence of diabetes mellitus, T1DM and T2DM, is increasing, it is estimated that diabetes mellitus affects over 150 million people worldwide (Libman and LaPorte, 2005; Lipman et al., 2006; Narayan et al., 2003). While

treatment with insulin improves glycemic control and prolongs survival, current standard diabetes treatment regimens with insulin replacement remain far from ideal. Transplantation of either the whole pancreas or isolated β -cells derived from the pancreas provide another modality for insulin replacement, but is often accompanied by undesirable side effects secondary to the chronic immunosuppressive treatment required for transplant (Campbell et al., 2007; Korsgren et al., 2005; Ryan et al., 2001).

Pluripotent stem cells differentiated into functional insulin-secreting cells offer the potential to treat diabetes by providing a source for insulin-secreting β -cells without the limitations of

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the current therapeutic modalities. Embryonic stem (ES) cells are a population of self-renewing, pluripotent cells derived from the inner cell mass (ICM) of mammalian blastocyst stage embryos (Evans and Kaufman, 1981; Martin, 1981), possessing the ability to differentiate into a wide variety of cell types, including β -cells (Keller, 2005). To be successful, however, β -cells generated from stem cells must possess physiologically appropriate regulation of insulin secretion (Bonner-Weir and Weir, 2005; Rolletschek et al., 2006), including the ability to sense ambient glucose concentrations, synthesize pro-insulin, and secrete insulin appropriately in response to physiological glucose concentrations without risk of neoplastic transformation (Fujikawa et al., 2005; Kroon et al., 2008; Schuit et al., 2002). At present, unsolved obstacles associated with differentiation of ES into β -cells include the lack of sufficient development of the insulin processing machinery and mechanisms responsible for sensing circulating glucose concentrations as well as maturation of the insulin secretory pathways (Fellous et al., 2007; Santana et al., 2006). Additional difficulties include the immunogenicity of differentiated ES cells (Drukker and Benvenisty, 2004) and the predisposition of undifferentiated cells to form teratomas (Fujikawa et al., 2005; Sipione et al., 2004).

Given the enormous potential to treat, and potentially cure, diabetes mellitus with this technology, much effort has been expended to develop differentiation protocols to generate functional β -cells. Soria et al. (2000) published a protocol for selection of insulin-producing cells from mouse ES cells using a neomycin selection system under the control of the regulatory regions of the human insulin gene. The resulting differentiated cells were able to correct streptozotocin-induced hyperglycemia in mice when transplanted under the kidney capsule. Lumelsky et al. (2001) utilized a five-stage process to differentiate mouse ES cells into insulin containing cells. This protocol begins with a highly enriched population of nestin-positive cells from embryoid bodies (EBs) plated into a serum-free medium (ITSFn). Cells were then expanded, and differentiated. When these cells were transplanted into streptozotocin-diabetic mice, the mice survived longer than the hyperglycemic sham-grafted control mice, however, euglycemia was not adequately restored (Lumelsky et al., 2001). Concerns have been raised as to whether the immunohistochemical staining of these cells represents endogenous insulin production or merely reflects insulin uptake from the media (Hansson et al., 2004; Rajagopal et al., 2003). Hence, assays to confirm C-peptide expression/secretion are vital to demonstrate *de novo* insulin synthesis (Serup, 2006) and thus, such assays serve as a reliable marker for β -cell differentiation.

Variations to the Lumelsky protocol have included introduction of the Pax4 gene into the ES cells, addition of phosphoinositide-3-kinase inhibitor prior to the final differentiation step (Blyszczuk et al., 2003; Hori et al., 2002), or the transfection of the undifferentiated mES cells with a Nkx6.1 promoter, a transcription factor involved in β -cell differentiation, followed by neomycin selection (Leon-Quinto et al., 2004). Another strategy incorporating activin A, *all-trans*-retinoic acid, and basic fibroblast growth factor (bFGF) treatment yielded C-peptide immunopositive cells within 2 weeks (Shi et al., 2005). Cells differentiated using this protocol demonstrated glucose-stimulated insulin secretion and prolonged the survival of mice with streptozotocin-induced

diabetes for up to 40 days posttransplantation. Although these results appear to be promising, challenges remain. For example, Fujikawa et al. (2005) showed that differentiated cells, immunopositive for both insulin and C-peptide, failed to restore euglycemia and were associated with teratoma formation after transplant into streptozotocin treated mice. It is uncertain whether the differentiation of β -cells from ES cells requires faithful replication of β -cell ontogeny beginning with endoderm differentiation, or whether it can be accomplished in another fashion. For example, D'Amour et al. (2006) differentiated human ESC into β -cells by mimicking *in vivo* pancreatic organogenesis through specific steps in this differentiation process. They then expanded their protocol to demonstrate that pancreatic endoderm derived from human ESCs generated glucose-responsive endocrine cells following implantation into immune-compromised mice (D'Amour et al., 2006; Kroon et al., 2008).

To assess the efficiency of the published β -cell differentiation protocols, we have utilized a mouse ES cell line in which the coding sequence for green fluorescent protein (GFP) was inserted into the C-peptide region of the mouse insulin II gene. We have previously demonstrated that the knock-in mice generated from these ES cells are viable, have normal glucose metabolism, and are fertile. We have also shown that the GFP can be detected within the β -cells and colocalizes with insulin (Ben-Yehudah et al., 2005). Here, we utilized these ES cells to determine if GFP (+) cells, indicating endogenous insulin gene transcription and translation, can be detected after differentiation using the methodology reported in four different published protocols (Blyszczuk et al., 2003; Hori et al., 2002; Lumelsky et al., 2001; Shi et al., 2005). These four protocols varied in duration, media components, and differentiation strategies (Table 1). In our hands, all four protocols produced GFP expressing cells at low frequency emphasizing the importance of identifying the very small population of differentiated insulin producing cells. Further, we explored whether these differentiated cells are β -cells, or rather other cells that functionally respond to glucose by the secretion of insulin. This novel research tool might be coupled with improved differentiation and survival protocols for even greater β -cell enrichment based on functional production of insulin.

Materials and Methods

Cell culture

Construction of the *InsII^{EGFP}* mouse line was previously described (Ben-Yehudah et al., 2005). The *InsII^{EGFP}* ES cells used in the experiments described in this manuscript were generated from one targeted G418-resistant ES cell clone by transfecting the cells with a circular plasmid (Pgk1-Cre) expressing the site-specific Cre recombinase to remove the selectable marker cassette from the targeted allele (Ben-Yehudah et al., 2005). These *InsII^{EGFP}* ES cells are heterozygous for the modified *InsII* allele.

Both the wild type (WT) GFP-negative and the "knock-in" GFP-positive, *InsII^{EGFP}* (+) murine ES cells were cultured on standard 100-mm TC plates coated with 1% gelatin. The cultures were maintained in DMEM containing 15% FBS, penicillin/streptomycin, L-glutamine, nonessential amino acids, nucleosides, β -mercaptoethanol, and supplemented with 1000 units/mL murine leukemia inhibitory factor (all

TABLE 1. COMPARISON OF PROTOCOLS USED FOR DIRECTED DIFFERENTIATION OF BETA CELLS

<i>Protocols</i>	<i>Blyszczuk 2004</i>	<i>Shi 2005</i>	<i>Hori 2002</i>	<i>Lumelsky 2001</i>	<i>Schroeder 2006</i>
Total Duration Stages	48 days EB formation EB plating EB replating Differentiation	9 days 8 h Induction expansion maturation	25 days 5 stages of replating	26 days Expansion generation of EBs selection for Nestin-positive cells expansion induction of specific differentiation	33 days Hanging drop-EB formation spontaneous differentiation specific differentiation
Media	DMEM IMDM DMEM/F12+ Na+B27+ITS+ P+P+T (differentiation media) α -monothioglycerol	DMEM DMEM/F12	K/O DMEM ITSFn N2+NA	KODMEM KOSMEM+ITS N2+B27	Differentiation I Differentiation II + FCS Differentiation – FCS
Treatments		bFGF+Activin RA RT-PCR	bFGF LY294002	bFGF	None except media
Means of assessment	RT-PCR immunofluorescence ELISA electrophysical transplantation	ELISA immunohistochemical transplantation	PCR, ELISA, transplantation immunohistochemistry insulin secretion grafting	Immunohistochemistry grafting	RT-PCR insulin elisa C-peptide RIA histochemistry transplantation
Conclusion	ES cells can become insulin-producers without selection of nestin-positive cells	ES cells can produce insulin in 2 weeks	ES cells can become homogeneous clusters of insulin-producing cells, similar to islets	ES cells can become insulin-secreting structures similar to pancreatic islets	ES cells form islet-like colonies

ITSFn, insulin–transferrin–selenium–fibronectin media; b, (ITS+progesterone+putrescine+B27).

from Chemicon, Tumeclula, CA). The cultures were observed every day, and the media was changed every other day. Cells were split every 5 days at no less than 1:4 and no more than 1:10 with our target concentration between 1.5×10^5 and 4.0×10^5 per mL. Cells were removed from the culture plates by rinsing 2 $\times$ with phosphate-buffered saline (PBS), followed by incubation with 0.1% trypsin at 37°C. The suspensions were centrifuged for 3 min/200 $\times$ g at room temperature (RT). Single-cell suspensions of the cell pellet were then plated at the proper concentration in freshly gelatin coated 100-mm TC plates. Differentiation was carried out according to the specific conditions described in each manuscript (Blyszczuk et al., 2003; Hori et al., 2002; Lumelsky et al., 2001; Shi et al., 2005) and summarized in Table 1, and in the supplementary material. (see online supplementary material at www.liebertonline.com).

Teratoma formation

InsII^{EGFP} (+) ES cells growing exponentially in culture were injected (5×10^5 cells in 100 μ L) into the testes of 7-week-old NOD-*Scid* (The Jackson Laboratory, Bar Harbor, ME) immunocompromised male mice. The injections were modified efferent duct injections into the interstitial space of testes (Ogawa et al., 1997) using micromanipulator and microinjectors. Tumor formation was monitored daily until the tumor was palpable, typically 12–16 weeks after the injection. At that time, mice were euthanized with CO₂ asphyxiation and the tumors dissected. Tumors were placed in 20 mL of 10% Formalin in PBS for several days to ensure adequate fixation. Teratomas were cut into smaller pieces 3–5 mm in diameter and returned to 10% formalin for further fixation followed by processing through a Peloris Processor (Viscon Biosystems, Norwell, MA) for dehydration and embedding. The samples were dehydrated by immersion in 70% ethanol for 45 min followed successively by 90, 95, and three 100% ethanol immersions each cycle lasting 45 min. This was followed by three 45-min cycles in xylene to clear the samples and three cycles for 45 min in paraffin wax to infiltrate the samples with paraffin. Finally, the samples were placed into blocks and immersed in paraffin for sectioning. Sections (0.4 μ m) were cut using a microtome and stained with hematoxylin and eosin (H&E).

Staining

Immunocytochemistry was performed using one of the following methods. Culture dishes containing undifferentiated colonies were fixed by addition of either 100% methanol (–20°C) for 15 min followed by a 15 min wash in PBS + 1% Triton X-100 (PBS-Tx, Sigma, St. Louis MO), or 2% paraformaldehyde in PBS for 40 min followed by quenching in PBS + 115 mM glycine for 30 min subsequently washed out with PBS-Tx. After fixation, nonspecific binding of the primary and secondary antibodies was blocked, when necessary, by a 20-min incubation in PBS with 3% nonfat dry milk. Primary antibodies used included anti-Oct-4 and anti-MafA (Santa Cruz Biotechnology, Santa Cruz, CA), anti-SSEA-1, -3, -4 (Developmental Studies Hybridoma Bank), and anti-insulin (Zymed Laboratories, San Francisco, CA). Primary antibodies were diluted in PBS + Tx and incubated on the coverslips for 40 min at 37°C in a humidified chamber. After a 15-min wash in PBS + Tx, a species appropriate fluorescently

labeled secondary antibody was added to the coverslips for an additional 40 min. After another 15 min wash in PBS + Tx, 5 μ M TOTO-3 (Molecular Probes, Eugene, OR) was added for 20 min to label the DNA. Coverslips were inverted onto slides and mounted in Vectashield antifade medium (Vector Labs, Burlingame, CA) to prevent photobleaching (Navara et al., 2001).

Slides were examined using a Leica TCS-SP2 laser scanning confocal microscope equipped with appropriate lasers for simultaneous imaging of up to four fluorophores. Digital data was archived to compact disk or DVD and prepared for publication using Adobe Photoshop software (Adobe Systems Inc.; MountainView, CA).

FACS sorting

At the termination of the specific differentiation protocol, cells (1×10^7 /mL) were harvested and single cell suspensions were created to allow subsequent analysis of GFP fluorescence and purification of this fluorescent subpopulation. Cells were sorted on a Becton Dickinson FACS Aria flow cytometer, using the LYSYS II program. The GFP fluorescence was detected at with a wavelength of 525 nm upon excitation of the argon laser (488 nm). A forward scatter versus side-scatter dot-plot determined cellular fractions of cells, gating out debris and clumps of cells. Cells doublets were eliminated using the forward scatter height versus side-scatterwidth dot-plot. The GFP fluorescent intensity was plotted against forward scatter to view the cellular fraction that had a green fluorescent property. Cells that had a green fluorescence above background were selected to be sorted. Wild Type (WT) cells were also collected and used as negative controls to monitor for autofluorescence. The cells were placed at a maximum concentration of 10^7 /mL and were sorted at a flow rate of 10,000 cells/sec. Cells were collected in media to maintain viability of the sorted *InsII^{EGFP}* (+) and WT cells. Cells were placed into 12 $\times$ 75 mm collection tubes to allow reculturing of the GFP (+) populations.

Glucose stimulated insulin secretion (GSIS)

After sorting, cells were subjected to an insulin secretion assays as previously described (Mathews and Leiter, 1999), and examined using a Rat/mouse insulin ELISA kit or by Rat Insulin RadioimmunoAssay (RIA kit) (Both from Millipore, St. Charles, MO) according to the manufacture's directions. The plates were read using an Absorbance ELX 800 Micro plate Reader (BioTek, Winooski, VT). Insulin RIA was read using a Wallac Wizard gamma counter.

Live cell imaging

Differentiated cells were sorted by FACS and plated onto optically clear Ibidi (Munchen, Germany) culture dishes and imaged using an Ultraview spinning microlens confocal microscope (Perkin-Elmer, Waltham, MA). Cells were imaged using a 60 $\times$ 1.4-NA objective and 488-nm excitation wavelength using a Krypton/Argon mixed gas laser (Melles Griot, Carlsbad, CA). Every 20 sec a z-stack encompassing the entire range of cells was collected using Metamorph Software (Molecular Devices, Sunnyvale, CA). For analysis, a maximum projection of each z-stack was calculated and individual vesicles tracked over time.

FIG. 1. *InsulinII*^{EGFP} ES cells display pluripotent markers and have the ability to form teratomas. Immunocytochemistry of *InsulinII*^{EGFP} cells with the positive pluripotent markers OCT4 (A), and SSEA1 (B), or the negative pluripotent markers SSEA3 (C) and SSEA4 (D) indicate that these cells are indeed pluripotent, indicated by the presence or absence of green fluorescence, respectively. For details see Material and Methods section. *InsulinII*^{EGFP} cells injected into immunocompromised mice formed teratomas with tissues representing all three germ layers: endoderm (gastrointestinal; green arrow, E), mesoderm (muscle; F) and ectoderm (neuronal ganglia; black arrow, E), and pancreatic tissue (G), and confirmed using immunohistochemistry and specific antibodies: antismooth muscle actin (SMA)-stained smooth muscle from the muscular layer of gastrointestinal epithelium (I); anti-CK7-stained gastrointestinal epithelium (H); and anti-NeuN-stained neuronal tissue (J). Bar = 25 μ m.

RT-PCR

Expression of the *InsII*-EGFP fusion transcript from the *InsII*^{EGFP} allele and expression of *Hprt1* were determined by RT-PCR assays. Total RNA was isolated from embryos and adult tissues using RNeasy MiniKit (Qiagen, Chatsworth, CA). During isolation of RNA, samples were treated with RNase-free DNase (Qiagen) to remove contaminating genomic DNA. cDNA synthesis was performed using 2 μ g total RNA and M-MLV Reverse Transcriptase (Promega, Madison, WI) in a total volume of 50 μ L. From each cDNA synthesis reaction 1 μ L was used as template for PCR reactions. *InsII*^{EGFP} cDNA was amplified using the following primers: GFP—AAC GAG AAG CGC GAT CAC AT and *InsII*—CTA GTT GCA GTA GTT CTC CA. *Hprt1* cDNA was amplified using the following primers: *HprtF*—TCT CAT GCC GAC CCG CAG TCC and *HprtR*—ATT CAA CTT GCG CTC ATC TTA.

Results

We recently described a mouse ES cell line in which the coding sequence for GFP was inserted into the C-peptide

region of the mouse insulin II gene. We demonstrated that the GFP signal can be detected within the β -cells and colocalizes with insulin when transgenic mice are generated with these cells (Ben-Yehudah et al., 2005). Here, we utilized these ES cells to determine if GFP (+) cells indicating endogenous insulin gene transcription and translation can be detected after *in vitro* differentiation.

We first verified that these transgenic cells maintained their undifferentiated state in culture using both *in vitro* and *in vivo* assays. As expected, immunostaining of the cells for markers of pluripotent cells, Oct-4 and SSEA-1, was positive, as indicated by the green fluorescence in undifferentiated colonies (Fig. 1A and B, respectively). In addition, these cells were negative for SSEA-3 and SSEA-4 (Fig. 1C and D, respectively), which are markers for differentiation. We also ascertained whether these ES cells would form teratomas in immunocompromised mice. After injecting 5×10^5 cells into testis of immunocompromised mice, teratomas developed within 1–3 months. Pathological examination following staining for H&E (Fig. 1E–H) and immunohistochemistry (Fig. 1I–L) showed that these cells form tissues representing all three lineages,

that is, gastrointestinal tissue (endoderm; green arrow, Fig. 1E) ganglia (ectoderm; black arrow, Fig. 1E), and muscle (mesoderm; Fig. 1F). Although rare, pancreatic tissue (endoderm, Fig. 1G) was also identified in these teratomas. Immunohistochemistry using specific antibodies confirmed these findings: CK7 stained gastrointestinal epithelium (endoderm; Fig. 1H), as well as surface of skin (ectoderm; not shown); smooth muscle actin (SMA)-stained smooth muscle from the muscular layer of gastrointestinal epithelium (mesoderm; Fig. 1I), and NeuN stained early neuroectodermic tissue (ectoderm; Fig. 1L).

We evaluated four previously published *in vitro* differentiation protocols selected on the basis of their reported β -cell differentiation outcomes (Blyszczuk et al., 2003; Hori et al., 2002; Lumelsky et al., 2001; Shi et al., 2005). Table 1 summarizes the differences in duration (9–48 days) and conditions among the four protocols. As the GFP protein was inserted into the C-peptide region of the *Insulin II* gene (Watkins et al., 2002), only cells in which the insulin gene was actively transcribed and translated would express GFP. For each protocol, both WT and *InsII^{EGFP}* (+) ES cells were grown simultaneously and received identical culture conditions and growth factor treatments. Cells were then analyzed and sorted by FACS. To control for autofluorescence, we sorted *InsII^{EGFP}* (+) and WT cells, and compared their GFP expression levels. For each experiment, we subtracted the percent of fluorescent GFP (+) cells in the control WT group (e.g., false positive cells due to autofluorescence), from the percent of GFP (+) cells in the *insulinII*-GFP group. Figure 2 shows an example of a single FACS experiment for each differentiation protocol. The average of ≥ 3 trials for each protocol is shown in Fig. 3A. The numbers of GFP (+) cells were increased in each of the differentiation protocols; this was independent of cell passage number (data not shown). However, directed differentiation was inefficient as not more than 3% of the cells were GFP (+) (range 0.25% for the Blyszczuk protocol to 2.4% for the Lumelsky protocol). To evaluate these GFP (+) cells for markers of insulin production, we stained with anti-insulin and anti-MafA antibodies (Aramata et al., 2007; Han et al., 2007). As seen in Figure 2, we could identify MafA-positive cells (red staining) in all protocols. Because we could only generate very few GFP positive cells in the Hori protocol, we could not identify large clusters of MafA-positive cells. Nevertheless, positive cells could be found. Staining with anti-insulin antibodies (Supplementary Fig. 1) showed that insulin-positive cells could be identified after differentiation.

After cell sorting, cell viability and growth potential were assessed. As shown in Figure 3, cells differentiated by either the Lumelsky protocol (B) or the Shi protocol (C–H) were viable for at least 2 months in culture following FACS sorting evident by their continuous proliferation and passaging. GFP expression was easily identifiable under the fluorescent microscope in these cells. This was similar to the pattern of GFP expression observed in the intact pancreas of the knock-in mouse (Ben-Yehudah et al., 2005).

We next followed the specific differentiation of the insulin producing cells under the phase contrast microscope. We could not observe any gross differences in cell morphology between the GFP (+) and WT cells during this process (Supplementary Fig. 2).

In an attempt to confirm that these differentiated cells are functional, we examined the ability to secrete insulin in re-

sponse to glucose by the secretion of insulin. Cells were differentiated according to the Lumelsky protocol, sorted for *InsII^{EGFP}* (+), and collected into low glucose medium. Cells were then subjected to a glucose stimulated insulin secretion assay (GSIS) (Mathews and Leiter, 1999), and insulin levels were tested using both an ELISA or RIA. As seen in Figure 4A, differentiated cells contain insulin (Basal), and could secrete insulin into the media in response to glucose stimulation (Glucose Stim) as well as to KCl (Insulin content) (Mathews and Leiter, 1999), as expected of functional β -cells. Because the RIA results were above the kit detection threshold and made direct comparisons difficult, we conducted an ELISA to confirm these results (Supplementary Fig. 3).

We next utilized the GFP to visualize the secretion of GFP from the GFP (+) sorted cells using live cell imaging. As can be seen in Figure 4B, vesicles in these positive cells move within the cell, and GFP positive vesicles are secreted shortly after transfer to a high glucose media (0:00).

It is possible that the outcome of this differentiation is not fully differentiated β -cells, but rather another type of cell that contains insulin and could secrete it in response to glucose. Therefore, we tested to see if other tissues in the transgenic *InsulinII^{EGFP}* mouse contain GFP. As expected, using RT-PCR and specific antibodies we could detect GFP in the pancreas of an adult transgenic mouse (Fig. 5). As a size control for the GFP, we used genomic DNA from transgenic mouse as described previously (Ben-Yehudah et al., 2005). *InsII* is expressed in the thymus (Mathews et al., 2003), and we detected thymic expression of GFP. In addition, we found that GFP was expressed in the head of an E12.5 mouse embryo where the brain would be found (Fig. 5), but not in the adult brain. This indicates that the GFP can be expressed in developing neurons, while not expressed in the fully differentiated cells. This expression of GFP in the developing brain fits with the fact that some of these differentiation protocols utilize and resemble neuronal differentiation (Hori et al., 2005; Lumelsky et al., 2001).

To further resolve the uncertainty regarding insulin secretion versus insulin uptake (Rajagopal et al., 2003), the WT and *InsulinII^{EGFP}* (+) cells differentiated following the Lumelsky or the Shi protocols (Lumelsky et al., 2001; Shi et al., 2005) were stained with anti-insulin antibodies (Fig. 6). As a positive control (Fig. 6A), we stained a section of a pancreas of an *InsulinII^{EGFP/EGFP}* homozygous knock-in mouse and could detect insulin-positive islets, that colocalized with GFP (seen by the yellow staining). Because only cells that synthesize insulin would express GFP, we compared the amount and location of the positive immunostaining for insulin with the GFP signal. In the *InsulinII^{EGFP}* (+) cells, the simultaneous absence of GFP fluorescence in the presence of insulin immunopositivity reflects insulin uptake rather than endogenous insulin gene transcription and translation. WT cells differentiated by the Lumelsky protocol (Fig. 6B) or the Shi protocol (Fig. 6C) frequently labeled with anti-insulin antibodies. However, anti-C-peptide staining was minimal (data not shown) indicating that most of the insulin immunopositive cells were not actively synthesizing insulin.

When the knock-in *InsulinII^{EGFP}* (+) differentiated ES cells were stained, three patterns could be identified (Fig. 6D–H): insulin-only staining, colocalization of insulin and GFP, and both GFP and insulin staining without colocalization. The first stage of the Lumelsky differentiation protocol

FIG. 2. $\text{InsulinII}^{\text{EGFP}}$ differentiated cells express markers of β -cells. Cells were differentiated according to the four differentiation protocols, and sorted using the FACS as described in the Materials and Methods section. Dot-plots depict GFP-positive cells after FACS analysis. P6 fraction denotes the GFP positive cells. An example of both WT cells and $\text{InsulinII}^{\text{EGFP}}$ cells sorted by FACS are described. To confirm the presence of insulin-producing cells, we stained differentiated cells with an anti-MafA antibody. We could identify positive (red) cells in all cultures. Indicating that these cells are indeed insulin-producing cells. Green tinge depicts the GFP expression of the cells. Cells were counterstained for DNA (blue). Cells differentiated by the Hori protocol resulted in a very low number of cells; hence, the small number of cells in this figure.

involves the formation of EBs. We noted that the size of the EBs affected the differentiation outcome for the cells. In small EBs (Fig. 6H), the entire EB seemed to colocalize GFP and insulin immunostaining, while large EBs could be classified into: (1) cells with false positive staining for insulin (Fig. 6F) similar to the staining in WT cells (Fig. 6B–C); (2) cells lacking insulin staining (Fig. 6E–F); and (3) cells demonstrating colocalization of GFP and insulin (Fig. 6D and G). Colocalization in this last group indicated that these were cells able to synthesize insulin. In addition, we found that isolated single cells generally represented false positives for insulin secretion (Fig. 6E).

Discussion

Appreciation of the potential for differentiation of ES cells into β -cells for insulin replacement therapy has provoked great interest and excitement. However, the development of successful protocols for differentiation of ES cells into β -cells has proven to be challenging. Multiple different protocols for differentiating ES cells *in vitro* into insulin immunoreactive cells have been published (Blyszczuk et al., 2003; Blyszczuk and Wobus, 2006; Bonner-Weir and Weir, 2005; Hori et al., 2002; Lumelsky et al., 2001; Santana et al., 2006; Schroeder et al., 2006a; Shi et al., 2005; Soria et al., 2000). Whether differentiation from a neuronal-like progenitor cell or from an endoderm progenitor cell influences outcome remains unresolved (Lumelsky et al., 2001; Noguchi, 2007; Rivas-Carrillo et al., 2007; Rolletschek et al., 2006; Scharfmann, 2006; Serup, 2006). To distinguish between insulin uptake from the media (Rajagopal et al., 2003) and insulin synthesis, we developed a murine ES cell line that expresses GFP within the C-peptide to monitor endogenous insulin synthesis (Ben-Yehudah et al., 2005). Using this ES cell line we compared four published differentiation protocols (Blyszczuk et al., 2003; Hori et al., 2002; Lumelsky et al., 2001; Shi et al., 2005).

The Blyszczuk protocol (Blyszczuk et al., 2003) was selected because it was the longest, which in theory, allows for the cells to differentiate over an extended time period. The Shi protocol (Shi et al., 2005), on the other hand, was the shortest. We chose to follow the Hori protocol (Hori et al., 2002) because of the different additives and the poly(L-ornithine) coated plates. Because the Lumelsky protocol (Lumelsky et al., 2001) was one of the first published protocols, as well as it induces the differentiation of nestin-positive cells as part of the differentiation process, we were interested to ascertain whether *de novo* insulin synthesis could be detected, especially due to the controversy regarding the findings of this study (Bonner-Weir and Weir, 2005; Keller, 2005; Rajagopal et al., 2003; Santana et al., 2006).

All four protocols produced GFP (+) cells, indicating that insulin-secreting cells in the final stages of differentiation were present. However, the percentage of GFP (+) cells was <3% of total cells for all protocols (Fig. 2J) regardless of the cell passage (data not shown). Our results are similar to those recently published using human ES cells (D'Amour et al., 2006; Jiang et al., 2007; Phillips et al., 2007). In fact, Jiang et al. (2007) commented on the relative inefficiency of published protocols to yield fully differentiated functional β -cells.

Our results further emphasize the continuing need to develop novel methods for selection of fully differentiated insulin secreting β -cells (Noguchi, 2007; Rivas-Carrillo et al.,

2007). Our construct can facilitate the strategy to develop such methods. We have demonstrated that cells differentiated by the Lumelsky protocol (Lumelsky et al., 2001) contain insulin (Fig. 6 and Supplementary Fig. 1) and respond to glucose stimulus (Fig. 4) in what appears to be a two phase manner (Ohara-Imaizumi and Nagamatsu, 2006), while maintaining a three-dimensional structure. These requirements have been shown recently to be crucial for functional β -cells (Cabrera et al., 2006; Michael et al., 2006; Morton et al., 2007; Ohara-Imaizumi et al., 2007).

Imaging of the biology of the β -cell has been a goal to elucidate the insulin secretion process (Rutter, 2004; Souza et al., 2006), and thus, to gain greater insights into the etiology of diabetes. In addition, these imaging tools could be implemented to follow the process of β -cell differentiation from precursor cells. A large number of studies have used the GFP as a marker for labeling the β -cells. Meyer et al. (1998) used an adenovirus construct with GFP under the Rat Insulin Promoter (RIP) to infect β -cell-containing islets. They were able to distinguish between infected and control islets using FACS. Hara et al. (2003, 2006) constructed a transgenic mouse carrying a MIP-GFP transgene, in which the GFP was under control of the mouse insulin promoter. This mouse has been used extensively to study the physiology and electrophysiology of the β -cell (Leung et al., 2005) and the pancreatic islet (Hara et al., 2006).

Although these mice accelerate our understanding of the insulin secretion process, they are not as efficient in serving as markers for differentiation. Such a marker would require the specific expression of insulin only in the fully differentiated beta cell. To this extent Fukazawa et al. (2006) developed an artificial promoter system with luciferase as a marker for the differentiated beta cells (Fukazawa et al., 2006). Although this is an intriguing and important study, it differs from our study because we did not use an artificial promoter to drive the expression of GFP. In our study, the GFP expression is driven by the endogenous insulin II promoter. Hence, our construct using the endogenous InsII promoter should reflect the natural expression pattern that occurs during differentiation.

The first differentiation of ES cells into β -cells was developed by Lumelsky et al. (2001). This protocol is based on the similarities between pancreas and brain. For example, the transcription factors neurogenin 3, Isl-1, and Pax-6 are expressed in both developing neuroectodermal and pancreatic tissue. Despite these similarities, major physiological differences exist between these tissues. Insulin is produced in low amounts in neuroectodermal-derived tissues and functions primarily as a growth factor during nervous system development. In contrast, insulin derived from pancreatic β -cells is a key metabolic regulator and anabolic factor. Importantly, the intracellular insulin content was low in the cells obtained using this protocol (Lumelsky et al., 2001). Further, the differentiated cells were unable to sustain euglycemia following transplantation into streptozotocin-induced diabetic mice. Microarray analysis demonstrated that cells differentiated following this protocol failed to demonstrate increased expression of β -cell specific functional genes such as islet amyloid polypeptide, insulin I, and insulin II (Kitano et al., 2006).

Subsequently, the original Lumelsky protocol (Lumelsky et al., 2001) was modified by other investigators. Modifications have included addition of LY294002, a phosphoinositide 3-kinase (PI3K) inhibitor (Hansson et al., 2004), nicotineamide,

FIG. 3. Insulin-producing cells can be identified after differentiation of InsulinII^{eGFP} cells and maintain GFP expression for long periods of time *in vitro*. Cells were differentiated according to the four differentiated protocols. An average of at least three experiments from each protocol were plotted in (A) and depict the percent change of GFP-positive control compared to WT as described in the Results section, error bars denote the standard error of the mean. (B–H). Cells were differentiated by both the Lumelsky protocol (Lumelsky et al., 2001) (B) and the Shi protocol (Shiet al., 2005) (C–H), sorted by FACS (see Materials and Methods section), and returned to culture and maintained for two months. (C,D-) GFP expression, (E,F-) brightfield, (G,H-) overlay. Cells expressed different levels of GFP (D,F,H) similarly to the GFP expression in the pancreas of a transgenic animal (Ben-Yehudah et al., 2005), while WT cells did not show green fluorescence (C,E,G). Bar = 25 μm .

FIG. 4. Cells differentiated using the Lumelsky protocol have functional secretion of insulin. **(A)** Glucose stimulated insulin secretion (GSIS). Cells were examined for their ability to secrete insulin in response to glucose. Cells were differentiated by the Lumelsky protocol, sorted by FACS, and allowed to recover overnight before performing GSIS. Basal—unstimulated cells. Medium basal—cell media. Glucose Stim—glucose stimulated. Medium stim—media containing glucose for GSIS. Insulin content—after KCl treatment. **(B)** Live cell confocal microscopy of mESC differentiated by the Lumelsky protocol. Cells were plated onto Ibidi dishes and switched to media containing low glucose the evening before the experiment. The next day the cells were imaged, and 59 sec prior to the addition of high glucose four vesicles could be seen within one cell (arrows). Three min and 22 sec after the addition of high glucose four vesicles still remained, but one vesicle (arrow) will be secreted by 6 min and 2 sec and two more are lost by 7 min and 2 sec (arrow). Time stamp format is min:sec

FIG. 5. *InsII^{EGFP}* expression is limited to the pancreas, thymus, and the developing brain. The top two panels are ethidium bromide stained gels of RT-PCR products of *InsII^{EGFP}* and *Hprt1* transcripts from a homozygous *InsII^{EGFP}* E12.5 embryo and adult mouse. The top panel shows *InsII^{EGFP}* expression in the embryonic head and adult pancreas and thymus. The right-most lane is a control lane showing the PCR amplification of genomic DNA. *Hprt1* expression is shown for comparison in the middle panel. The bottom panel shows the absence of PCR products, and thus the absence of DNA contamination, from the RNA samples prior to cDNA synthesis. The right-most lane is a control lane showing the PCR amplification of genomic DNA.

FIG. 6. Identification of insulin positive cells in differentiated *InsII^{eGFP}* cell culture. Differentiated *InsII^{eGFP}* ES cells express GFP, while WT cells can be falsely assessed as β -cells. (A) Positive control for the anti-insulin staining, as described previously (Ben Yehudah et al., 2005). Sections of a pancreas from an *InsII^{eGFP/eGFP}* mouse were stained with anti-insulin antibodies (red) and colocalization with the positive GFP cells can be seen as a yellow tinge. The different levels of staining have been discussed previously (Ben Yehudah et al., 2005). WT cells (B–C) or *InsII^{eGFP}* ES cells (D–H) were differentiated using the Lumelsky protocol (Lumelsky et al., 2001) (B, E–H) or the Shi protocol (Shi et al., 2005) (C–D), and stained with anti-insulin antibodies. Green cells denote cells expressing GFP. Red—insulin. All images are laser scanning confocal micrographs. Bar = 25 μ m.

laminin, retinoic acid and Activin A (Bonner-Weir and Weir 2005; Hori et al., 2002; Santana et al., 2006; Shi et al., 2005). Recent cotransplant experiments in which ES cells were exposed to mouse embryonic pancreas or conditioned media increased the efficiency of β -cell differentiation (Brolen et al., 2005; Vaca et al., 2006). However, as demonstrated by our study, the yield of insulin secreting cells is low regardless of the protocol utilized.

We have demonstrated the importance of using our Insulin II-GFP to assess β -cell differentiation. Using these ES cells, insulin gene transcription could be distinguished from false positive insulin production determined only by immunostaining. Use of such cells may facilitate the development of improved differentiation protocols because these cells provide a cost- and time-saving tool to detect and localize insulin gene transcription and translation. While the use of transgenic reporters may influence outcomes, GFP does not interfere with the pluripotent state of the cells (shown by immunostaining and teratoma formation including pancreatic tissue in Fig. 1) as well as β -cell differentiation *in vitro* (the production of green cells) and *in vivo* (normal and viable insulin GFP mice) (Ben-Yehudah et al., 2005).

It should also be emphasized that this reporter is an unequivocal intracellular marker of C-peptide expression. As cellular functions of C-peptide are not clearly defined, this reporter not only allows detection of insulin expression, but also can serve as a tool to study the properties and functions of C-peptide within the transgenic animal, because one can follow native C-peptide expression noninvasively and stoichiometrically.

Due to the lack of organs for transplantation or for β -cell isolations, there is great need for β -islets from a renewable

source (Bonner-Weir and Weir, 2005). As ES cells are considered to be a potential source of β -cells, much work has been carried out in recent years to develop better protocols that would increase both the number as well as quality of fully differentiated ES cells into β -cells (Blyszczuk and Wobus, 2006; Ta et al., 2006). These results demonstrate that multiple obstacles remain to be overcome before stem cell therapy from ESC is a reality for patients with diabetes mellitus. These hurdles include specific and sensitive markers to assure functional β -cells, development of extracellular components to enable proper three-dimensional architecture of the cells, guarantee that differentiated cells are free from the risk for teratoma formation (Gruen and Gabel, 2006; Kroon et al., 2008; Santana et al., 2006), and the generation of human ES cell lines free from animal feeder layers and animal serum that would decrease risks of immunorejection and infection. This model system will help overcome these hurdles by marking differentiated β -cells able to synthesize insulin for additional studies (Gruen and Gabel, 2006; Santana et al., 2006).

Conclusion

We have compared four published protocols for differentiating mouse ES cells into insulin producing β -cells. These studies were carried out with a reporter protein, in which GFP was inserted into the C-peptide portion of the insulin II gene. This reporter allows identification of insulin-producing cells without directly tagging mature insulin. We confirmed that published protocols result in the production of insulin expressing β -cells. However, their yield of insulin expressing β -cells is low and variable. Therefore, new protocols for this

process would be beneficial; the *InsulinII*-GFP ES cells offer a unique and important tool to optimize newer differentiation procedures.

Note Added before Resubmission

Since our initial review process, a new report has compared the same protocols of differentiation (Boyd et al., 2008). Their results indicate the need for further investigation of these differentiation protocols, which is in agreement to our results and underlines the use of reporters such as the *InsulinII*^{eGFP} to study this process.

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A.B.-Y. and S.F.W. conceived of the research and wrote the manuscript together with CSN. Cells were differentiated by C.W. and A.B.-Y., and characterized by A.B.-Y. and C.S.N., and teratomas were produced by M.S. and analyzed by C.A.C. Live imaging was done by C.S.N. and A.B.-Y. RT-PCR by J.R.C. and B.S. Insulin secretion assays and insulin measurements were performed by D.I.L. and C.E.M.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

References

- Aramata, S., Han, S.I., and Kataoka, K. (2007). Roles and regulation of transcription factor MafA in islet beta-cells. *Endocr. J.* 54, 659–666.
- Ben-Yehudah, A., Reinhart, B., Navara, C., et al. (2005). Specific dynamic and noninvasive labeling of pancreatic beta cells in reporter mice. *Genesis* 43, 166–174.
- Blyszczuk, P., and Wobus, A.M. (2006). *In vitro* differentiation of embryonic stem cells into the pancreatic lineage. *Methods Mol. Biol.* 330, 373–385.
- Blyszczuk, P., Czyz, J., Kania, G., et al. (2003). Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. *Proc. Natl. Acad. Sci. USA* 100, 998–1003.
- Bonner-Weir, S., and Weir, G.C. (2005). New sources of pancreatic beta-cells. *Nat. Biotechnol.* 23, 857–861.
- Boyd, A.S., Wu, D.C., Higashi, Y., et al. (2008). A comparison of protocols used to generate insulin-producing cell clusters from mouse embryonic stem cells. *Stem Cells*. [E-pub ahead of print].
- Brolen, G.K., Heins, N., Edsbacke, J., et al. (2005). Signals from the embryonic mouse pancreas induce differentiation of human embryonic stem cells into insulin-producing beta-cell-like cells. *Diabetes* 54, 2867–2874.
- Cabrera, O., Berman, D.M., Kenyon, N.S., et al. (2006). The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proc. Natl. Acad. Sci. USA* 103, 2334–2339.
- Campbell, P.M., Senior, P.A., Salam, A., et al. (2007). High risk of sensitization after failed islet transplantation. *Am. J. Transplant.* 7, 2311–2317.
- D'Amour, K.A., Bang, A.G., Eliazar, S., et al. (2006). Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat. Biotechnol.* 24, 1392–1401.
- Drukker, M., and Benvenisty, N. (2004). The immunogenicity of human embryonic stem-derived cells. *Trends Biotechnol.* 22, 136–141.
- Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154–156.
- Fellous, T.G., Guppy, N.J., Brittan, M., et al. (2007). Cellular pathways to beta-cell replacement. *Diabetes Metab. Res. Rev.* 23, 87–99.
- Fujikawa, T., Oh, S.H., Pi, L., Hatch, H.M., Shupe, T., and Petersen, B.E. (2005). Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *Am. J. Pathol.* 166, 1781–1791.
- Fukazawa, T., Matsuoka, J., Naomoto, Y., et al. (2006). Development of a novel beta-cell specific promoter system for the identification of insulin-producing cells in *in vitro* cell cultures. *Exp. Cell Res.* 312, 3404–3412.
- Gruen, L., and Gabel, L. (2006). Concise review: scientific and ethical roadblocks to human embryonic stem cell therapy. *Stem Cells* 24, 2162–2169.
- Han, S.I., Aramata, S., Yasuda, K., et al. (2007). MafA stability in pancreatic beta cells is regulated by glucose and is dependent on its constitutive phosphorylation at multiple sites by glycogen synthase kinase 3. *Mol. Cell Biol.* 27, 6593–6605.
- Hansson, M., Tonning, A., Frandsen, U., et al. (2004). Artificial insulin release from differentiated embryonic stem cells. *Diabetes* 53, 2603–2609.
- Hara, M., Wang, X., Kawamura, T., et al. (2003). Transgenic mice with green fluorescent protein-labeled pancreatic beta-cells. *Am. J. Physiol. Endocrinol. Metab.* 284, E177–E183.
- Hara, M., Dizon, R.F., Glick, B.S., et al. (2006). Imaging pancreatic beta-cells in the intact pancreas. *Am. J. Physiol. Endocrinol. Metab.* 290, E1041–E1047.
- Hori, Y., Gu, X., Xie, X., et al. (2005). Differentiation of insulin-producing cells from human neural progenitor cells. *PLoS Med.* 2, e103.
- Hori, Y., Rulifson, I.C., Tsai, B.C., et al. (2002). Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. *Proc. Natl. Acad. Sci. USA* 99, 16105–16110.
- Jiang, J., Au, M., Lu, K., et al. (2007). Generation of insulin-producing islet-like clusters from human embryonic stem cells. *Stem Cells* 25, 1940–1953.
- Keller, G. (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev.* 19, 1129–1155.
- Kitano, M., Kakinuma, M., Takatori, A., et al. (2006). Gene expression profiling of mouse embryonic stem cell progeny differentiated by Lumelsky's protocol. *Cells Tissues Organs* 183, 24–31.
- Korsgren, O., Nilsson, B., Berne, C., et al. (2005). Current status of clinical islet transplantation. *Transplantation* 79, 1289–1293.
- Kroon, E., Martinson, L.A., Kadoya, K., et al. (2008). Pancreatic endoderm derived from human embryonic stem cells generate glucose-responsive insulin-secreting cells *in vivo*. *Nat. Biotechnol.* 26, 443–452.
- Leon-Quinto, T., Jones, J., Skoudy, A., et al. (2004). *In vitro* directed differentiation of mouse embryonic stem cells into insulin-producing cells. *Diabetologia* 47, 1442–1451.

- Leung, Y.M., Ahmed, I., Sheu, L., et al. (2005). Electrophysiological characterization of pancreatic islet cells in the mouse insulin promoter-green fluorescent protein mouse. *Endocrinology* 146, 4766–4775.
- Libman, I.M., and LaPorte, R.E. (2005). Changing trends in epidemiology of type 1 diabetes mellitus throughout the world: how far have we come and where do we go from here. *Pediatr Diabetes* 6, 119–121.
- Lipman, T.H., Jawad, A.F., Murphy, K.M., et al. (2006). Incidence of type 1 diabetes in Philadelphia is higher in black than white children from 1995 to 1999: epidemic or misclassification? *Diabetes Care* 29, 2391–2395.
- Lumelsky, N., Blondel, O., Laeng, P., et al. (2001). Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 292, 1389–1394.
- Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* 78, 7634–7638.
- Mathews, C.E., and Leiter, E.H. (1999). Resistance of ALR/Lt islets to free radical-mediated diabetogenic stress is inherited as a dominant trait. *Diabetes* 48, 2189–2196.
- Mathews, C.E., Pietropaolo, S.L., and Pietropaolo, M. (2003). Reduced thymic expression of islet antigen contributes to loss of self-tolerance. *Ann. N.Y. Acad. Sci.* 1005, 412–417.
- Meyer, K., Irminger, J.C., Moss, L.G., et al. (1998). Sorting human beta-cells consequent to targeted expression of green fluorescent protein. *Diabetes* 47, 1974–1977.
- Michael, D.J., Ritzel, R.A., Haataja, L., et al. (2006). Pancreatic beta-cells secrete insulin in fast- and slow-release forms. *Diabetes* 55, 600–7.
- Morton, R.A., Geras-Raaka, E., Wilson, L.M., et al. (2007). Endocrine precursor cells from mouse islets are not generated by epithelial-to-mesenchymal transition of mature beta cells. *Mol. Cell Endocrinol.* 270, 87–93.
- Narayan, K.M., Boyle, J.P., Thompson, T.J., et al. (2003). Lifetime risk for diabetes mellitus in the United States. *JAMA* 290, 1884–1890.
- Navara, C.S., Benyumov, A., Vassilev, A., et al. (2001). Vanadocenes as potent anti-proliferative agents disrupting mitotic spindle formation in cancer cells. *Anticancer Drugs* 12, 369–376.
- Noguchi, H. (2007). Stem cells for the treatment of diabetes. *Endocr J.* 54, 7–16.
- Ogawa, T., Arechaga, J.M., Avarbock, M.R., et al. (1997). Transplantation of testis germinal cells into mouse seminiferous tubules. *Int. J. Dev. Biol.* 41, 111–122.
- Ohara-Imaizumi, M., Fujiwara, T., Nakamichi, Y., et al. (2007). Imaging analysis reveals mechanistic differences between first- and second-phase insulin exocytosis. *J. Cell Biol.* 177, 695–705.
- Ohara-Imaizumi, M., and Nagamatsu S. (2006). Insulin exocytotic mechanism by imaging technique. *J. Biochem.* 140, 1–5.
- Phillips, B.W., Hentze, H., Rust, W.L., et al. (2007). Directed differentiation of human embryonic stem cells into the pancreatic endocrine lineage. *Stem Cells Dev.* 16, 561–578.
- Rajagopal, J., Anderson, W.J., Kume, S., et al. (2003). Insulin staining of ES cell progeny from insulin uptake. *Science* 299, 363.
- Rivas-Carrillo, J.D., Okitsu, T., Tanaka, N., et al. (2007). Pancreas development and beta-cell differentiation of embryonic stem cells. *Curr. Med. Chem.* 14, 1573–1578.
- Rolletschek, A., Kania, G., and Wobus, A.M. (2006). Generation of pancreatic insulin-producing cells from embryonic stem cells—“proof of principle,” but questions still unanswered. *Diabetologia* 49, 2541–2545.
- Rutter, G.A. (2004). Visualising insulin secretion. The Minkowski Lecture 2004. *Diabetologia* 47, 1861–1872.
- Ryan, E.A., Lakey, J.R., Rajotte, R.V., et al. (2001). Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 50, 710–719.
- Santana, A., Ensenat-Waser, R., Arribas, M.I., Reig, J.A., and Roche, E. (2006). Insulin-producing cells derived from stem cells: recent progress and future directions. *J. Cell Mol. Med.* 10, 866–883.
- Scharfmann, R. (2006). How to make beta cells from embryonic cells. *Diabetologia* 49, 2535–2536.
- Schroeder, I.S., Kania, G., Blyszczuk, P., et al. (2006a). Insulin-producing cells. *Methods Enzymol.* 418, 315–333.
- Schroeder, I.S., Rolletschek, A., Blyszczuk, P., et al. (2006b). Differentiation of mouse embryonic stem cells to insulin-producing cells. *Nat. Protoc.* 1, 495–507.
- Schuit, F., Flamez, D., De Vos, A., et al. (2002). Glucose-regulated gene expression maintaining the glucose-responsive state of beta-cells. *Diabetes* 51 (Suppl 3): S326–S332.
- Serup, P. (2006). Embryonic stem cell-based diabetes therapy—a long road to travel. *Diabetologia* 49, 2537–2540.
- Shi, Y., Hou, L., Tang, F., et al. (2005). Inducing embryonic stem cells to differentiate into pancreatic beta cells by a novel three-step approach with activin A and all-trans retinoic acid. *Stem Cells* 23, 656–662.
- Sipione, S., Eshpeter, A., Lyon, J.G., et al. (2004). Insulin-expressing cells from differentiated embryonic stem cells are not beta cells. *Diabetologia* 47, 499–508.
- Soria, B., Roche, E., Berna, G., et al. (2000). Insulin-secreting cells derived from embryonic stem cells normalize glycaemia in streptozotocin-induced diabetic mice. *Diabetes* 49, 157–162.
- Souza, F., Freeby, M., Hultman, K., et al. (2006). Current progress in non-invasive imaging of beta cell mass of the endocrine pancreas. *Curr. Med. Chem.* 13, 2761–2773.
- Ta, M., Choi, Y., Atouf, F., et al. (2006). The defined combination of growth factors controls generation of long-term-replicating islet progenitor-like cells from cultures of adult mouse pancreas. *Stem Cells* 24, 1738–1749.
- Vaca, P., Martin, F., Vegara-Meseguer, J.M., et al. (2006). Induction of differentiation of embryonic stem cells into insulin-secreting cells by fetal soluble factors. *Stem Cells* 24, 258–265.
- Watkins, S., Geng, X., Li, L., et al. (2002). Imaging secretory vesicles by fluorescent protein insertion in propeptide rather than mature secreted peptide. *Traffic* 3, 461–471.

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