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Islet grafting and imaging in a bioengineered intramuscular space[†]

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Abstract

Background—Since the hepatic portal system may not be the optimal site for islet transplantation, several extrahepatic sites have been studied. Here we examine an intramuscular transplantation site, bioengineered to better support islet neovascularization, engraftment, and survival, and demonstrate that at this novel site, grafted beta cell mass may be quantitated in a real time non-invasive manner by PET imaging.

Methods—Streptozotocin induced rats were pretreated intramuscularly with a biocompatible angiogenic scaffold received syngeneic islet transplants 2 weeks later. The recipients were monitored serially by blood glucose and glucose tolerance measurements and by PET imaging of the transplant site with [11C] dihydrotetrabenazine. Parallel histopathologic evaluation of the grafts was done using insulin staining and evaluation of microvasculature.

Results—Reversal of hyperglycemia by islet transplantation was most successful in recipients pretreated with bioscaffolds containing angiogenic factors as compared to those who received no bioscaffolds or bioscaffolds not treated with angiogenic factors. PET imaging with [11C] dihydrotetrabenazine, insulin staining and microvascular density patterns were consistent with islet survival, increased levels of angiogenesis, and with reversal of hyperglycemia.

Conclusions—Induction of increased neovascularization at an intramuscular site significantly improves islet transplant engraftment and survival compared to controls. The use of a non hepatic transplant site may avoid intrahepatic complications and permit the use of PET imaging to

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measure and follow transplanted beta-cell mass in real time. These findings have important implications for effective islet implantation outside of the liver, and offer promising possibilities for improving islet survival, monitoring, and even prevention of islet loss.

Keywords

Islet transplantation; Imaging; Bioengineering

Introduction

Although the application of steroid-free immunosuppression protocols and improvements in islet isolation and dosing, developed by the Edmonton group resulted in a great resurgence of optimism for clinical islet transplantation(1), the long term clinical outcomes have been disappointing since insulin independence at one year of 100% fell to less than 10% at five years(2).

The current standard for clinical islet transplant is intraportal infusion of purified islets from one to three donor pancreata(3). Multiple sites for transplantation have been explored, including the kidney capsule, splenic capsule, omentum, testes, and peritoneal cavity, but the intraportal site remains most popular as it presents the least invasive alternative and produces normoglycemia with the fewest number of transplanted islets (4,5). Problems with the intraportal site, however, indicate that it is far from optimal. Intraportal islets are subject to substantially higher concentrations of toxic immunosuppressants (6,7), activation of complement and coagulation cascades - the so-called instant blood-mediated inflammatory reaction (IBMIR)(8), and exposure to inflammatory mediators released during activation of alloimmune rejection pathways(9). These problems complicate the already inefficient islet engraftment process (10) and suggest that new more efficient sites and methods for islet allografting are needed.

In this report we tested intramuscular islet implantation using a highly purified biocompatible alginate scaffold to form a microenvironment conducive to islet survival. Besides offering malleability and freedom from many of the conditions that imperil intraportal islets, the intramuscular site offers other advantages. First, the intraportal embolization procedure itself carries significant risk of perihepatic hematoma, portal branch thrombosis and hemorrhage (2,11). Comparatively, an intramuscular implantation would be less invasive. Second, once infused, intraportal islets are accessible only by systemic therapy. In contrast, local interventions could potentially be made in the clinic at an intramuscular site.

Finally, monitoring islet grafts after infusion remains a problem. Metabolic tests only detect graft dysfunction when substantial islet mass has already been lost, after which graft-saving intervention is no longer possible. Attempts to develop other markers for early detection of rejection have been unsuccessful (12). Intramuscular implantation offers the opportunity to biopsy allografts or, alternatively, allow use of a beta cell imaging technique which relies on PET detection of a [^{11}C] dihydrotetrabenazine (DTBZ) radioligand bound to vesicular monoamine transporter type 2 (VMAT2), a biomarker of beta cell mass (BCM) (13,14). This technique shows promise for in situ islet imaging in man (15) but has never been tested in an extrapancreatic site. Therefore we tested the feasibility of this approach in our model of intramuscular islet transplantation.

Materials and Methods

Animals and Study Design

All animal studies were reviewed and approved by the Columbia University Institutional Animal Care and Use Committee. Male Lewis rats were obtained from Harlan Sprague Dawley, Inc (Indianapolis, Indiana) weighing between 200 and 250g and served as islet donors and transplant recipients in this study. Briefly, rats were divided into 5 groups of 12 rats each depending on the treatment they would receive (Table 1). Two weeks prior to scheduled transplantation (transplant day -14), groups one and two underwent surgery for implantation of scaffolds and group three underwent a sham operation. Four days prior to transplantation (day -4), all animals were rendered chemically diabetic with streptozotocin (STZ, Sigma Aldrich, St. Louis, Missouri) at a dose of 50mg/kg via the penile vein under isoflurane anesthesia. Animals were considered diabetic if blood glucose values were greater than 300 mg/dL for three consecutive days. Finally, on day 0, animals were transplanted with syngeneic islets unto the submuscular scaffold.

The principle outcome measure used in these studies was the four-hour fasting blood glucose levels. These measurements were performed throughout the post transplant period day +5 to day +60. Islet implantation success was defined as reversal of diabetes with fasting glucose < 110mg/dL on day +5 through +60. Islet implantation success rate was defined as the percentage of animals in each group who could maintain a fasting blood glucose <110mg/dL throughout the post transplantation period. Statistical significance of the differences in blood glucose levels among the different treatment groups was determined by a repeated measure ANOVA with Tukey's HSD post-hoc testing using the blood glucose measurements obtained during the two month post transplant window.

Scaffold Preparation and Implantation

Three-dimensional scaffolds were prepared from purified low molecular weight alginate (Sigma, 0682, St. Louis, Mo). High M Alginate was purified using a modified protocol based on charcoal extraction, serial filtration, dialysis and alcohol precipitation (16). Subsequently, alginate was modified with 2 mg cyclic RGDfK peptide per gram alginate using sulfo-NHS and EDC cross-linker according to a modified protocol(17). Cyclic RGDfK (f = D-phenylalanine) is a synthetic, protease resistant, alpha(V)beta (3)- and alpha(5)beta(1) integrin selective peptide(18). Resulting cRGDfK-alginate was re-dissolved at 2% in sterile distilled water. To generate scaffolds, 400 µl cRGDfK-alginate was loaded in a 4.2 cm² cell culture insert (0.4 µm pores, pore density of 1.6×10⁶/cm, polyethylene terephthalate membrane) (BD number 353090, BD Falcon, BD Biosciences, New Jersey, USA). Loaded inserts were placed in 6-well tissue culture plates and 3 ml of 4.3% calcium gluconate was added to each bottom well. Plates were placed at 4° Celsius for 24 hours to solidify cRGDfK-alginate by diffusion of calcium ions through the insert membrane. Following solidification, scaffolds were washed 3 times in 3 ml sterile distilled water to remove excess calcium gluconate and kept moist in sterile distilled water until implantation.

Growth factor enriched scaffolds were prepared following the same protocol, with the addition of 100 ng/ml recombinant human VEGF₁₆₅ (PHG0145, Invitrogen, Carlsbad, USA) and 100 ng/ml recombinant human PDGFbb (P3201, Sigma Aldrich, St Louis, USA) to cRGDfK alginate, calcium gluconate and sterile distilled water solutions.

On day -14, animals in groups 1, 2, and 3 were given isoflurane gas anesthesia and after opening the skin the muscle was dissected to the peritoneum. A newly created pocket accommodated a 10mm scaffold. Scaffold was then implanted in animals in groups one and two before closing the pocket and skin in separate layers. Animals in group three (sham) were closed without scaffold implantation.

Islet Isolation

On transplant day 0, fresh islets were isolated from Lewis donor rats using collagenase digestion and Ficoll separation, as previously described (19).

Islet yield and viability

Islet yield was quantified by hand counting of a dithizone-stained islet isolate (19). Islet viability was assessed with double staining with SYTO 13/Ethidium bromide (EB) as previously described (20).

Insulin Stimulation Index

Retrospectively, islet quality was confirmed with insulin stimulation index as previously described by Eizirik et al (21). Briefly, 200 isolated, hand-picked islets were washed twice with low-glucose (1.7mM) media (no glucose added RPMI 1640 w/o phenol red, supplemented with 0.1% BSA and glucose). From those, 5 groups of 20 islets measuring 100 to 150 μ m in diameter were placed in separate 6ml polypropylene tubes. Next, islets were sequentially pre-incubated with low-glucose media (1.67 mM), incubated with low-glucose media, and incubated with high-glucose media (16.7mM). After each incubation (1hr), the media was removed from the islets and frozen for ELISA analysis.

Islet Implantation

After isolation, approximately 2400 islets were resuspended from the final centrifugation pellets into volumes of approximately 0.4-0.5mL of HBSS. High islet quality was confirmed before each injection by measurement of viability (>90%) and retrospectively by examination of insulin stimulation indices (all greater than 4). After anesthesia with isoflurane, the midline skin of study animals was dissected away from the abdominal musculature and in animals in groups 1 and 2, scaffolds were identified by sight and palpation. Islets with approximate purity of 90% were injected onto scaffolds in an intramuscular wheal via an 18-gauge needle. Animals from groups 3 and 4, which had not been pre-implanted with scaffolds, had islets injected intramuscularly in a similar fashion. Group 5, as a control, received no islets.

Graft Monitoring: Metabolic Function and Histologic Examination

Transplanted animals were weighed biweekly and were monitored with daily blood glucose measurements (four-hour fasting) over the first two weeks after transplantation followed by bi-weekly measurements thereafter. Six out of twelve animals from each treatment group were sacrificed on transplant day 0 for histopathologic examinations of the scaffolds and neovascularization. Sections were obtained through the scaffold for animals in groups 1 and 2 and through muscle for animals in groups 3, 4 and 5. Paraffin tissue sections were stained with Gill's hematoxylin/eosin (H&E) and factor VIII related antigen (A0082, Dako, USA) 1 in 200 using the Vectastain ABC kit (Vector Laboratories, USA) according to the manufacturer's instructions in order to examine vascularity of the tissue. Vessels within five separate high-power fields at 400 $\times$ magnification were counted.

Insulin staining—From the remaining six animals in each group, tissue samples were obtained in a similar fashion at two months after transplantation. Those samples were additionally stained for insulin (I2018, Dako, USA) 1 in 1000 using the ABC kit to demonstrate the presence of islets.

IPGTT—Intraperitoneal glucose tolerance testing (IPGTT) was conducted on study rats two months after transplantation and prior to removal of the scaffolds after which fasting blood glucose levels were repeated. Glucose boluses of 1g glucose/kg body weight were

administered to unanesthetized animals, and blood glucose was measured at 0, 30, 60, 90, and 120 minutes after injection. Area under the curve (AUC) for glucose excursion was calculated by the trapezoidal rule for comparisons of the extent of diabetes.

Beta Cell Imaging

For BCM imaging of islet transplants, stereochemically resolved (+)-9-O-desmethyl- α -dihydrotetrabenazine precursor of [11C]DTBZ was obtained from MonomerChem Laboratories (RTP, NC). (+)- α -[11C] DTBZ was synthesized by [11C] methylation of the appropriate precursor and the product purified by HPLC (22). The purity of [11C]-DTBZ preparations varied from 98.5 to 99.9 % of the desired (+) - product. Specific activities of carbon-11 labeled radiotracers were >2000 mCi/ μ mol at time of injection.

Prior to gel implantation and STZ treatment, two control rodents were imaged using a Concorde microPET-R4 (Siemens - CTI Molecular Imaging, Knoxville, TN, USA). Four additional rodents were imaged 2-4 weeks after STZ treatment followed with islets transplant, two of them pretreated with VEGF/PDGFa scaffold implantation and remaining two pretreated with a sham operation. PET scanning and image reconstruction was performed as previously described (23). Region of interest analysis was performed with PMOD software (Zurich, Switzerland). Regions of interest were placed using coronal, transverse, and sagittal reconstructions. Reconstructed PET images were used to identify and measure the time course of radioligand activity within each region of interest.

The concentration of VMAT2, as biomarker of BCM, in the region of interest was estimated by calculation of a distribution volume ratio (DVR) using the Logan reference region method (24) and PMOD software. In previous studies we identified the kidney cortex as an appropriate VMAT2 free reference regions to which ligand uptake in the pancreas could be compared (14,23,25). For islet transplants to the intramuscular space, we found that the kidney cortex overestimated non specific binding in the muscle and substituted a ROI contralateral to the islet transplant within the abdominal muscle wall.

Results

Restoration of Normoglycemia and Islet Implantation Success

The goal of islet transplantation is not only the survival of islets in a new environment but also resumption of function and restoration of normoglycemia in otherwise hyperglycemic and diabetic animals. Measurements of fasting blood glucose levels were performed throughout the post transplant period and are presented in Figure 1A. The islet implantation success was defined as fasting glucose <110mg/dL on day +5 through +60 post implantation. Islet implantation success rate was defined as the percentage of animals in each group with fasting blood glucose <110mg/dL (N=6) (Figure 1B). That correction of hyperglycemia was associated with islet transplantation was confirmed by the observation that when the scaffolds were removed on day 60, all animals reverted to hyperglycemia. As all recipients received syngeneic grafts, not allografts, there was some improvement in hyperglycemia in animals that received the syngeneic grafts without a scaffold or a non-modulated scaffold, but not in sham operated or untransplanted controls. Successful reversal was achieved in all animals (6/6) transplanted with islets into the fully enriched scaffold (group 1: gel + VEGF/PDGF) but in partial reversal in only 50% (3/6) of animals with the same scaffold but without VEGF or PDGF (Gel alone, group 2); and partial reversal in only 33% (2/6) of animals that received syngeneic islet grafts without surgical pretreatment (group 4). All control animals (sham operated – group 3; or not transplanted – group 5) remained fully hyperglycemic ($p < 0.05$) as expected.

As a more sensitive metric of restoration of islet mass and function, we measured the glucose excursions following glucose challenge by IPGTT (Figure 2A). Calculation of area under the curve (AUC) of blood glucose excursion showed that the AUC was significantly lower for the gel+VEGF/PDGF group than for the other groups (2-4 fold) (Figure 2B). Animals in the Gel + VEGF/PDGF group also gained weight significantly better than animals in the other groups. This confirms that islet function and overall anabolic control were better in Group 1 animals ($p<0.05$) (Figure 3C).

Development of fibrovascular tissue and robust vasculature at the implantation “bed”

We hypothesized that success of islet engraftment and their functional capacity depends on proper neovascularization at the time of implantation and afterwards. Therefore, we evaluated the development of the vascular bed in the transplant site just before islet implantation. Scaffolds were surgically removed and evaluated for vessel development 2 weeks after their implantation, just before islet transplantation would have occurred. Figure 3, Top and Middle rows, shows hemotoxylin and eosin stained tissue containing fibrovascular tissue penetrating scaffolds placed between muscular layers. Preserved tissue was then stained for factor VIII related antigen, which is specific for endothelial cells and identifies blood vessels (Figure 4A). The number of capillaries stained with factor VIII related antigen per high power field was significantly higher in the gel + VEGF/PDGF group compared to the other groups by 2-5 fold, $p<0.05$ (Figure 4B). These histopathologic results, along with functional success, confirm the significance of neovascularization within the implantation “bed.”

Confirmation of the presence and function of islets 2 months after implantation

To confirm the role of the grafted islets in glucose control, on day 60 after transplantation, all tissue and scaffolds were removed and the normoglycemic animals promptly became hyperglycemic and diabetic. When the tissue was stained for insulin (Figure 3, bottom row) cells staining positively for insulin are seen within the scaffold, especially in proximity to vessels at the scaffold-muscle interface which suggests preferential islet engraftment in proximity to the blood vessels. We did not evaluate the direct neovascularization of the beta cells in this study.

Visualization of the implanted islets by PET

Since vesicular monoamine transporter type 2 (VMAT2) has been proposed as a biomarker for beta cells as it is highly expressed relative to other cells in the pancreas (13) we used PET imaging to quantitate VMAT2 at islet transplant site. This method, effective for imaging rodent beta cells in situ (14), cannot be applied to islets infused into the liver because liver and bile are routes of DTBZ excretion and have a high background signal (Figure 5).

Following intramuscular islet transplantation, PET scans with [11C] DTBZ demonstrated that the transplanted and viable beta cell mass can be visualized and quantified. Successful transplantation, defined by restoration of normoglycemia, was visualized in rodents in the gel+VEGF/PDGF group (Figure 5- A2, B2, C2). As a measure of VMAT2 density in tissue and by inference, beta cell mass, we calculated a distribution volume ratio (DVR), which is a linear function of VMAT2 target availability. The DVR presented in **Panel D**, was higher for the rodent from gel+VEGF/PDGF group (**D2**) in the islet transplant site in comparison to the control shown in **D1**. In contrast, transplanted islets in the animals in the sham group, which remained hyperglycemic due to unsuccessful transplant, were not visible on any intramuscular plane (Figure 5- A3) while the DVR (**D3**) in the region of interest, was much lower than for viable islets (**D2**), and as low as the corresponding region in controls without islets (**D1**).

Imaging results were correlated to the islets' ability to maintain normoglycemia (Figure 5 A2 vs A3). The native pancreas in animals treated with STZ had lower tracer binding, as revealed by the time -activity curves and calculation of the distribution volume ratios relative to the control animal prior to treatment with STZ (Figure 5- C2 and C3 versus control C1 and D2 and D3 versus control D1). These measurements additionally suggest that reversal of hyperglycemia was not due to residual beta cell mass in the native pancreas of the rodent with islet transplanted into the scaffold and confirm STZ induced impairment of native pancreas in animals with a functional islet transplant which is critical in maintenance of normoglycemia.

Discussion

Our use of synthetic scaffolds and an intramuscular islet transplantation site is not without precedent. Weber et al (26) and Axen et al (27) validated the potential use of the intramuscular site over twenty-five years ago. Subsequent studies, however, showed poor function attributable to inadequate vascularization as well as dispersal of the intramuscular grafts (28,29). To address these problems, we impregnated scaffolds with growth and anti anoikosis factors. Specifically, we used vascular endothelial-derived growth factor (VEGF) and platelet-derived growth factor (PDGF) in combination to promote ingrowth of functionally sound vessels (30). In addition to these, we covalently modified alginate with cyclic extracellular matrix signaling molecule modeled after the repeated arginine-glycine-aspartic acid peptide motif (RGD) recognized in fibronectin molecules (cyclic-RGD). This peptide has been shown to enhance cellular adhesion and prevent transmembrane apoptotic signaling via integrins (31-33).

Synthetic scaffolds have been successfully used as transplanted-cell carriers that maintain cell viability and function (34). When scaffolds are prepared from highly purified polysaccharides like alginate, they are endotoxin, pathogen and mitogen free allowing for long-term cell survival in the absence of acute or chronic foreign body reactions (FBR). Alginate capsules have been widely used as inert material for encapsulated islet protection from inflammatory and immunological response of the body after implantation (35,36).

The alginate used for the preparation of three dimensional scaffolds in the current study consists of cRGDfK-modified poly-mannuronic acid chains cross-linked with Ca^{2+} for solidification. Poly-mannuronic acid was used due to its favorable physical properties such as low viscosity and high biocompatibility after sufficient purification (35). Physical stability of cross-linked alginate, such as that used in capsules or scaffolds, depends on many different factors such as mannuronic acid and glucuronic ratio, cation used for cross-linking (i.e. Ca^{2+} , Ba^{2+}), local pH, presence of a protective poly-L-lysine layer and local concentration of cation chelators (37). Thus, alginate capsules, stabilized with poly-L-lysine, can be stable *in vivo* for periods longer than 1 year after intraperitoneal implantation (38).

In contrast, we observed degradation of the scaffold over the follow-up period. We believe that Ca^{2+} leeches out of the scaffold or is actively absorbed by invading vasculature, leading to changes in three dimensional structures. Stability of cross-linked alginate can be altered by modifying aforementioned factors. In the current system, degradability is a desired property that promotes further in growth of blood vessels creating the fibrovascular tissue "bed" for transplanted islets which permits improved supply of oxygen and nutrients compared to unmodified muscle. Scaffold degradation could also be due to the use of cRGDfK-modified alginate in combination with vascular growth factors. After implantation of the modified scaffold, fibrovascular tissue is stimulated by locally released vascular growth factors which penetrate the space within the scaffold leading to neovascularization. Although we did not measure the persistence of the slowly released angiogenic factors in

this study, it is probable that their persistence is not necessary, once they trigger the use of local host growth factors in the area. This process is additionally enhanced by the presence of the cRGDfK peptide supporting adherence and migration of endothelial cells. Increase in neovascularization may additionally enhance Ca^{2+} resorption and scaffold disintegration which permits further penetration by new endothelial cells (Fig. 3). Islets infused into such milieu may be offered more optimal conditions for engraftment. Although we did not identify directly neovascularization of the transplanted islets, after 2 months we found islets lodged in proximity of the vessels and predominantly at the border of the scaffold, surrounding muscular tissue where the concentration of the vessels was the highest.

The selection of anti anoikis peptides and growth factors in our scaffolds was based on a group of findings from the cell therapy and transplantation literature. Growth factors such as basic fibroblast growth factor (bFGF), VEGF and platelet derived growth factor (PDGF), when gradually released from synthetic scaffolds, synergistically enhance neovascularization of the transplant site thereby improving engraftment of the cells(39-42). This approach has been effective in vivo for transplants of hepatocytes, myocytes, and stem cells within subcutaneous and intramuscular sites (42-44).

Other groups have successfully utilized similar approaches in induction of angiogenesis (29,35). Inoue et al (45,46) and Weir et al (47) used bFGF and VEGF respectively to improve neovascularization for use with devices. In our system, VEGF₁₆₅ and PDGFbb were integrated with cRGDfK-modified alginate via electrostatic interaction during the solidification phase. In our experiments we used the initial concentration of vascular growth factors (both 100 ng/ml) based on physiologically active concentrations (1-10 ng/ml) and the gradual release gradient. These growth factors not only stimulate development of new vessels but also augment the islets' own vasculature(35,42).

It has been shown *in vitro* that extracellular signaling with arginine-glycine-aspartic acid (RGD) peptides efficiently prevents apoptosis of islets after isolation from the pancreas (48). Scaffolds synthesized with RGD peptide also provide extracellular signaling necessary for proper angiogenesis(39). Linear GRGDSP peptides are less stable and prone to proteolytic degradation. Therefore, we used a protease resistant cyclic RGDfK peptide in all scaffolds to enhance islet survival.

Since in our study we used an intramuscular space which is already abundant in a rich blood supply, the addition of the slowly released factors synergistically potentiated neovascularization. Histologic examination at the time of transplantation showed a 6-fold increase in vascularity of the tissue surrounding VEGF/PDGF-enriched scaffolds as compared to controls. Islets transplanted onto such neovascularized scaffolds were most effective in converting the animals to normoglycemia.

At the same time as we tested the feasibility of bioengineering an extrahepatic site for islet transplantation, we also evaluated if this approach allowed application of a non invasive beta cell imaging method which we developed for evaluation of islets in-situ (14,15,23,25). Our results suggest that, while there is a need for further optimization of the "bioengineering" phases of the protocol, the use of an extrahepatic site has the potential to overcome many of the shortcomings of intrahepatic islet transplantation. In current study we purposely used a relatively high number of islets to reverse hyperglycemia because we wished to also test the beta cell imaging protocol. The parameters which need to be optimized in the future will include the concentration of vascular growth factors, size and density of the scaffold, timing of the islet injection after scaffold implantation, and determination of the lowest number of islets needed to restore normoglycemia as compared to that needed for the intraportal route.

The selection of an intramuscular space and use of a bioscaffold for islet implantation may have additional advantages. Future clinical applications of this approach might include monitoring of the transplanted BCM in real time using PET imaging with quantitation of VMAT2, which may permit modulation of immunosuppression and early diagnosis of allograft rejection. Allogeneic islet transplantation, at least in the near term, will continue to be hampered by the shortage of donor tissue and allograft rejection. It has been demonstrated that adult beta cells have some self renewal potential(49). Scaffolds such as those employed in our study might be useful depots for cocktails of beta cell specific growth factors. Lastly, these scaffolds seem to be ideal locations for delivery of targeted immune intervention, such as co-stimulatory blockade or antisense RNA. Here only a small region will be affected and might preempt the need for traditional high-dose systemic immunosuppression.

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Abbreviations used

BCM	beta cell mass
DVR	Distribution Volume Ratio
Dithizone	Diphenylthiocarbazone
DTBZ	dihydropyridine
EB	ethidium bromide
EDC	diethylcarbamate
ELISA	enzyme linked immunoassay
H&E	Gill's hematoxylin/eosin
HBSS	Hanks balanced salt solution
IBMIR	instant blood-mediated inflammatory reaction
IPGTT	intraperitoneal glucose tolerance testing
PBS	phosphate buffered salt solution

PDGF	platelet-derived growth factor
PET	positron emission tomography
RGD	repeated arginine-glycine-aspartic acid peptide motif
STZ	streptozotocin
sulfo-NHS	sulfo N hydroxysuccinamide
VEGF	vascular endothelial-derived growth factor
VMAT2	vesicular monoamine transporter type 2

Figure 1. Blood Glucose Levels in Transplanted Rodents

(A) Figure presents mean fasting serum glucose level in animals within the 60 days follow-up. Mean fasting glucose level oscillates below 110mg/dL for animals from group 1 (gel + VEGF/PDGF), whereas in other groups it was statistically higher, ANOVA, $p < 0.05$. As an additional control, removal of the scaffolds in normoglycemic animals from the gel + VEGF/PDGF group on day +60 led to prompt return of diabetes and hyperglycemia. Error bars represent S.E.M. (B) Islet grafting success rate. Number of animals out of six total achieving euglycemia following syngeneic islet transplantation is shown on the Y axis. Euglycemia was defined as four-hour fasting glucose < 110 mg/dL on post transplant days +5 through +60. The statistical significance of the differences in grafting success rate among the different treatment groups is shown and was determined by ANOVA using the blood glucose measurements obtained in the 55 day post transplant window.

Figure 2. Restoration of Glucose metabolism following islet transplantation; IPGTT testing and weight measurements

Panels A and B, Animals with islets transplanted into scaffolds containing vascular growth factors (Gel+ VEGF/PDGF) had a smaller glucose excursion in GIPTT than animals in other four groups. Area under the curve was 2-4 times smaller then in other groups (ANOVA, $p < 0.05$). The areas under the curve were determined using the trapezoid rule. Panel C, Body weight after Islet Tx. Rodents in the Gel+VEGF/PDGF group gained significantly more weight ($p < 0.01$) than other groups as determined by repeated measure ANOVA and Tukey's post-hoc analysis.

Figure 3. Histology of the tissue containing the scaffold on day 0, just before islet transplant
Top and middle row. The development of the vascular bed in the transplant site was evaluated microscopically just before islet implantation in our experimental animals. Scaffolds were surgically removed and tissues removed for preparation of paraffin sections. Hemotoxylin and eosin stained tissue containing fibrovascular tissue penetrating scaffolds placed between muscular layers (Figures labelled Gel and Gel+VEGF/PDGF) is shown with the grey arrows. Normal muscular tissue anatomy was seen in Figures labeled “No surgery” and “Sham” slides. Magnification 400×. **Bottom row.** Sections of the rectus muscle with the scaffold containing RGD peptide, VEGF and PDGF 60 days after islet transplantation was examined by H&E and insulin staining. Islets staining positive for insulin (shown by black arrows) were found within the scaffolds containing VEGF and PDGF in animals which reverted from hyperglycemia after islet transplantation providing another proof for successful islets implantation and function. The majority of the islet were found in the proximity of the border between muscle and the VEGF/PDGF, the same location were rich vascularization was found (Fig 4)

Figure 4. Evaluation of the vascularization within the tissue based on histology and staining for factor VIII related antigen

Panel A. Tissue containing scaffold was surgically removed 2 weeks after the implantation just before islet injection. In order to identify endothelium in blood vessels histological were stained for factor VIII related antigen. **Panel B.** The average number of capillaries stained positively was quantified per high power field (magnification 400 $\times$) and was significantly (2-5 fold) higher in the gel+VEGF/PDGF group compared to the other groups as determined by a repeated measure ANOVA and Tukey's post-hoc analysis ($p < 0.01$). The error bars represent the standard error of the mean.

Figure 5. Imaging of native pancreas and islet transplants with [11C] DTBZ

Rodents were imaged prior to or following islet transplantation to the intramuscular space. Dynamic PET data obtained from each scan was reconstructed and representative abdominal coronal plane images from transplanted animals are shown. Display ranges of images are not equivalent.

A Panels are abdominal images from:

- (A1)- control rodent from group 5, prior to STZ treatment, no islet tx;
- (A2)- rodent from group 1 STZ pretreated, with implanted scaffold containing VEGF and PDGFa, and islet isograft;
- (A3)- rodent from sham group (3) treated with STZ and an islet isograft. A plane of liver, an organ of [11C] DTBZ catabolism, is shown with the letter “L” on A1, and the islet tissue isograft, distributed around the gel insert is visualized by PET scans with [11C] DTBZ and marked by a white arrow labeled with the letters “iTx” on A2.

B Panels are more dorsal abdominal coronal planes showing the native pancreas, indicated by a white arrow or the letter “P” (B1), from the same series of rodents shown in A Panels:

- (B1) animal from Group 5, prior to STZ treatment;
- (B2) animal from Gel+ VEGF/PDGF group (Group 1);
- (B3) rodent after islet transplant from sham group (Group 3)

C Panels show the time activity curves of [11C] DTBZ uptake in Liver, Kidney (the native pancreas reference region), native pancreas, islet transplant (iTx) and muscle wall and ipsilateral (to pancreas) muscle wall (iTx reference regions) from the same series of animal that in Panel A and B: (C1)- group 5, (C2)- group 1, (C3)- group 3 animals.

D Panel shows quantitation of VMAT2 density in the islet tissue transplanted to intramuscular space of the abdominal wall (iT_x) as well as that measured in the native pancreas for the rodents imaged in Panels A, B, and C. Measurements are expressed as distribution volume ratios (DVR). Blood glucose concentrations on the day of imaging are shown in the last column.

Table 1
Experimental grouping as per pretransplantation procedures

Group	Group	Day -14	Day -4	Day 0
1	Gel ^I + VEGF/ PDGF	implantation of bioscaffold containing RGD peptide, VEGF and PDGF	STZ	Islet Tx
2	Gel	bioscaffold containing RGD peptide, no VEGF, no PDGF	STZ	Islet Tx
3	Sham	intramuscular space opened and closed surgically without any scaffold implantation	STZ	Islet Tx
4	No surgery	no pretreatment surgery was performed	STZ	Islet Tx
5	No islets	no pretreatment surgery was performed and no islets were transplanted	STZ	No Islet tx

^I Gel - alginate scaffold with cyclic arginine-glycine-aspartic acid (RGD) peptide; Tx - transplant; STZ - streptozotocin.