Diabetes Is Reversed in a Murine Model by Marginal Mass Syngeneic Islet Transplantation Using a Subcutaneous Cell Pouch Device

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Background. Islet transplantation is a successful β-cell replacement therapy for selected patients with type 1 diabetes mellitus. Although high rates of early insulin independence are achieved routinely, long-term function wanes over time. Intraportal transplantation is associated with procedural risks, requires multiple donors, and does not afford routine biopsy. Stem cell technologies may require potential for retrievability, and graft removal by hepatectomy is impractical. There is a clear clinical need for an alternative, optimized transplantation site. The subcutaneous space is a potential substitute, but transplantation of islets into this site has routinely failed to reverse diabetes. However, an implanted device, which becomes prevascularized before transplantation, may alter this equation. **Methods.** Syngeneic mouse islets were transplanted subcutaneously within Sernova Corp's Cell Pouch (CP). All recipients were preimplanted with CPs 4 weeks before diabetes induction and transplantation. After transplantation, recipients were monitored for glycemic control and glucose tolerance. **Results.** Mouse islets transplanted into the CP routinely restored glycemic control with modest delay and responded well to glucose challenge, comparable to renal subcapsular islet grafts, despite a marginal islet dose, and normoglycemia was maintained until graft explantation. In contrast, islets transplanted subcutaneously alone failed to engraft. Islets within CPs stained positively for insulin, glucagon, and microvessels. **Conclusions.** The CP is biocompatible, forms an environment suitable for islet engraftment, and offers a potential alternative to the intraportal site for islet and future stem cell therapies.

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Intrahepatic transplantation of isolated pancreatic islets of Langerhans is a successful therapy for selected patients with type 1 diabetes mellitus, complicated by glucose lability or frequent hypoglycemic events.[1] Despite initial high rates of insulin independence at 1 year after transplantation, long-term decline in graft function has been observed in 5 years after transplantation. More recently, 6 international centers have presented encouraging data indicating marked improvement in long-term durable function of at least 50% insulin independence at 5 years[2] providing renewed optimism for islet replacement strategies. The inherent procedural risks associated with intraportal islet infusion, hemorrhage and thrombosis, loss of graft function from immediate innate inflammatory processes, intrahepatic hypoxia, the requirement for life-long antirejection protocols and the future potential of stem cell technologies has stimulated interest in alternative transplant sites.[3-6]

Development of alternative, extrahepatic sites for islet implantation has been extensively examined preclinically and in

pilot clinical studies including the spleen,[7,8] renal subcapsular space,[9,10] muscle,[11,12] gastric submucosal space,[13] intestinal submucosal,[14,15] venous sac,[16] omentum,[17] bone marrow,[18] and peritoneum.[19] The subcutaneous site has been suggested as an attractive surrogate due to the ease of access, availability of suitable space for transplant, potential for biopsy, imaging, and retrievability.[20] However, transplantation into the unmodified subcutaneous site has routinely failed to reverse the diabetic state in animal models,[20,21] hypothesized in part to be a result of inadequate neovascularization.

A novel, scalable implantable macro-polymer device, the Cell Pouch (CP) was developed by Sernova Corp. (London, Ont, Canada), as a potential new method to induce formation of neovascularized tissue chambers for transplant of therapeutic cells within the subcutaneous space. This device is specifically designed to be nonimmunoisolating to promote vascularization and can allow for the addition of cellular immune protection within the device chambers. Herein, we assess the efficacy of the prevascularized CP for its potential to transform the subcutaneous site suitable for islet engraftment and long-term function in a well-established syngeneic, marginal, and full islet mass murine model.

MATERIALS AND METHODS

Subcutaneous Implantation of the CPs

Four to five weeks before islet transplant, to induce neovascularization, a single chamber mini-CP (Sernova Corp. London, ON) was implanted subcutaneously into the lower abdominal quadrant of approximately 25 g male BALB/c mice (Jackson Laboratories, Canada). This biocompatible device was contract manufactured (product and process development was conducted in accordance with the manufacturer's Quality System compliant to ISO 13485:2003, MDD 3.42.EEC, US FDA Quality System Regulations 21 CFR 820 and Canadian Medical Device Regulation and sterilized by ethylene oxide processing) from medical-grade materials approved for use in permanent implants. Briefly, to place the CP, a small transverse incision is made, allowing for a small pocket to be created inferior to the incision line. Once an adequate space is created, the CP is implanted into the space such that the opening is in the cranial position. The incision is closed with surgical staples (Autoclip; Becton Dickinson, Sparks, MD). The mini-CP is 7 mm wide and 15 mm long with macropores for vascularized tissue incorporation that after 4 to 5 weeks implantation provides a void space of approximately 75 μL for therapeutic cell transplant.

Mouse Pancreatectomy and Islet Isolation

Pancreatic islets were isolated from 8 to 12-week-old male BALB/c mice (Jackson Laboratories, Canada). Animals were housed under conventional conditions having access to food and water ad libitum. The care for the mice was in accordance with the guidelines approved by the Canadian Council on Animal Care. Before pancreatectomy, the common bile duct was cannulated with a 27-G needle, and the pancreas was distended with 0.125 mg/mL cold Liberase TL Research Grade enzyme (Roche Diagnostics, Laval, QC, CA) in Hank's Balanced Salt Solution (Sigma-Aldrich Canada Co., Oakville, ON, CA). Islets were isolated by digesting the pancreases in a 50-mL Falcon tube placed in a 37°C water bath for 14 minutes with light shaking. Subsequent to the

digestion phase, islets were purified from the pancreatic digests using histopaque-density gradient centrifugation (1.108, 1.083, and 1.069 g/mL; Sigma-Aldrich Canada Co., Oakville, ON, Canada).

Diabetes Induction and Syngeneic Mouse Islet Transplantation

Three to 5 days before transplantation, CP implanted and kidney capsule (KC) control mice were rendered diabetic by chemical induction with intraperitoneal streptozotocin (Sigma-Aldrich Canada Co., Oakville, ON, Canada), at 185 mg/kg in acetate phosphate buffer, pH 4.5. The animals were considered diabetic when their blood glucose levels exceeded 15 mmol/L for 2 consecutive daily readings.

Immediately after isolation, either a full islet mass (500 islets ± 10% per diabetic recipient mouse) or marginal mass (200 islets ± 10% per diabetic recipient) with purity of 90% ± 5% was aspirated into polyethylene-50 tubing using a microsyringe and centrifuged into a pellet suitable for transplantation.

A pilot experiment examined the efficacy of full islet dose (500 islets) to reverse diabetes when transplanted into the CP as compared to KC islet grafts. After this, a marginal islet mass study (200 islets) was conducted comparing efficacy of islets transplanted into CP with those transplanted into under the KC or subcutaneously alone (SC). The SC grafts served as a primary nonvascularized control to compare outcomes against CP implanted islets. Additionally, KC marginal islet mass grafts served as a secondary control, as described previously, as an alternative standard method for islet implantation in mice.[22]

To transplant islets into the CP, a small incision was made in the skin to gain access to the cranial portion of the device. Subsequently, the plug was removed revealing a vascularized tissue chamber into which the islet preparation was infused. The CP was closed by approximating the 2 layers of the cranial portions of the CP with 4-0 vicryl suture. The cutaneous incision was subsequently closed with a surgical staple (Autoclip; Becton Dickinson).

Evaluation of Islet Graft Function

Islet graft function was assessed twice weekly in recipients through nonfasting blood glucose measurements (mmol/L), with a portable glucometer (OneTouch Ultra 2; LifeScan, Canada) in all groups tested. Graft function and reversal of diabetes were defined as 2 consecutive readings less than 11.1 mmol/L and maintained until study completion. In addition, glucose tolerance tests were conducted on euglycemic mice 100 days after transplantation, to further assess metabolic capacity. Recipients were fasted overnight before receiving an intraperitoneal glucose bolus (3 g/kg). Blood glucose levels were monitored at baseline (time 0), 15, 30, 60, 90, and 120 minutes after injection, allowing for area under the curve (AUC-blood glucose) to be calculated and analyzed between transplant groups.

Graft Retrieval

To confirm graft-dependent euglycemia, animals with functional grafts had their islet transplants explanted by nephrectomy or CP removal. The KC transplant recipients were placed under anesthesia, and their graft-bearing kidney was exposed. A LT200 Ligaclip (Johnson & Johnson, Inc., Ville St-Laurent, QC, CA) was used to occlude the renal vessels and ureter. The left kidney was dissected from the animal.

The explanted graft was preserved in 10% buffered formalin for immunohistochemistry. Animals were monitored after nephrectomy for 5 days; a return to hyperglycemia confirmed graft function over naive pancreas β-cell regeneration.

The CP explants were carried out by a small skin incision. The ventral surface of the CP was dissected from the dermis while maintaining the integrity of the encompassing neo-vascularized tissue. The dorsal side of the islet-engrafted device was dissected to allow for its complete removal. Postexplantation CPs were placed in 10% buffered formalin for histological analysis, and animals were monitored for hyperglycemia.

Histological Assessment of CPs

Immunohistochemistry was used to identify overall structure detail using hematoxylin-eosin, endothelial cells for the assessment of vascularization using anti-von Willebrand Factor antibody, anti-insulin, and antiglucagon antibodies to identify the presence of islets within the CP. Immediately after explantation, CP tissues were fixed in 10% buffered formalin for 48 hours and then washed in phosphate-buffered saline, with a final wash in 70% ethanol. The tissue was dissected, embedded in paraffin, and sectioned at a thickness of 5 μm. Representative tissue sections were stained with hematoxylin-eosin. Additionally, tissue sections were stained using immunofluorescence. The sections were deparaffinized and treated with antigen heat retrieval (Target Retrieval Solution, Dako) followed by washing with tris-buffered saline (TBS) supplemented with tween-20 (TBS-T). Sections were blocked using 10% goat serum in TBS-T for 1 hour room temperature. Sections were treated with a primary antibody of either rabbit anti–von Willebrand factor (Millipore AB7356) diluted 1:100 (TBS with 1% goat serum), guinea pig anti-insulin (Dako A0564) diluted 1:1000 (TBS with 1% goat serum), or rabbit antiglucagon (AbD Serotec AHP534) diluted 1:100 (TBS with 1% goat serum) for 15 hours at 4°C. Sections were washed with TBS-T followed by secondary antibody treatment consisting of goat antirabbit (Molecular Probes A-11034; Alexa Fluor 488) diluted 1:1000 (TBS-T with 1% goat serum) or goat anti–guinea pig (Molecular Probes A-11075; Alexa Fluor 568) diluted 1:1000 (TBS-T with 1% goat serum) for 1 hour at room temperature. Samples were washed with TBS-T, counterstained with DAPI (1:1000). To reduce autofluorescence background, sections were treated with 0.3% sudan black (in 70% ethanol) for 2 minutes, washed with TBS and coverslipped with an antifade mounting medium. Microscopy and image analysis were accomplished using Aperio-Scan Scope Console for light microscopy and Zeiss Axio Imager Z1 for fluorescent microscopy.

Statistical Analysis

Nonfasting blood glucose data are represented as the mean ± SEM and blood glucose AUC analysis; for intraperitoneal glucose tolerance test (IPGTT), data were conducted through nonparametric analysis of variance and unpaired t test using GraphPad Prism (GraphPad Software, La Jolla, CA). Tukey post hoc tests were used after the analysis of variances. Kaplan-Meier survival function curves were compared using the log-rank (Mantel-Cox) statistical method. P less than 0.05 was considered significant.

RESULTS

Efficacy of Full Murine Islet Mass Transplanted Into CPs

In initial pilot studies, CP devices were implanted subcutaneously into the lower left abdominal quadrant of recipient mice (n = 3) 4 to 5 weeks before islet transplantation, after which full mass syngeneic murine islets were implanted (500 islets per CP). The device is designed with macropores to encourage entry of tissue and microvessels around plugs that once removed, form vascularized tissue-lined chambers for the transplantation of therapeutic cells (Figure 1).

The CPs were well tolerated by the recipient mice, without complications (Figure 1E). At the time of transplantation, a limited cutaneous incision allowed access to the upper portion of the CP, which was observed to be well vascularized. In parallel, similar 500 islets from the same isolation were transplanted under the left KC of diabetic mice.

In the initial pilot full mass study, after an initial delay in engraftment by 20 days after transplantation, CP-islet recipients demonstrated similar glycemic control to the KC-islet recipients (n = 4), which was maintained until graft explantation at 40 days after transplantation (Figure 2B).

Immunohistochemical and histopathological analysis for connective tissue, insulin and glucagon were performed on retrieved full mass islet grafts 40 days after transplantation. Islet grafts within the CP stained positive for insulin and glucagon, indicating the presence of insulin producing β-cells and α-cells, respectively. Furthermore, islets were well vascularized and surrounded by viable stroma (Figure 2B, C).

Efficacy of Marginal Islet Mass Transplanted Into CPs

To investigate efficiency of islet engraftment in the prevascularized subcutaneous site, CPs (n = 20 transplants) were placed 4 to 5 weeks ahead of marginal mass islet transplantation (200 islets per mouse). To compare engraftment efficacy, 2 additional groups of diabetic recipients were transplanted with 200 islets under the KC (n = 7) and 500 islets into the subcutaneous space without a device (SC n = 6). KC-islet recipients became euglycemic, 6 of 7 (87%), on average 23.0 ± 9.7 days postsyngeneic transplant. The CP-islet recipients became euglycemic, 19 of 20 (95%) by 40.5 ± 5.0 days after transplantation. By contrast, all SC-islet recipients failed to reverse diabetes (0 of 6, $P < 0.001$, log-rank, compared with CP) despite receiving a full islet dose (500 islets) (Figure 3A). Despite a modest delay in engraftment, the CP-islet recipient mice demonstrated similar nonfasting glycemic profiles to the KC-islet recipients ($P = 0.63$, log-rank), maintaining glucose homeostasis for the duration of the study (Figure 3B).

To further evaluate islet engraftment efficacy, a glucose tolerance test was performed on euglycemic recipients 100 days after transplantation. Islets transplanted either under the KC or within the CPs demonstrated a physiological response to an IPGTT, as observed by a rapid glucose clearance and return to normoglycemia after a glucose bolus (Figure 4A). Islets transplanted into either the KC or the CP engrafted with similar efficacy as observed through their parallel IPGTT blood glucose AUC (KC, 1773 ± 93.5 vs CP, 1953 ± 115.6 mmol/L/120 min, $P = 0.55$) (Figure 4B). As a means of metabolic comparison, a naive nondiabetic group of mice also underwent IPGTT. As anticipated with an extraportal site, there was a shorter delay in metabolic
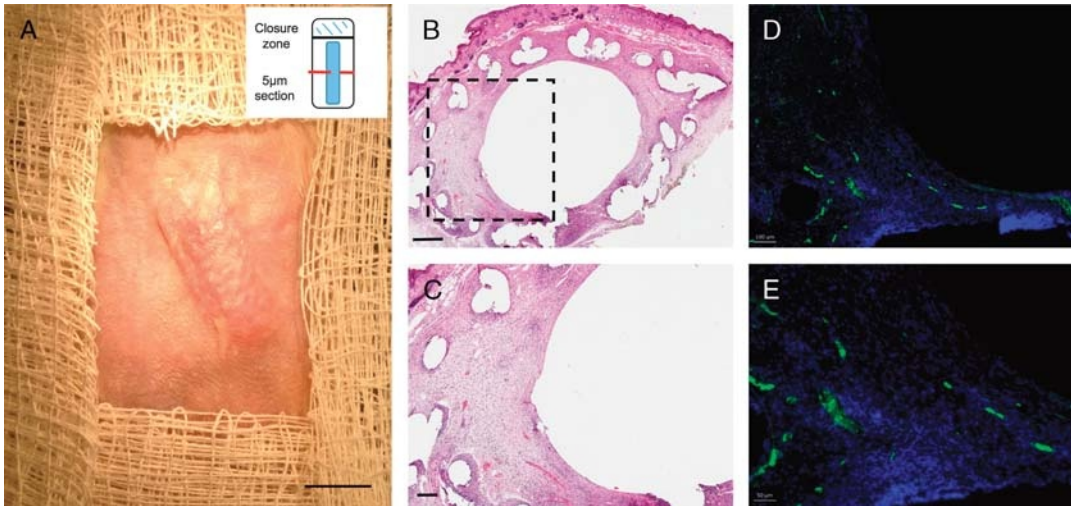
FIGURE 1. Representative sample of the tissue incorporated CP 4 to 5 weeks after implantation. A, Macroscopic overview of implanted CP into the left lower abdominal quadrant of recipient BALB/c mice (insert schematically depicts device cross-section for histological assessment). Minimal device profile is apparent 4 weeks after implantation, at the time of syngeneic mouse islet transplant. Scale bar represents 5 mm. B-C, Cross-section of CP demonstrating vascularized tissue ingrowth at the time of islet transplantation. Scale bar represents 500 and 200 μm, respectively. D-E, Positive fluorescent staining for blood vessels (green, vWF) within a cross-section of the CP at the time of transplantation. Scale bar represents 500 and 200 μm, respectively. vWF indicates von Willebrand factor.
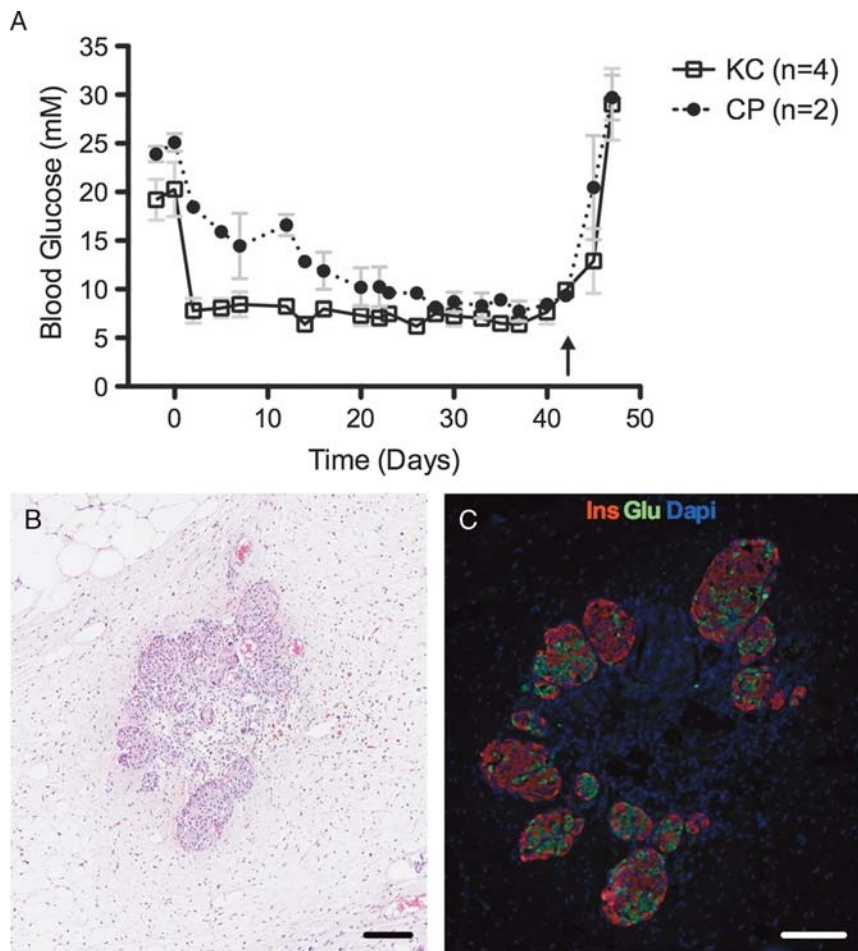
FIGURE 2. Restoration of glycemic control post-syngeneic mouse islet transplantation (full mass, 500 islets). A, Reversal of diabetes was maintained in all recipients in both the subcutaneously implanted mini CP (n = 2) and renal subcapsular (KC) (n = 4) transplant groups until graft bearing devices or kidneys were procured (arrow). On recovery device explantation or nephrectomy, all recipients rapidly reverted back to a pretransplant hyperglycemic state. B, Cross-section hematoxylin-eosin staining (5×) of an explanted CP approximately 40 days after transplantation, depicting the islet graft within the tissue chamber of the device supported by a vascularized tissue. C, Fluorescent staining (10×) of a serial section from the transplanted CP, staining positive for insulin (red), glucagon (green), and nuclei.[1] Scale bars represent 100 μm.
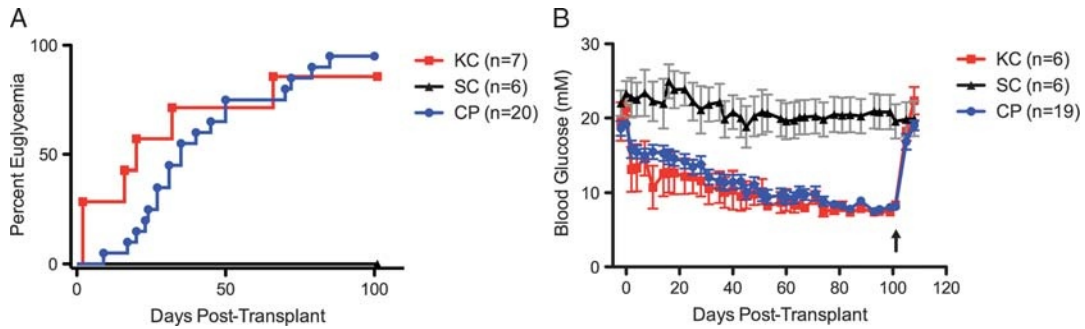
FIGURE 3. Long-term glycemic control post-syngeneic islet transplantation (marginal mass,– 200 islets). A, Reversal of diabetes rates, percent euglycemia, between islet-KC recipients (KC, n = 7) and mini-CP (blue, n = 20) recipients were comparable 100 days after transplantation. One recipient from each transplant group failed to achieve euglycemia after transplantation. None of the SC recipients (n = 6) became euglycemic after transplantation B, Nonfasting blood glucose measurements of euglycemic recipients after transplantation. Recipient of marginal islet-KC and CP transplants maintained robust glycemic control until the time of graft procurement (arrow), at which point all recipients reverted back to pre-transplant glycemic values, indicating graft dependent euglycemia, whereas all SC recipients remained hyperglycemic after transplantation.

response compared to CP-islet recipients (AUC naive, 1367 ± 63.8 vs CP, 1953 ± 115.6 mmol/L per 120 min, $P < 0.01$) (Figure 4B).

Long-term graft dependent euglycemia was confirmed through islet-graft bearing procurement by either nephrectomy or CP explantation, 100 days after transplantation. All islet recipients returned to pretransplant hyperglycemia within 1 week of graft retrieval (Figure 3B).

Histological examination of procured islet grafts within CPs revealed viable intact islets contained within a vascularized stroma (Figure 4C). The islets within the CP stained positively for insulin, in addition to endothelial cells of newly developed microvessels associated with the islets (Figure 4D). These data demonstrate that the CP provides a subcutaneous microenvironment that is suitable for long-term functional islet engraftment.
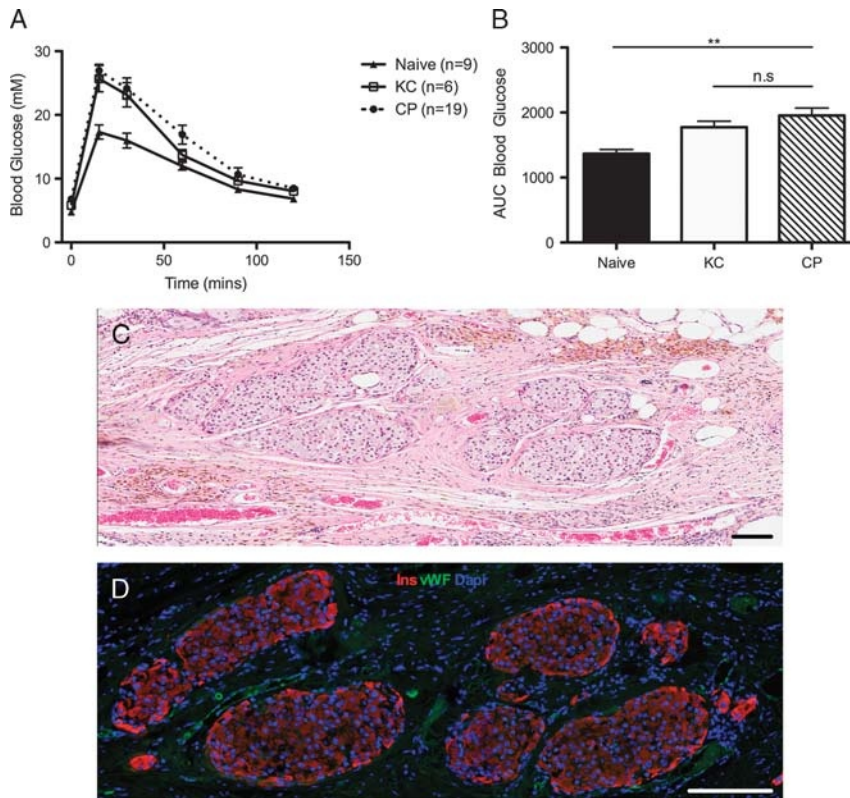
FIGURE 4. Intraperitoneal glucose tolerance test of syngeneic mouse islets transplanted under the kidney capsule or into the mini-CP, 100 days after transplantation. A, Blood glucose prolife post-dextrose bolus of naive (n = 9), islet-KC recipients (n = 6) and islet-CP recipients (n = 19). B, Blood glucose area under the curve[1] analysis did not differ between the kidney capsule (KC, n = 16) and islet-CP (blue, n = 19) recipients. Naive represents nondiabetic, nontransplant BALB/c mice (n = 9), which were more tolerant to the glucose tolerance test than the islet-CP recipients as expected (**$P < 0.01$, ANOVA). Mice were administered 3 mg/kg 50% dextrose intraperitoneal. Blood glucose measurements were monitored at t = 0, 15, 30, 60, 90, and 120 minutes. Histological analysis of cross-sections from long-term syngeneic marginal islet mass grafts. C, Hematoxylin-eosin staining of an islet graft within the CP recipient, 100 days after transplantation (8×). D, Fluorescent staining of a serial section depicting an islet graft within the CP staining positive for insulin (red), blood vessels (green) and nuclei[1] (20×). Scale bar represents 100 μm. ANOVA indicates analysis of variance.

DISCUSSION

Aside from the instant blood-mediated inflammatory response, infusion of islets into the liver is associated with potential limitations, including risk of hemorrhage, thrombosis, limited transplant capacity, and the inability to effectively track, image and biopsy the cellular graft. In addition, the liver is currently regarded as an unsuitable site for nonestablished cellular products, including insulin-producing stem cells or xenogeneic cells because the graft cannot be removed without recourse to hepatectomy.

These concerns have stimulated research efforts directed toward alternative transplant sites. Although multiple tissue sites have been examined, the subcutaneous space has potential beneficial attributes as an alternative site due to its relatively transplantable large surface area, its accessibility, potential for biopsy, imaging and graft retrieval without compromise to collateral organs. Previous small animal studies in which islets were placed directly into the subcutaneous space have universally failed to provide sufficient graft function,[20,23,24] an observation confirmed within the present study. This may in part be related to the location within the subcutaneous space where the islets have been placed. Although the subcutaneous space contains areas with relatively low vascularity, associated with reduced tissue oxygen tension, other more richly vascularized areas are better suited for transplant.[20] In the subcutaneous site, modified by the use of polymer encapsulated islets,[24,25] growth factors,[26] matrices,[27,28] and macroimmunoisolating devices,[29-32] improvements in islet graft efficacy have been noted elucidating some of the features likely required to achieve durable insulin independence within this tissue.

Subcutaneous devices, involving immune isolation are typically comprised of semipermeable membranes, impermeable to immune system cells, yet permeable to low molecular weight molecules. Although the concept purports to solve the issue of administering lifelong immunosuppression, several issues may limit the clinical utility of this approach. The reliance on the device surface area for sufficient passive diffusion of metabolic products and nutrients[33] can limit the number of cells that can be sustained within the device. In addition, the lack of porosity minimizes the potential for development of vascularized tissue resulting in potential hypoxia, graft nonfunction and a persistent foreign body reaction at the device tissue interface further hindering oxygen diffusion and glucose sensing.[33,34] To circumvent this phenomenon, several groups have implemented a means to immunoisolate islets within a macrodevice tethered to an external oxygen supply[26,31]; however, durable insulin independence has yet to be demonstrated, and it does not eliminate the issues with externally placed devices, such as ongoing potential for infection.

As an alternative approach, the CP evaluated in this study is designed with large pores to enable host peridevice vascularized to form tissue chambers around a scaffold into which therapeutic cells are transplanted. The concept of this macrodevice is to harness the foreign body response to induce development of neovascularized tissue chambers for transplantation of therapeutic cells including islet. The device is macroporous and was specifically designed not to immunoisolate therapeutic cells. Hence, we did not explore allogeneic islet transplantation in these experiments. However, the CP can accommodate microimmunoprotection through colocalized transplanted immunomodulatory cells, encapsulated cellular products or stem cell–derived cells for local immune protection, while being able to be safely explanted if required. After transplantation, the cells become housed in an environment enabling the development of intraislet capillary networks. The nonmetallic properties of this device allow for imaging, such as magnetic resonance to track vessel ingrowth before transplantation as well as engraftment.

This study demonstrated that syngeneic islets at a marginal islet dose transplanted into prevascularized CPs within the subcutaneous space, function similar to islets transplanted under the KC, whereas islets transplanted into SC space without the CP universally fail to reverse diabetes. As expected, both CP and KC islet transplant recipients were more glucose intolerant compared to naive (nondiabetic, nontransplanted) mice, indicating that islet grafts consist of a beta cell mass that is less than that contained within the endogenous normal pancreas. Notwithstanding, islets transplanted within the CP are sufficiently potent to fully reverse diabetes long term (>100 days), indicating the development of a durable, vascularized islet suitable environment.

Posttransplant insulin independence was delayed somewhat in the recipients of the islet-CP transplant in comparison to recipients of islets transplanted under the KC possibly due to the establishment of new islet microvessels. A similar delay has been observed in both experimental and clinical islet transplantation in the nondevice, intramuscular site.[20,35] With isolation from the pancreas, islets are rendered avascular, removed from endogenous extracellular matrix and cellular contacts and denervated.[36] These and other factors, such as endotoxin contamination,[36] instant blood-mediated inflammatory response,[37] recruited autoimmunity and alloimmunity,[38] have been suggested as factors in early graft loss and delayed insulin independence independent of the site of transplantation; however, islets within the CP would be protected from the blood-mediated inflammatory reactions occurring after intraportal islet delivery.

In the immediate posttransplant period, even when islets are deposited into the intraorgan vasculature, at least 2 weeks are required before graft revascularization.[39] During the transient period of hypoxia, insulin metabolism, processing, and release in conjunction with glucose sensing may be reduced or even halted in the heterotopic microenvironment. These factors, in addition to the reestablishment of the intraislet vascular environment may in part, be related to the delay in insulin independence when islets were transplanted into the CP.

The slight delay to insulin independence may result in improved islet engraftment and sustained long-term outcome at a reduced islet dose. This observed delay should not be a concern from a clinical perspective because in subjects with longstanding diabetes, it may take 1 to 2 months for insulin independence to be achieved even with the conventional portal vein transplant technique.

The emerging field of cellular transplantation involving human-derived engineered stem cells is providing potential therapeutic treatment options to benefit far more patients than donor cells can provide, especially for diseases, such as type 1 diabetes. The future of these therapies, aside from manufacturing aspects to improve safety, depends on a suitable environment for the cells and cellular engraftment.

The current study demonstrates that the CP placed in the subcutaneous space provides a suitable environment for

therapeutic islets as effective as the renal subcapsular transplantation, at least in the murine model. Furthermore, in subsequent assessment, it has been shown that the CP scaled for human use can provide glucose control in large animal transplantation models of diabetes (Sernova unpubl. results).

Thus, the CP system placed subcutaneously also meets the requirements for scalable human islet transplantation. Further studies, which include evaluation in the clinic are now required to demonstrate that the CP can indeed serve as a potential alternative to clinical intraportal islet transplantation, and provide a vehicle for future placement of alternate cellular therapies in replacement and regenerative medicine. Indeed, the data presented herein in addition to Sernova's large animal data (unpublished) formed the experimental basis of a first-in-human trial using identical CP technology currently underway in patients at the University of Alberta.

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