Correction of hyperglycemia in type 1 diabetic models by transplantation of encapsulated insulin-producing cells derived from mouse embryo progenitor

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Abstract

Shortage of cadaveric pancreata and requirement of immune suppression are two major obstacles in transplantation therapy of type 1 diabetes. Here, we investigate whether i.p. transplantation of alginate-encapsulated insulin-producing cells from the embryo-derived mouse embryo progenitor-derived insulin-producing-1 (MEPI-1) line could lower hyperglycemia in immune-competent, allogeneic diabetic mice. Within days after transplantation, hyperglycemia was reversed followed by about 2·5 months of normo- to moderate hypoglycemia before relapsing. Mice transplanted with unencapsulated MEPI cells relapsed within 2 weeks. Removal of the transplanted capsules by washing of the peritoneal cavity caused an immediate relapse of hyperglycemia that could be reversed with a second transplantation. The removed capsules had fibrotic overgrowth but remained permeable to 70 kDa dextrans and displayed glucose-stimulated insulin secretion.

Following transplantation, the number of cells in capsules increased initially, before decreasing to below the starting cell number at 75 days. Histological examination showed that beyond day 40 post-transplantation, encapsulated cell clusters exhibited proliferating cells with a necrotic core. Blood glucose, insulin levels, and oral glucose tolerance test in the transplanted animals correlated directly with the number of viable cells remaining in the capsules. Our study demonstrated that encapsulation could effectively protect MEPI cells from the host immune system without compromising their ability to correct hyperglycemia in immune-competent diabetic mice for 2·5 months, thereby providing proof that immunoisolation of expansible but immune-incompatible stem cell-derived surrogate β-cells by encapsulation is a viable diabetes therapy.

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Introduction

Islet or pancreas transplantation is a promising approach to treat type 1 diabetes (Shapiro *et al.* 2000, Halban 2004, Vaithilingam *et al.* 2008, Ichii & Ricordi 2009). However, the limited supply of cadaveric pancreata and the need for long-term immunosuppression have limited the use of this approach. Therefore, alternative methods of cell replacement therapy by generating surrogate β-cells (Halban 2004, Bonner–Weir & Weir 2005, Ricordi & Edlund 2008) that could circumvent the need for long-term immunosuppression will greatly enhance the utility of this approach. Insulin-producing cells can be derived from adult cells including islet precursor cells, acinar cells, bone marrow cells, and liver cells (Ferber *et al.* 2000, Bonner–Weir & Sharma 2002, Ianus *et al.* 2003). The other promising approach is to differentiate embryonic stem (ES) cells into insulin-producing cells (Soria *et al.* 2001, Halban 2004, Fujikawa *et al.* 2005). Soria *et al.* (2000) first reported that ES cells could be induced to such

cells. Multi-stage protocols were used to differentiate ES cells into insulin-producing cells (Shi *et al.* 2005, D'Amour *et al.* 2006). However, the efficiency of generating these insulin-producing cells and their insulin content as reported to date is generally low, their functions are also too poor to fulfill the requirement criteria of surrogate β-cells in replacement therapy.

We have previously generated more than a dozen functional insulin-producing cell lines from progenitor cells of early gastrulating mouse embryos (Li *et al.* 2009b) and also from mouse ES cells (Li *et al.* 2009a). Although these cell lines were derived from different precursors, they all displayed similar properties in terms of gene expression profile and responsiveness to glucose stimulation (Li *et al.* 2009a,b, Chen *et al.* 2010). One of the lines, mouse embryo progenitor-derived insulin-producing-1 (MEPI-1), has been extensively characterized. The cells express β-cell-specific genes and contain typical insulin secretory granules with much higher insulin content than previously reported insulin-producing

cell lines. These cells secrete equimolar ratio of insulin and C-peptide when stimulated by glucose, tolbutamide, or glibenclamide through a mechanism similar to that of adult pancreatic β-cells. These stimuli trigger the closure of K_{ATP} channels leading to the depolarization of the cell membrane and opening of the voltage-gated Ca^{2+} channels in these cells. In addition, these cells can be stably expanded to a large number with no loss in insulin content (Li *et al.* 2009*b*). *In vivo*, MEPI-1 cells engraft and reverse streptozotocin (STZ)–induced hyperglycemia in SCID mice without producing teratomas (Li *et al.* 2009*b*). However, transplantation of these cells in STZ–induced immune-competent diabetic mice only transiently and partially corrected hyperglycemia, apparently due to acute immune rejection (unpublished data).

The use of encapsulation technology to circumvent immune rejection has been widely reported (Lim & Sun 1980, Calafiore *et al.* 1997, van Schilfgaarde & de Vos 1999, Orive *et al.* 2003, Omer *et al.* 2005, Zimmermann *et al.* 2007, Fort *et al.* 2008, Murua *et al.* 2008, Weber *et al.* 2010). For example, isolated islets have been microencapsulated in barium alginate before peritoneal transplantation in diabetic animals (Lim & Sun 1980, Omer *et al.* 2005). Encapsulation can physically isolate a cell mass from an outside environment and protect the encapsulated cells from contact and attack by immune cells and immunoglobulins. At the same time, the permeability of the capsule membranes allows for accessibility to small molecules such as glucose, insulin, and other molecules that are essential for cell survival (Calafiore *et al.* 1997, de Groot *et al.* 2004, Zimmermann *et al.* 2005).

In this study, MEPI-1 cells were microencapsulated in barium alginate before peritoneal implantation in STZ–induced hyperglycemic mice to examine their *in vivo* functionality. Our results indicated that transplantation of encapsulated MEPI-1 cells into immune-competent diabetic mice was able to correct hyperglycemia for about 2·5 months. Importantly, this hyperglycemia correction outcome could be reproduced by repeated implantations of encapsulated cells. Thus, the generation of expansible, secretory-competent insulin-producing cells from ES/progenitor cells together with the immunoisolation technology opens new perspectives in cell replacement therapy for diabetes.

Materials and Methods

MEPI-1 cell culture

MEPI-1 cells (originally from C57 strain mouse) were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in humidified air with 5% CO_2 (Li *et al.* 2009*b*). Cells were seeded at a concentration of 1×10^6/ml, and the medium was changed every 48 h. Cells were sub-cultured by weekly trypsinization.

Microencapsulation of MEPI-1 cells in barium alginate

About 7·5 million MEPI-1 cells were harvested and cultured in suspension in RMPI 1640 overnight to form cell clusters. After two washes with saline, the cells were mixed with 1·5 ml alginate SLG 100 (NovaMatrix, Sandvika, Norway). Afterwards, the alginate–cell suspensions were transferred into a 5 ml syringe, which was mounted on a syringe pump (Harvard Apparatus, Holliston, MA, USA) attached to an electrostatic encapsulation system (Nisco, Zurich, Switzerland). After encapsulation, the capsules were transferred with the barium gelling solution into a 50 ml centrifuge tube and sedimented down. The hardening solution was then removed, and capsules were washed three times with saline and once with cell culture medium. There were about 3% empty capsules that were washed away during the three washes. Eventually, 5 million encapsulated cells in around 1500 capsules were achieved. Thus, the encapsulation yield was 67%. There were one to three cell clusters per capsule. The newly made capsules were cultured overnight before transplantation.

Peritoneal transplantation of microcapsules

Immune-competent male Balb/c mice of 8 weeks were purchased from local laboratory animal holding facilities, and their use and experimental procedures were approved by IACUC of the institutions. The mice were subject to two injections (15 mg/kg in the first and 12·5 mg/kg in the second on 2 days later) of STZ to induce hyperglycemia (Fujikawa *et al.* 2005, Li *et al.* 2009*a,b*). An aliquot of capsules containing 2·5 million MEPI-1 cells in about 1·5 ml saline solution was peritoneally transplanted into each STZ–induced diabetic mouse with hyperglycemia constantly exceeding 350 mg/dl in this study. Transplantation of 5 million encapsulated cells resulted in a high frequency of severe hypoglycemia with 60% of mice dying within 40 days. On the other hand, it took a long time to correct hyperglycemia when 1 million encapsulated cells were transplanted. Blood glucose and body weight were monitored before and after implantation.

Washing out of transplanted microcapsules

Transplanted microcapsules were washed out from abdominal cavity by flushing the cavity with sterile saline solution at different stages: 1) when glycemic level was corrected to normal and kept stable, and 2) when hyperglycemia reappeared.

Assessment of insulin content and secretion

Insulin content and secretion of encapsulated MEPI-1 cells in both freshly made and washed-out capsules were examined as described previously (Asfari *et al.* 1992, Li *et al.* 2009*b*). Capsules were placed in 24-well plates and cultured overnight. After pre-incubation with Krebs-Ringer bicarbonate HEPES (KRBH) buffer (Asfari *et al.* 1992) for 30 min at
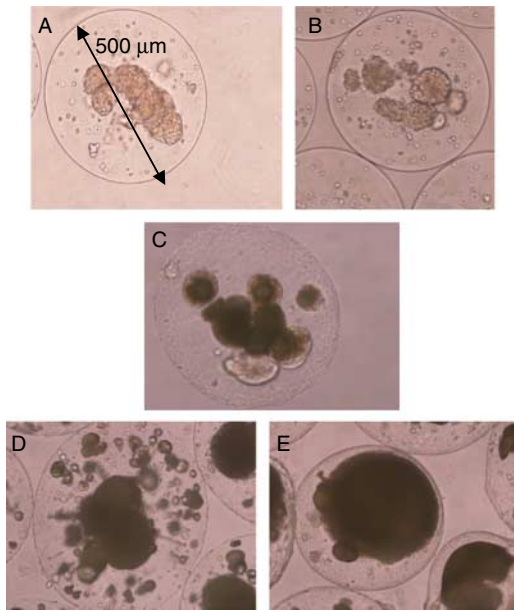
Figure 1 Barium alginate-made capsules both *in vitro* and *in vivo*. (A) Newly made capsules and capsules after 30 days (B) and 100 days (C) in cell culture. Capsules in (D) and (E) were washed out after peritoneal transplantation of 30 and 100 days respectively. 100× magnification.

37 °C, the capsules were incubated in KRBH buffer containing test agents for another 30 min. The supernatants were removed for measurements of secreted insulin, and the capsules were extracted by acid–ethanol for determining the insulin content. Insulin was assayed by RIA (Linco Research, St Charles, MO, USA).

DNA content determination

Capsules in 48-well plates were washed, and 200 μl Hoechst 33258 (1 μg/ml) was added into each well. Fluorescence was measured by a fluorescence plate reader (Tecan M200) at the excitation wavelength of 346 nm and emission wavelength of 460 nm. DNA concentration of each sample was quantified against a DNA standard curve.

Determination of plasma insulin

At specified time points, blood was collected from the mouse heart in the presence of heparin, an anticoagulant. Insulin in the plasma was determined by RIA (Linco Research).

Oral glucose tolerance test

Mice were fasted for 18 h, and then glucose (1·5 g/kg of body weight) was orally administrated to each mouse. Blood was collected at 0, 10, 20, 30, 50, 70, and 90 min from tail vein and measured with glucose meter (Bayer Diabetes Healthcare).

Permeability detection

Capsules were incubated with dextrans (250 μg/ml) and shaken at room temperature for 2 h before observation under fluorescence microscope. TRITC-dextran (4·4 kDa) and FITC-dextrans (20 and 70 kDa; Sigma) were used for the assessment of capsule permeability.

Immunohistochemistry

5–Bromo-2′–deoxy–uridine staining Capsules were incubated at 37 °C in cell culture medium with 20 μM 5-bromo-2′-deoxy-uridine (BrdU) for 24 h and then fixed in ethanol–glycine (70% absolute ethanol/30% 50 mM glycine). After washing and incubating in 30% sucrose at 4 °C with shaking overnight, the capsules were frozen in liquid nitrogen and then cryosectioned (3 μm). Subsequently, the sections were treated in 4 M HCl, permeabilized in 0·1% Triton X-100, and blocked with 4% BSA. The sections were then incubated with monoclonal anti-BrdU antibody, followed by goat anti-mouse IgG-TRITC antibody (Sigma). Finally, sections were mounted using Vectashield with 4′,6-diamidino-2-phenyindole, dilactate (DAPI, Sigma) for counter staining of nuclei before observation by fluorescence microscopy.

Insulin staining Capsule cryosections (3 μm) were fixed in 3·7% formaldehyde and permeabilized with 0·2% saponin. After blocking with 4% BSA, sections were incubated with guinea pig anti-insulin antibody (Chemicon–Millipore, Billerica, MA, USA) and then with FITC-conjugated anti-guinea pig IgG (Sigma). Afterward, sections were mounted using Vectashield with DAPI for counter staining of nuclei before analysis.

H&E staining Cryosections (5 μm) were fixed in 100% ethanol for 1 min and then stained in Harris' hematoxylin for
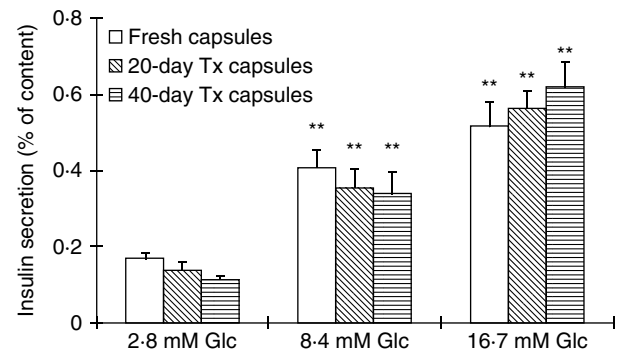

Figure 2 Insulin secretion from encapsulated MEPI-1 cells. Newly made and washed-out capsules after 20- or 40-day transplantation (Tx) in STZ-treated mice were incubated with basal or two stimulating concentrations of glucose (Glc) for 30 min. Results of insulin secretion were expressed as percentages of insulin content to correct for variation in the capsule number among wells. Data are mean±s.e.m. from three independent experiments. **$P < 0.01$ vs 2·8 mM Glc.

2 min. Subsequently, sections were dipped in 1% acid alcohol for 1 s, followed by counter staining in eosin for 3 s and visualized under microscope.

Cytokine measurement

Peritoneal fluids and culture media were collected at different intervals. Bio-Plex Pro assays in 96-well plates (Bio-Rad) were used to detect 23 cytokines simultaneously according to the manufacturer's manual.

Statistical analysis

Data were expressed as mean ± S.E.M. and analyzed by two-tailed t-test or ANOVA. $P < 0.05$ is considered significant.

Results

Stable microcapsules *in vitro and* in vivo

The average diameter of cell microcapsules was 500 μm (Fig. 1A). Capsules were mechanically stable after culture

in vitro or following transplantation *in vivo* of 30 (Fig. 1B and D) and 100 days (Fig. 1C and D). MEPI-1 cell clusters within microcapsules grew larger and denser with the time.

Glucose-stimulated insulin secretion in encapsulated cells

MEPI-1 cells in freshly made capsules after culture overnight retained their ability to increase insulin secretion upon glucose stimulation (Fig. 2), as previously reported for non-encapsulated cells grown as monolayers (Li *et al.* 2009*b*). Furthermore, cells in washed-out capsules from transplanted mice at 20 and 40 days were also able to enhance insulin release when stimulated by high glucose like those newly encapsulated cells (Fig. 2).

Reversing hyperglycemia in diabetic mice by transplantation of encapsulated MEPI-1 cells

Two injections of STZ rendered immune-competent mice stably hyperglycemic within days. Transplantation of unencapsulated MEPI-1 cells under the kidney capsule in these mice only transiently corrected hyperglycemia (Fig. 3A, solid circles).
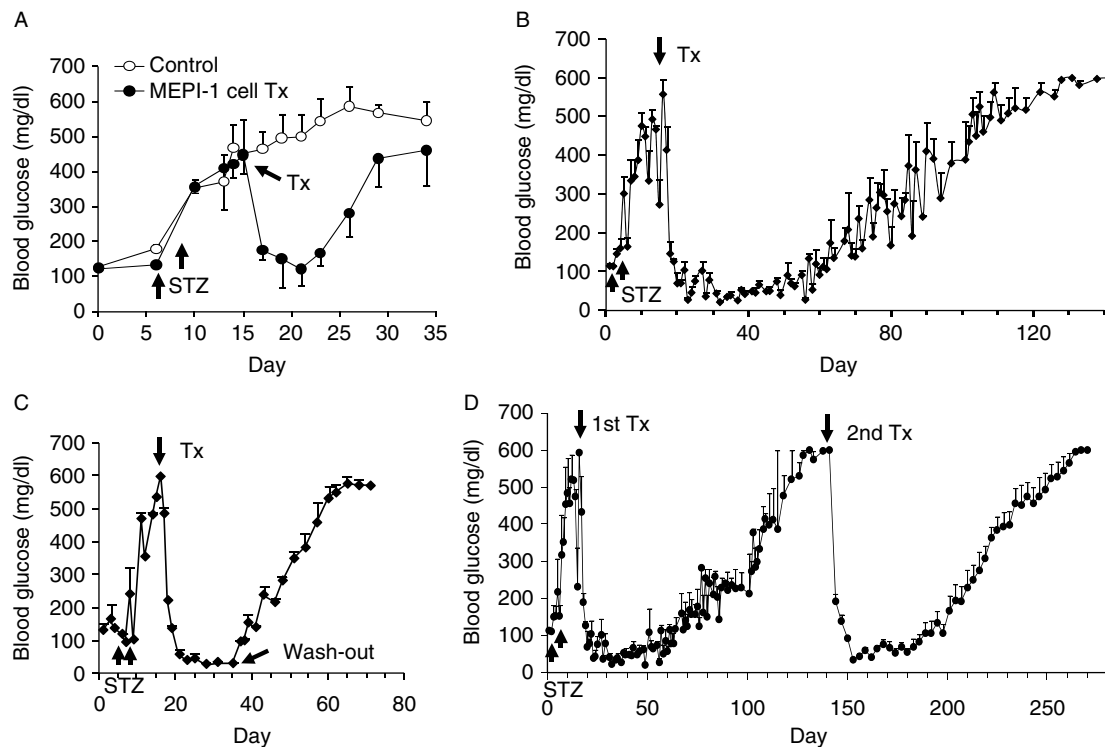

Figure 3 Reversing hyperglycemia in STZ-induced immune-competent diabetic mice by transplantation (Tx) of encapsulated MEPI-1 cells. (A) Transplantation of unencapsulated MEPI-1 cells into kidney subcapsule at 2·5 million cells per mouse resulted in transient correction of hyperglycemia. The control group was subject to sham surgery. (B) Peritoneal transplantation of encapsulated MEPI-1 cells (2·5 million cells) reversed hyperglycemia in STZ-treated mice. The mice developed hypoglycemia and relapsed to hyperglycemia 2·5 months later. (C) Encapsulated MEPI-1 cells were peritoneally transplanted in STZ-treated mice, and capsules were washed out after hyperglycemia was stably corrected. (D) Second transplantation in STZ-induced diabetic mice that had a relapse of hyperglycemia after the first transplantation effectively reversed hyperglycemia. Data are mean ± S.E.M. from 3 (in A and C), 18 (in B), and 7 (in D) independent observations.
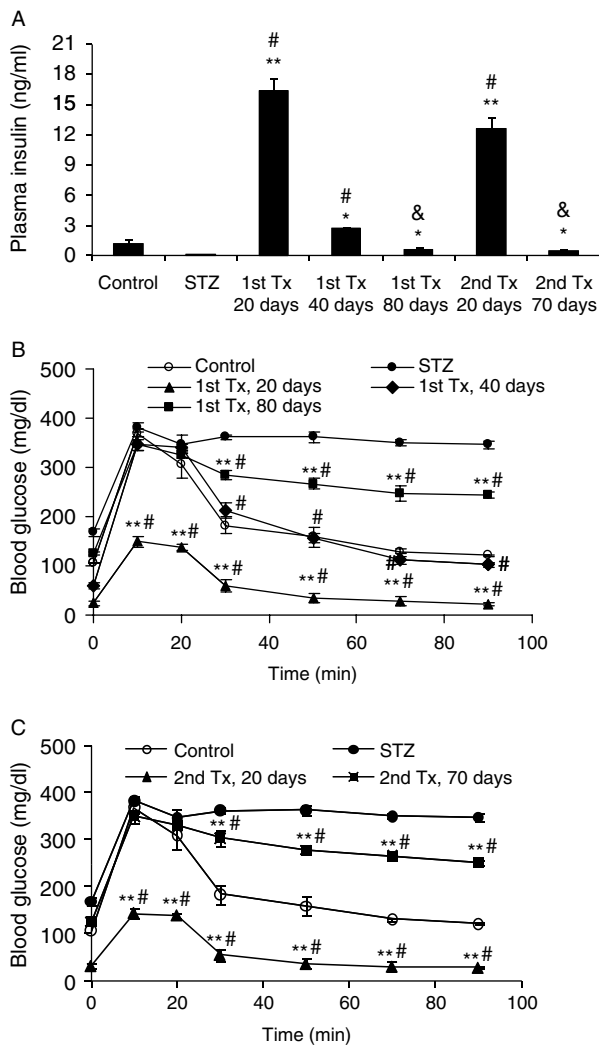
Figure 4 Plasma insulin and OGTT in mice at different intervals after transplantation of encapsulated MEPI-1 cells. (A) Blood was collected from non-treated mice (control), mice treated with STZ alone (STZ), and STZ-treated mice followed by peritoneal implantation of encapsulated MEPI-1 cells at 20, 40, and 80 days after first Tx, and at 20 and 70 days after second Tx. *$P<0.05$ and **$P<0.01$ versus control; $^{\&}P<0.05$ and $^{\#}P<0.01$ versus STZ. (B) OGTT was performed in STZ-treated mice at 20, 40, and 80 days after first Tx of encapsulated MEPI-1 cells, in comparison with control and STZ-treated mice. (C) OGTT was performed on mice at 20 and 70 days after second Tx. Results are mean ± S.E.M. from three independent experiments. **$P<0.01$ versus control and $^{\#}P<0.01$ versus STZ in both (B) and (C).

Histological examination revealed a significant infiltration of immune cells with few insulin-positive cells at the implantation site 1 week after transplantation (data not shown), indicating acute immune rejection. In contrast, peritoneal transplantation of encapsulated MEPI-1 cells in diabetic mice quickly restored normoglycemia (70–150 mg/dl, $n=18$) that lasted for ~4 days (Fig. 3B). Thereafter, the mice developed

varying degrees of hypoglycemia, defined as blood glucose level below 70 mg/dl. On average, the mice had low blood glucose level of 58.3 ± 6.1 mg/dl for 38.5 ± 3.8 days (Fig. 3B). Despite the hypoglycemia, they exhibited similar physical activity and body weight to the non-diabetic controls. Following the hypoglycemia period, glycemic levels gradually increased to the euglycemic ranges (110.4 ± 4.5 mg/dl over 32.6 ± 4.1 days on average). About 3 months after implantation, all the mice started to become hyperglycemic, eventually reaching pre-implantation level (Fig. 3B). The average duration between capsule transplantation and relapse of hyperglycemia (>300 mg/dl) was 83.8 ± 4.3 days (126 days for the longest), and the average glycemic levels were 112.1 ± 7.8 mg/dl during this period. Independent experiments revealed that when implanted capsules were removed with saline washing of the peritoneal cavity before mice relapsed to hyperglycemia, a sharp increase in glycemic levels ensued (Fig. 3C), further indicating that encapsulated cells were secreting insulin into the blood stream. Transplantation of the same number of empty capsules into STZ-induced diabetic mice ($n=4$) had no effect on hyperglycemia (data not shown).

Another set of experiments was performed to evaluate the efficacy of repeated transplantations in restoring normoglycemia after mice had relapsed to hyperglycemia, i.e. 600 mg/dl (Fig. 3D). The second implantation of encapsulated MEPI-1 cells not only reversed hyperglycemia as efficiently as the first, it also replicated the kinetics in glycemic profile of the first implantation, i.e. normoglycemia, hypoglycemia, euglycemia, and hyperglycemia (Fig. 3D). In addition, the average glycemic levels before relapse were similar to that observed in the first transplantation (114.7 ± 6.7 mg/dl, $n=7$), although the durations between capsule transplantation and hyperglycemia relapse were a bit shorter (71.6 ± 4.1 days).

Plasma insulin level and oral glucose tolerance test in capsule-transplanted diabetic mice

To assess more directly the effects of transplantation, plasma insulin levels were measured. Insulin levels were 1.14 ± 0.17 ng/ml in non-diabetic mice and undetectable in STZ-treated mice (Fig. 4A). Insulin levels at 20 days after the first transplantation of encapsulated MEPI-1 cells were markedly elevated (16.33 ± 1.29 ng/ml), but were less elevated at 40 days, and were below the normal level at 80 days after transplantation (0.66 ± 0.09 ng/ml; Fig. 4A). During the second transplantation, the plasma insulin levels were also initially elevated (12.67 ± 1.1 at day 20) but later declined to 0.54 ± 0.10 ng/ml at 70 days. In addition, oral glucose tolerance tests (OGTTs) were carried out at various days after transplantation to assess the function of implanted encapsulated MEPI-1 cells. The overall changes in OGTT patterns were similar after the first and second transplantations (Fig. 4B and C). OGTT in the initial period after transplantation (at day 20) revealed a stronger suppression of
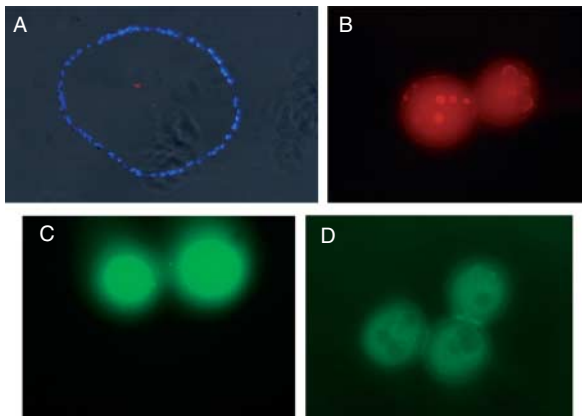
Figure 5 DAPI staining and permeability of washed-out capsules. (A) DAPI staining of a capsule washed out after 100 days of implantation in STZ-treated mice with relapsed hyperglycemia (600 mg/dl) showed fibrotic overgrowing at its surface. (B–D) Capsules were washed out from diabetic mice after 100 days of implantation and then incubated with TRITC-dextran of 4·4 kDa (B), FITC-dextran of 20 kDa (C) and 70 kDa (D). Staining of fluorescent dextrans was examined after washing. Images are representative of five experiments with similar results.

glycemic rises compared to that in non–diabetic mice. At day 40, OGTT in capsule-implanted mice was compatible with that in non-diabetic mice. However, OGTT at day 80 after first transplantation or day 70 after second implantation was severely impaired, similar to that displayed by diabetic mice, indicating the loss of function of cells in transplanted capsules.

Normal permeability of capsules after implantation in mice

To investigate the cause of the failure of encapsulated MEPI–1 cells to maintain the function beyond 80 days after implantation, the permeability of capsules was examined. DAPI staining showed a layer of cells covering the washed–out capsules at 100 days post transplantation when the mice had relapsed to a hyperglycemic stage (Fig. 5A), suggesting fibrotic overgrowth. However, when these washed–out capsules were incubated with florescence-labeled dextrans of 4·4–70 kDa, they remained permeable to them (Fig. 5B–D).

MEPI-1 cell growth in capsules

The population doubling time of MEPI–1 cells cultured in monolayer was stable at about 2·4 days throughout with more than 30 passages (Li *et al.* 2009*b*). As the barium alginate-based capsules could only be dissolved in toxic chemical solution, e.g. overnight incubation with 1 M sodium citrate, the growth kinetics of encapsulated cells was assessed indirectly by determining the DNA and insulin contents in the capsules. The changes in DNA and insulin contents in the washed-out capsules at given intervals after peritoneal implantation were directly correlated (Fig. 6A). They both increased to peak at about 20–30 days. Thereafter, they declined to below the

starting levels after 75 days. Using these data, the cell population doubling time in capsules was calculated to be about 2·4 days before day 5 and then became longer with time (Supplementary Figure 1, see section on supplementary data given at the end of this article), indicating that the increase of MEPI-1 cell number in capsules slowed with time. In addition, we also estimated the population growth kinetics of encapsulated cells after transplantation by counting of DAPI- or BrdU-stained cells in histological sections of clusters at five time points. The kinetic changes in the number of cells determined by both staining approaches were very similar to those obtained by assessment of insulin and DNA contents (Fig. 6B and C). However, the percentage of mitotic cells did
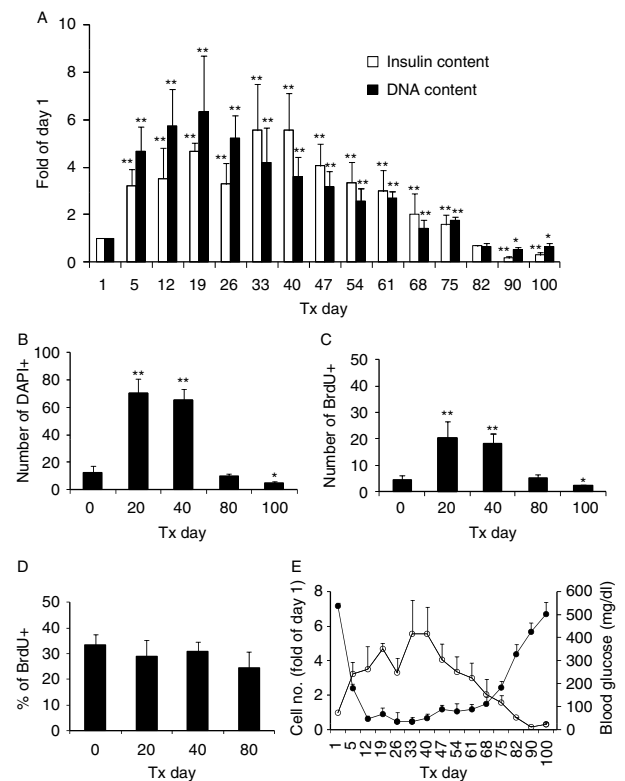

Figure 6 Growth of encapsulated MEPI-1 cells after transplantation in STZ-treated mice and the relationship with glycemic levels. (A) Capsules were washed out at different intervals after peritoneal transplantation. Average insulin and DNA contents of capsulated MEPI-1 cells per capsule were measured and expressed as fold change relative to that in fresh capsules (day 1). (B and C) Number of DAPI- and BrdU-stained cells in histological sections and (D) the proliferation rates over the transplantation period. Data are mean±S.E.M. from counting five sections of individual encapsulated cell clusters in each case. (E) Changes between the cell number (calculated from data of insulin content) per capsule (open circles) and glycemic levels (closed circles) from day 1 to day 100 after transplantation into STZ-induced diabetic mice were anti-parallel. Based on insulin content, the number of cells per newly made capsule was about 2000. Data are mean±S.E.M. from five independent experiments. *$P<0·05$ and **$P<0·01$ versus day 1.

not alter significantly over the transplantation period (Fig. 6D). Interestingly, *in vitro* cultures of MEPI cell capsules exhibited similar kinetics in population doubling time (Supplementary Figure 1), DNA and insulin contents (Supplementary Figure 2A, see section on supplementary data given at the end of this article), number of DAPI- and BrdU-positive cells (Supplementary Figure 2B and C), and proliferation rates (Supplementary Figure 2D).

An anti-reciprocal pattern was observed between MEPI-1 cell number in capsules and glycemic levels following implantation (Fig. 6E). The number of cells per capsule increased initially and then decreased to below the original after 75 days of transplantation (Fig. 6E; open circles). Accordingly, the high blood glucose levels declined when the cell number in capsules was increasing initially, but started to increase as the encapsulated cell number decreased to or below the original transplanted cell number (Fig. 6E; closed circles). These results indicated that the number and viability of MEPI-1 cells in capsules are critical in maintaining glycemic control.

Histology of encapsulated MEPI cells

Histological examinations were performed on encapsulated MEPI-1 cells at various time points after peritoneal implantation or *in vitro* culture. Capsules were washed out of the peritoneal cavity during the period of mild hypoglycemia (day 20 and 40) and hyperglycemia (day 80). At day 20, both H&E and DAPI staining showed an even distribution of nuclei in the encapsulated cell clusters either after implantation (Fig. 7) or after culture *in vitro* (Supplementary Figure 3, see section on supplementary data given at the end of this article) up to 20 days. Progressive central necrosis was observed in the cell clusters after 40 days in either condition. Additionally, BrdU incorporation assay indicated that there was cell proliferation both in the center and at the periphery of the clusters until at least day 20 after implantation (Fig. 7) or *in vitro* culture (Supplementary Figure 3). However, cell proliferation was only observed in the periphery of clusters in capsules beyond 40 days in either case. When insulin immunofluorescence was performed, strong signal was seen in the cell clusters at 20 days following implantation of the capsules (Fig. 7) or *in vitro* culture (Supplementary Figure 3). Beyond 40 days, insulin staining in the cell cluster became progressively weaker, and at day 80 after implantation when hyperglycemia had relapsed, insulin-positive cells in the washed-out capsules were few.

Peritoneal cytokine production in capsule-implanted mice

After transplantation of encapsulated MEPI-1 cells, peritoneal fluid was collected at different intervals. Using a multiplex assay system, production of 23 cytokines could be determined simultaneously. The results revealed varying degrees of
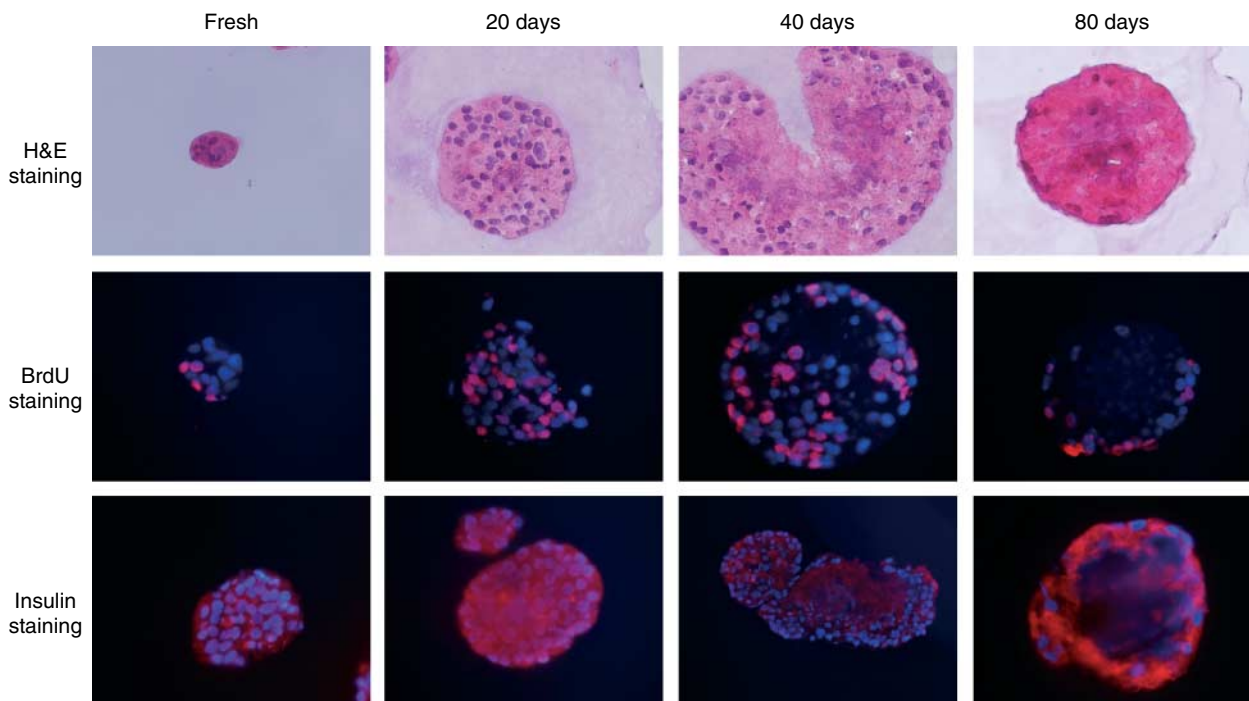

Figure 7 Histology of encapsulated MEPI-1 cells after transplantation. H&E staining, BrdU staining, and insulin staining were performed on capsules washed out at different intervals after peritoneal implantation in STZ-treated mice. Images are representative of five experiments.

increase for 12 cytokines, and among them, interleukin 12, macrophage inflammatory protein-1β, and rantes levels were most highly elevated (Supplementary Table 1, see section on supplementary data given at the end of this article). These cytokines all exhibited a marked increment initially after transplantation, followed by a decline. In addition, the first transplantation induced a much larger increment of cytokines than the second transplantation. In contrast, most increases in cytokine production from encapsulated cells cultured *in vitro* were trivial, and most cytokines were undetectable (Supplementary Table 1).

Discussion

In this study, we demonstrated that peritoneal transplantation of encapsulated MEPI-1 cells into immune-competent, STZ-induced diabetic mice could correct hyperglycemia for 2·5 months. Although the capsule-transplanted mice eventually relapsed to hyperglycemia due apparently to the death of implanted cells, a second transplantation of encapsulated cells could effectively correct the hyperglycemia again. This study demonstrated that the expansible secretory-competent insulin-producing cells generated from ES/progenitor cells in a reproducible manner could be immunoisolated to circumvent immune rejection without compromising their therapeutic efficacy in regulating glycemic level in a mouse model. It also demonstrated that sequential multiple transplantations of the immunoisolated cells were individually effective at regulating glycemic level. Together, this study provides new strategic perspectives in cell replacement therapy for diabetes.

Transplantation of encapsulated islets to reverse hyperglycemia in diabetic animals has been reported by many researchers (Mikos *et al*. 1994, Efrat 2002, Orive *et al*. 2003, Kizilel *et al*. 2005, Zimmermann *et al*. 2005, Vaithilingam *et al*. 2008, Abalovich *et al*. 2009). However, this approach has been severely limited by the lack of islet donors. We recently reported a novel strategy to generate a potentially infinite supply of surrogate insulin-producing β-cells. This strategy essentially describes a reproducible protocol that yielded three groups of insulin-producing cell lines from differentiation of mouse early embryo culture, an embryo-derived progenitor RoSH cell line or an ES cell-derived lineage-restricted E-RoSH cell line respectively (Li *et al*. 2009a,b). Despite being generated from different precursors, these insulin-producing cell lines are highly similar in terms of their gene expression profile, signaling events, and insulin secretion responses to glucose stimulation (Li *et al*. 2009a,b, Chen *et al*. 2010). These cells are highly homogenous and expansible, and could directly address the problem of limited β-cell source for transplantation. In addition, they are capable of reversing hyperglycemia in immune-deficient diabetic mice (Li *et al*. 2009a,b). In the current study, we have demonstrated that one of these cell lines (MEPI-1) could exert similar therapeutic effect after being encapsulated and transplanted in immune-competent diabetic animals. Our results revealed

that the freshly encapsulated or transplanted encapsulated MEPI-1 cells retained their ability to increase insulin secretion upon glucose stimulation like the unencapsulated cells as described previously (Li *et al*. 2009a,b). This demonstrated that encapsulation or transplantation of the encapsulated cells did not affect the functional capacity of the cells.

One important issue regarding the use of ES/progenitor-derived insulin-producing cells in cell replacement therapy for type 1 diabetes is their long-term survival and function after encapsulation and transplantation. Two sets of transplantation experiments using MEPI-1 cells were performed to assess this issue. In the first set of experiments, one group of STZ-induced hyperglycemic mice was peritoneally transplanted with 2·5 million encapsulated cells, whereas the control group (also STZ-treated) was subject to sham surgery only. After transplantation, normoglycemia was restored within a few days followed by the development of hypoglycemia in varying degrees for a considerable period. During this period, OGTT was normalized or glucose was cleared faster than the non-diabetic mice, correlating well with the hyperinsulinemia observed. These results indicated that encapsulated MEPI-1 cells could correct hyperglycemia effectively. This effect lasted for an average of 2·5 months in 18 mice tested. Then, the mice relapsed to the elevated glycemic levels before transplantation. In the second set of experiments, a second transplantation was conducted after the relapse to hyperglycemia in mice implanted with encapsulated MEPI-1 cells. This second transplant basically elicited a similar profile of glycemic changes, OGTT, and blood insulin levels as observed in the first transplant. This demonstrated that repeated transplantations could elicit similar corrective effects on hyperglycemia. Therefore, transplantation of microcapsules using highly expansible surrogate β-cells represents an effective therapeutic approach for long-term treatment of type 1 diabetes.

Like transplantation of encapsulated islets (van Schilfgaarde & de Vos 1999, Vaithilingam *et al*. 2008, Abalovich *et al*. 2009), the relapse of hyperglycemia observed 2·5 months after transplantation of encapsulated MEPI-1 cells in diabetic mice might involve three major scenarios.

One is related to the change in permeability due to the fibrotic overgrowth on the surface of transplanted microcapsules (Siebers *et al*. 1997, van Schilfgaarde & de Vos 1999, de Vos *et al*. 2002). Host can respond to the transplanted biomaterials and initiate an inflammatory reaction. The membrane layer of our washed-out capsules was apparently thicker than the newly made capsules. DAPI staining revealed a layer of cell nuclei around these capsules. These cells revealed positive Masson's trichrome staining of collagen (data not shown), indicative of fibrotic overgrowth. This might reduce the permeability of transplanted capsules, and thus prevent the delivery of insulin secreted from and the supply of nutrients into encapsulated MEPI-1 cells. However, despite this overgrowth, membrane permeability was not compromised, as dextrans with molecular weight up

to 70 kDa could still pass through the washed-out capsules. This molecular cut-off point is sufficient for the traffic of insulin and necessary nutrients (Calafiore *et al.* 1997, Zimmermann *et al.* 2005). Therefore, the fibrotic overgrowth did not contribute significantly to the relapse of hyperglycemia.

Another potential event causing the failure of transplantation of encapsulated cells is the immune reaction. Although alginate capsules can insulate cells from contact and attack by immune cells, immune reaction may still occur through humoral route by the release of cell debris (Calafiore *et al.* 1997, Siebers *et al.* 1997). It has been speculated that the failure of islet transplantation could be attributable to several factors including erforin, cytokines, nitric oxide, poor nutrition, and hypoxia (Siebers *et al.* 1999, Gray 2001, de Groot *et al.* 2003, 2004). Indeed, our study found that at least 12 cytokines were increased to different degrees in the peritoneal fluid of capsule-implanted mice. These cytokines are involved in various inflammatory reactions. Their induction was obvious initially after transplantation but declined thereafter. Furthermore, the first transplantation evoked a larger increment in the level of cytokines than that by the second transplantation. These data indicated that although the transplantation of encapsulated MEPI-1 cells could elicit an inflammatory reaction, the reaction did not persist or escalate beyond the initial reaction. Moreover, if cytotoxic mediators, such as cytokines, were responsible for the graft necrosis, the periphery and not the center would be more susceptible to necrotic death. Instead, we observed in this study that cells in the center of the cluster were necrotic, while those at the periphery of clusters were viable. This phenomenon was also observed in the transplantation of encapsulated islets especially when larger islet clusters were used (de Groot *et al.* 2003, Langlois *et al.* 2009). Furthermore, in long-term *in vitro* culture of cell capsules where there was no cytokine induction, there was also central necrosis like that observed *in vivo*. Since cytokines were detected only in animals after transplantation of capsules and not in the medium capsules cultured *in vitro*, it is highly likely that the cytokines were produced by immune cells in peritoneum. However, the possibility that immune cells could induce the encapsulated cells to produce cytokines cannot be completely excluded. Nonetheless, immune reaction was not a likely cause of cell death and since cell death occurred mainly in the center of the cell cluster, we speculate that access to oxygen and nutrients is likely to be limiting to cells in the center of the cell cluster as the cluster grows, suggesting that chronic hypoxia and poor nutrition are the more likely major causes of cell death in microcapsules. Resolving this limiting access to oxygen and nutrients may help prolong the functional lifespan of our substituted β-cells *in vivo*.

The third scenario that could cause the relapse of hyperglycemia is related to the growth of transplanted cells in the capsule. We observed that MEPI-1 cell clusters within microcapsules continued to proliferate. During a 100-day

observation, the number of viable and functional MEPI-1 cells per transplanted or cultured capsule initially increased and then decreased to below the starting cell number as determined by DAPI- and BrdU-staining assays and their DNA and insulin contents. These parallel changes in the functional cell number for either transplanted or cultured capsules were further supported by histological examinations. Both H&E and DAPI staining showed increased cell mass of the clusters within the capsules initially after transplantation, but this was followed later by the development of a necrotic core in the cell clusters. We also observed that the cell number per capsule was directly related to blood insulin levels, OGTT responses, and glycemic control effectiveness. Thus, the sharp decline in cell number after about 3 months with a corresponding elevation in glycemic level strongly suggested that insufficient cells could be the major cause of the relapse to hyperglycemia.

Although this study provides proof that immunoisolation by encapsulation provided a viable strategy for the use of immune-incompatible stem cell–derived surrogate β-cells for diabetes therapy, at least one potentially deleterious challenge remains. As previously observed with the transplantation of these cells in immune-compromised mice (Li *et al.* 2009*a*,*b*), diabetic animals transplanted with these encapsulated cells also exhibited hypoglycemia and hyperinsulinemia. We have previously attributed these phenomena to the immature state of MEPI-1 cells and other ES cell–derived insulin-producing cells, which caused these cells to continue to proliferate and have a higher basal insulin release rate (Li *et al.* 2009*a*,*b*). Indeed, hyperinsulinemia and hypoglycemia were also seen in animals transplanted with neonatal islet cells (Aguayo-Mazzucato *et al.* 2006) and hES cell–derived endocrine cells (Kroon *et al.* 2008). Although the proliferation rate of MEPI-1 cells was slower after encapsulation compared to that of unencapsulated cells grown as monolayers, these cells unlike adult β-cells continue to proliferate to reach a mass where access to the supply of nutrients and oxygen by diffusion becomes limiting such that cells in the central core are deprived of nutrients and oxygen resulting in cell death. We further observed that the development of central necrosis did not inhibit the proliferation of surviving cells on the periphery of the cell clusters. Therefore, the apparent loss of cells in the transplanted capsules over time was due primarily to external environmental factors such as lack of oxygen and nutrient supply and not to intrinsic biological factors such as differentiation or de-differentiation. Moreover, the continued proliferation and maintenance of high basal insulin release resulting in hypoglycemia and hyperinsulinemia suggest that transplantation and encapsulation did not affect the developmental maturity of the cells. Therefore, the viability of using ES cell–derived surrogate β cells for diabetes therapy will further require the generation of more mature insulin-producing cells from ES cells with a slower proliferation rate and lower basal insulin secretion rate that together may delay the relapse of hyperglycemia and minimize hypoglycemia.

Here, we like to note that although the continued proliferation of insulin-producing cells contributed to the induction of hypoglycemia after transplantation, this proliferation rate was essential in producing the large number of cells required for transplantation. Thus, understanding how proliferation of these cells is regulated could potentially provide means to manipulate the proliferation rate to optimize cell production or function. The immaturity of our cells could be due to a failure to express or turn off the appropriate key regulators such as the failure to express MafA which is up–regulated in mature β cells (unpublished data; Kataoka *et al*. 2002). Thus, correction of these aberrant regulators prior to transplantation may help in the maturation of these cells and reduce proliferation and lower basal insulin secretion. Alternatively, development of new encapsulation technology that will release inhibitors of cell proliferation may also improve and extend the therapeutic effectiveness of these surrogate β-cells.

In conclusion, our present results suggest that transplantation of alginate microcapsules of expansible, ES/progenitor-derived surrogate β-cells could circumvent host immune response and provide a promising model of investigating the stem cell technology for the treatment of type 1 diabetes.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-10-0378.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S S designed and performed the experiments shown in Figs 1, 2, 3B and C, 4A, 5, 6 and 7 and in the Supplementary data, analyzed the data, and wrote the manuscript. G Y and X B and X F performed the experiments shown in Figs 3A and 4B and C, and analyzed the data. S K L gave advice, analyzed the data, and wrote the manuscript. G L initiated the study, designed the experiments, analyzed the data, and wrote the manuscript.

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