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An elastin-based vasculogenic scaffold promotes marginal islet mass engraftment and function at an extrahepatic site

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Abstract

In islet transplantation, one of the major obstacles to optimal engraftment is the loss of islet natural vascularization and islet-specific extracellular matrix (ECM) during the islet isolation process. Thus, transplanted islets must re-establish nutritional and physical support through formation of new blood vessels and new ECM. To promote this critical process, we developed an elastin-based vasculogenic and ECM-promoting scaffold engineered for extrahepatic islet transplantation. The scaffold by design consisted of type I collagen (Coll) blended with 20wt% of elastin (E) shown to promote angiogenesis as well as *de novo* ECM deposition. The resulting “CollE” scaffolds had interconnected pores with a size distribution tailored to accommodate seeding of islets as well as growth of new blood vessels. *In vitro*, CollE scaffolds enabled prolonged culture of murine islets for up to one week while preserving their integrity, viability and function. *In vivo*, after only four weeks post-transplant of a marginal islet mass, CollE scaffolds demonstrated enhanced vascularization of the transplanted islets in the epididymal fat pad and promoted a prompt reversal of hyperglycemia in previously diabetic recipients. This outcome was comparable to that of kidney capsular (KC) islet transplantation, and superior to that of islets transplanted on the control collagen-only scaffolds (Coll). Crucial genes associated with angiogenesis (VEGFA, PDGFB, FGF1, and COL3A1) as well as *de novo* islet-specific matrix deposition (COL6A1, COL4A1, LAMA2 and FN1) were all significantly upregulated in islets on CollE scaffolds in comparison to

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those on Coll scaffolds. Finally, CollE scaffolds were also able to support human islet culture *in vitro*. In conclusion, CollE scaffolds have the potential to improve the clinical outcome of marginal islet transplantation at extrahepatic sites by promoting angiogenesis and islet-specific ECM deposition.

Graphical Abstract

Keywords

angiogenesis; scaffold; islets; transplantation; diabetes

1. Introduction

Intrahepatic islet transplantation is a promising therapy for insulin-dependent diabetic patients [1]. However, data suggests that the liver is not an optimal site for islet transplantation due in part to the instant blood-mediated inflammatory response (IBMIR) associated with this implantation site, and that insulin independence after intrahepatic islet transplantation is not durable [2]. In fact, at this site, significant loss of islet mass immediately following their intrahepatic implantation often leads to inadequate short-term graft function and compromised long-term survival. These issues have limited a widespread application of this therapy [3, 4]. Furthermore, regardless of the implantation site, the process of islet isolation per se severs the islet cells from their natural vasculature and their specialized surrounding extracellular matrix (ECM) [5]. Thus, once transplanted, islets must re-establish nutritional and physical support through the formation of new blood vessels and *de novo* matrix deposition [6].

Various extrahepatic transplant sites have been explored to avoid the IBMIR associated with intrahepatic islet implantation. These alternative sites include subcutaneous spaces [7–9], and laparoscopically accessible intraperitoneal locations such as the small bowel mesentery (SBM) [10, 11], the omentum [12, 13], and the epididymal fat pad (EFP) [14, 15]. Although the accessibility of these extrahepatic sites is appealing, these sites also have their own limitations, including inadequate vascular supply and sometimes rigorous inflammatory responses, which in turn may influence islet engraftment, survival, and function [6]. Synthetic or natural polymer-based scaffolds have been shown to be effective in providing temporary support to islet engraftment at extrahepatic transplantation sites [16]. The highly porous structure of such scaffolds facilitates cell infiltration, nutrient exchange, as well as vascularization [17–19]. In particular, the ability to further bioengineer such scaffolds offers the intriguing possibility of physically defining a biocompatible and tunable extrahepatic site for islet transplantation, as well as providing a versatile platform for future incorporation of islet-promoting interventions [16, 20, 21].

The peri-islet basement membrane associated with adult human islets consists of collagens (collagen I, collagen III, collagen IV, and VI), laminin and fibronectin [18]. Thus, an ideal scaffold would either mimic the main biochemical cues of the peri-islet basement membrane or promote *de novo* islet-specific matrix deposition after transplantation to ensure islet engraftment and function. For this purpose, both synthetic and natural polymer-based scaffolds have been experimented [18, 22].

Among synthetic polymer-based scaffolds for islet transplantation, poly(lactic-co-glycolide) (PLG) scaffolds have been extensively tested in extrahepatic islet transplantation. It is recognized, however, that they have several limitations, mainly due to the lack of bioactive cues and therefore the need for engineering specific ECM proteins and/or growth factors to ensure cell adhesion, survival and neovascularization in these scaffolds [5, 23]. In addition, during PLG degradation, acidic byproducts are produced, causing lowering of ambient pH and substantial pro-inflammatory responses *in vivo* [24]. Furthermore, the hydrophobic nature of PLG also interferes with cell infiltration, limiting post-transplantation vascular regeneration [25]. In an attempt to obviate these obstacles, PLG scaffolds for islet transplantation have often been engineered with pro-angiogenic factors such as PDGF-BB [14], VEGF [26] and FGF-2 [27, 28], with varying degree of success.

Among natural scaffolds for islet transplantation, collagen-based scaffolds have demonstrated to be extremely valuable, largely due to their unique biocompatibility and bioactivity [29]. Collagen scaffolds have been reported to be immunomodulatory [30, 31], particularly by engaging alternatively activated macrophages [32]. In addition, it has been reported that collagen scaffolds are able to support extrahepatic islet engraftment, while reducing the number of islets necessary to revert diabetes [33, 34], probably due to the ability of the interconnected porosity supporting angiogenesis [35, 36] needed for islet engraftment and overall graft function.

Recently, in murine models other than islet transplantation, collagen-based scaffolds functionalized with soluble elastin have been shown to significantly enhance neovascularization without the need for any heterologous growth factors or angiogenic molecules [37–39]. Collagen and elastin are the most abundant components of the ECM of almost every tissue in the body, which provides essential cues for attachment, migration and organization of cells of the vasculature [37]. Thus, in this study, we fabricated and tested a biomimetic scaffold consisting of type I collagen and elastin in its ability to facilitate extrahepatic islet transplantation and diabetes reversal by a marginal islet mass by promoting neovascularization of the islet grafts.

2. Materials and methods

2.1. COLLE scaffold fabrication

1 g of bovine type I collagen (Nitta Casings Inc.) was dissolved in acetate buffer (pH 3.5) to reach a concentration of 10 g/L. The collagen fibers were assembled through a pH-driven process with titrating in a sodium hydroxide solution (0.1 M) to a pH of 5.5. The collagen was washed three times with de-ionized (DI) water. Soluble bovine elastin (Sigma Aldrich) was added to the collagen slurry (20 wt%) and homogenized to ensure homogeneous

blending of collagen with the elastin. The resulting collagen slurry was cross-linked with the bis-epoxide crosslinker 1,4-butanediol diglycidyl ether (BDDGE) (Sigma-Aldrich). The slurry was embedded in a BDDGE aqueous solution (2.5 mM) for 48 h, at a BDDGE/collagen ratio of 1 wt%. The cross-linked collagen was washed 3 times with DI water. The resulting collagen/elastin slurry was casted in a cylindrical mold (5 mm in diameter) to fabricate 0.5 × 0.3 cm cylindrical ColIE scaffolds. The same procedure was used to fabricate plain collagen scaffolds without elastin (Coll) as controls. The slurries were casted through a multistep freeze-drying method. Briefly, the slurry was placed on a cooling metal shelf at −80°C for 1h for small pore (20–50 μm) formation, and then at −30°C for 1h for large pore (200–400 μm) formation. Finally, the scaffolds were lyophilized under a chamber pressure of 20 mTorr.

2.2. ColIE scaffold characterization

The overall morphology and structure of ColIE scaffolds were evaluated through a Nikon AZ100 Multizoom microscope system (1X magnification), while the details of the pores were imaged through a Nikon A1R confocal microscope exploiting the autofluorescence of type I collagen at 358 nm. The size of the pores and pore size distribution was assessed by an automated measuring tool within the NIS Elements software (Nikon). The overall porosity of the scaffolds was calculated by using an ethanol infiltration method as described [40]. Values are expressed as means ± standard deviation (n = 5). The swelling properties (PBS uptake) of the scaffolds were determined as previously described [40]. Briefly, lyophilized scaffolds were weighted before (dry) and after soaking in PBS at 37 °C for various periods of time. The uptake ratio was defined as % of swelling. Values were expressed as means ± standard deviation (n = 5).

2.3. Islet Isolation and scaffold seeding for *in vitro* studies

All murine studies were approved by the Northwestern University Animal Care and Use Committees. Male C57BL/6 mice (Jackson Laboratories, Bar Harbor ME) 8–12 weeks old were used as islet donors. Islet isolation was performed as previously described [41]. Briefly, donor mice were anesthetized with an intraperitoneal injection of 50 mg/kg Ketamine (Henry Schein)/Xylazine (Anased, Lloyd Labs). After a midline abdominal incision, the common bile duct was cannulated and injected with a cold solution of collagenase (type XI; Sigma Chemical Company, St Louis, MO) in Hank's balanced salt solution. The pancreas was dissected, removed, and digested at 37°C for 15 min. After filtration through a mesh screen, the filtrate was applied to a discontinuous Biocoll separating gradient (Cedarlane). Isolated islets were hand-counted and 70 islets were seeded on each scaffold using a fine glass pipette under a surgical microscope. Scaffolds were examined after seeding to ensure a seeding efficiency of >95%. Scaffolds were then incubated at 37°C in 5% CO₂ and 95% air for 20 min, before adding serum-free RPMI 1640, supplemented with 1% L-Glutamine and 1% Penicillin/Streptomycin, (Gibco), and used for transplantation.

2.4. *In vitro* viability and function of murine pancreatic islets on Coll and ColIE scaffolds

AlamarBlue™ assay was performed over 7 days of islet cultures according to manufacturer's protocol (Invitrogen) to assess the combined effects of proliferation and metabolism on total cellular respiration. LIVE/DEAD® Cell Viability assay was also

performed over 7 days of islet cultures according to manufacturer's protocol (Invitrogen) to further assess the viability of islets. The glucose-stimulated insulin secretion was determined by ELISA, following a modified protocol of the NIH-approved human islet insulin secretion [42]. Briefly, free islets (2D control), or islets seeded on Coll and ColLE scaffolds were first equilibrated in a 2.8 mM glucose solution in Krebs buffer for 1h at 37°C. After equilibration, the samples were first incubated for 1h in a 2.8 mM glucose solution, followed by a 1h incubation in a 28 mM glucose solution. The supernatants after both incubations were recovered, and the amount of insulin present in the supernatants was quantified by ELISA (ThermoFisher). The stimulation index was calculated as the ratio of insulin produced during the high glucose incubation over insulin produced during the low glucose incubation.

2.5. Induction of diabetes

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) between 8 and 12 weeks of age were used as both islet donors and transplant recipients. Four days before islet transplantation, recipient mice were injected intraperitoneally with 220 mg/kg of streptozotocin (Sigma, St. Louis, MO) to chemically induce irreversible diabetes (29). Nonfasting blood glucose levels were measured in whole blood samples obtained from the tail of the animals using a One Touch Basic glucose monitor (Lifescan, Milpitas, CA). Mice were used in these studies only if they had blood glucose measurements greater than 300 mg/dL on two consecutive days before transplantation. The blood glucose levels of donor mice were also checked before islet isolation to verify that they were metabolically normal.

2.6. Islet transplantation

After islet isolation from donor mice, 70 islets were seeded on scaffolds (as described in paragraph 2.3). A midline incision was made, and the EFP identified and spread on the abdominal surface. Islet-seeded scaffolds were wrapped in the EFP which was then returned to the abdominal cavity. The wound was closed in two layers. Mice were given analgesics pre- and post-operatively as needed and monitored for signs of infection or distress. Mice were allowed free access to food and water postoperatively and their blood glucose level was monitored throughout the duration of the study.

2.7. Assessment of islet graft function

After transplantation, nonfasting blood glucose and weight measurements were taken between 12:00 and 17:00. Grafts were considered to be functional if glucose levels were maintained at less than 200 mg/dL. Intraperitoneal glucose tolerance tests (IPGTTs) were performed at 12 weeks after transplantation to assess graft's ability to respond to glucose challenges. After an 8 h fast, 2 g/kg of 50% dextrose (Abbott Labs, North Chicago, IL) was injected intraperitoneally. Blood glucose levels were measured at baseline (before injection), 15, 30, 60, and 120 min after glucose injection. The day after IPTGTT test, grafts were removed and the blood glucose levels were monitored for an additional 72 h to ensure return of hyperglycemia, at which point mice were euthanized.

2.8. Histology

Following euthanasia, grafts were harvested and immersed in 10% formalin buffer for 48 h and then embedded in paraffin according to established protocols (n = 3) [8]. 4µm sections were deparaffinized and stained using a kit for Masson's trichrome staining (Abcam; ab150686) according to manufacturer's protocol. Stained slices were mounted with Cytoseal XYL mounting medium (Thermo Scientific) and imaged with an ECLIPSE Ci-E histological microscope (Nikon). Images were processed and analyzed using the NIS Elements software (Nikon).

2.9. Immunofluorescence

After obtaining 4µm sections, epitopes were unmasked through heat-induced epitope retrieval standard protocols (Abcam). Slices were labelled with the following primary antibodies: (i) anti-insulin (guinea pig polyclonal, ab7842) (ii) anti-glucagon (rabbit polyclonal, ab133195) and anti-CD31 (mouse monoclonal, ab24590), and subsequently stained with specific secondary antibodies (goat anti-guinea pig IgG H&L, Alexa Fluor® 555, ab150186; donkey anti-rabbit IgG H&L, Alexa Fluor® 647, ab150075; goat anti-mouse IgG H&L, Alexa Fluor® 488, ab150113). Slides were imaged by a Nikon A1R confocal microscope, and images were analyzed using the NIS Elements software (Nikon).

2.10. Quantitative PCR

The grafts were collected at 4 and 12 weeks post transplantation. The RNA was isolated from the specimens with the RNeasy Plus Micro Kit (Qiagen) according to the manufacturer's protocol. RNA was quantified using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). The cDNA was synthesized from 500 ng total RNA, using the iScript retrotranscription kit (Bio-Rad Laboratories). Transcribed products were analyzed using Taqman fast advanced master mix (Thermo-Fisher Scientific) for the following mouse genes: VEGFA (Mm00437306_m1), PDGFB (Mm00440677_m1), FGF1 (Mm00438906_m1), COL3A1 (Mm00802300_m1), COL6A1 (Mm00487160_m1), COL4A1 (Mm01210125_m1), LAMA2 (Mm00550083_m1), and FN1 (Mm01256744_m1). For each gene, the probe recommended by the manufacturer was used. The reactions were carried out in a StepOne Plus real-time PCR system (Applied Biosystems). Results were first normalized to expression of the housekeeping gene GAPDH (Mm99999915_g1), and then expressed as "relative fold expression" in comparison to the expression level of the same gene from the control kidney subcapsular islet graft.

2.11. Human islets isolation and 3D culture on Coll and ColIE scaffolds

Human islets were obtained from Northwestern University Human Islet Transplant Program (IRB exemption: STU00207825). The islets were obtained through an optimized procedure previously described [42]. A deceased donor pancreas was processed under cGMP conditions using enzymatic digestion with SERVA Collagenase NB GMP Grade and Neutral protease NB GMP Grade (Nordmark, Germany). Islets were purified by density-gradient separation with Cobe 2991 cell processor. Subsequently, isolated islet were kept in culture for 7 days in 95% air, and 5% CO₂ at 25°C. Islets were of high purity (> 95%) and viability (> 97% based on FDA/PI staining). Islet equivalents (IEQs) were determined by

multiplication of islet numbers with the correlating volume correction factors, with 1 IEQ = one islet of a diameter of 150 μm [43]. 500 IEQ were seeded on each scaffold for testing. Islets were labeled with DRAQ5 according to manufacturer's protocol (Thermo ScientificTM), and imaged through fluorescence stereomicroscopy and confocal laser microscopy. Images were analyzed using the NIS Elements software (Nikon). Cell viability was evaluated by AlamarBlueTM assay, and insulin production was measured following the same protocol as described in Section 2.4.

2.12. Statistical analysis

All values were reported as the mean $\pm$ SD. Differences between two experimental groups were compared using an unpaired Student t test. In experiments with multiple groups, differences between two groups within the experiment were compared using a one-way ANOVA multiple comparison test. A value of $p < 0.05$ was considered statistically significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results

3.1. Physical characterization of CollE scaffolds

The overall morphology of CollE scaffolds, in comparison to control Coll scaffolds, was characterized by fluorescence stereomicroscopy. The scaffolds appeared highly porous (Fig. 1A and 1B). The detailed architecture and pore structure were evaluated by confocal laser microscopy, which revealed an anisotropic interconnected porous structure, with an average pore size of $121 \pm 88 \mu\text{m}$ and $92 \pm 64 \mu\text{m}$ for Coll and CollE scaffolds, respectively, with no statistically significant difference. The pore size distribution was found to be broad, with a minimum pore size of 20.4 μm for Coll scaffolds and 15.5 μm for CollE scaffolds; and a maximum pore size of 461.7 μm for Coll scaffolds and 390.6 μm for CollE scaffolds (Fig. 1C). The porosity of the scaffolds was calculated to be 80% (± 2.0) for Coll scaffolds and 79% (± 0.6) CollE scaffolds (Fig. 1D), again with no significant difference between the two types of scaffolds. The scaffolds displayed a swelling of up to 100% in slightly over 1 hr, and up to 200% over 6 hrs, also with no significant difference between the two types of scaffolds (Fig. 1E).

3.2. *In vitro* characterization of CollE scaffolds with murine pancreatic islets

Coll and CollE scaffolds were compared *in vitro* to assess seeding efficiency, and their ability to support islet cell viability and function. An identical number of murine islets (70 islets/scaffold) was seeded on day 0 the Coll or Coll E scaffolds. As shown in Fig. 2A, functionalization of the collagen scaffolds with elastin (CollE) resulted in a slightly better islet retention at day 1 compared to Coll scaffolds (Fig. 2B, * $p < 0.05$), although islet cells appeared homogeneously distributed on both scaffolds (Fig. 2A, day 1). At day 7, islets appeared to maintain their globular shape on both scaffolds (Fig. 2A, inset). However, again, CollE scaffolds seemed to retain islets more efficiently than Coll scaffolds, where the overall area fraction of islet cells was significantly higher in CollE scaffolds compared to Coll scaffolds (Fig. 2B, **** $p < 0.0001$).

The viability of murine islets after 7 days of culture was assessed by LIVE/DEAD assay (Fig. 3A). Here, we compared islets in 2D control culture and islets cultured on Coll or ColIE scaffolds. Imaging by confocal laser microscopy revealed that after 7 days of culture, large islets in 2D culture contained a higher number of dead cells (in red), whereas dead cells were distinctly rare in Coll and ColIE scaffolds. The viability at 7 days was calculated as percentage of live cells (green signal) over total signal (green and red), and was found to be 50% (± 18), 94% (± 6) and 98% (± 1) for 2D, Coll and ColIE scaffold islets, respectively (Fig. 3B). Islet viability was also assessed by the more quantitative Alamar Blue test (Fig. 3C), which revealed a significant difference between the viability of islets cultured on ColIE scaffolds vs. those cultured in 2D (** $p < 0.01$). In contrast, there was overall no statistically significant difference between the viability of islets cultured on Coll scaffolds vs. that of islets cultured in 2D ($p = \text{ns}$). Lastly, islet function was assessed by quantification of insulin production in response to low (2.8 mM) and high (28 mM) glucose concentrations on day 7 of culture. As shown in Fig. 3D, insulin production by islets cultured on ColIE scaffolds was significantly higher than that by islets cultured in 2D or on Coll scaffolds, both at low and high glucose concentrations (**** $p < 0.0001$). The stimulation index was also significantly higher by islets on ColIE scaffolds than islets from the other two groups (* $p < 0.05$) (Fig. 3E).

3.3. *In vivo* characterization of ColIE scaffolds by marginal islet transplantation in an extrahepatic transplantation site

ColIE scaffolds were next tested *in vivo* in a syngeneic extrahepatic murine islet transplantation model. A marginal mass of 70 murine islets was seeded on each ColIE scaffold followed by transplantation in the epididymal fat pad of diabetic recipient mice. An equal number of islets was seeded on each Coll scaffold and similarly transplanted for comparison. A kidney capsular (KC) transplant with the same marginal islet mass was used as the gold standard.

During the first two weeks post-transplantation, a significant difference was observed between the blood glucose levels in recipients transplanted with islets seeded on ColIE scaffolds vs. those seeded on Coll scaffolds (Fig. 4A, **** $p < 0.0001$). Specifically, islets seeded on ColIE scaffolds were able to revert recipient hyperglycemia with a kinetics comparable to that by KC islet transplantation (Fig. 4A). In contrast, blood glucose levels of recipients transplanted with islets seeded on Coll scaffolds remained above 300 throughout this period (Fig. 4A), although they eventually normalized over one month after transplantation (Fig. 4B). Consistent with this, at 3 month post-transplantation, mice from all groups responded undistinguishably to glucose challenges (Fig. 4C).

At 4 weeks and 12 weeks post-transplantation, islet grafts were retrieved, and insulin and glucagon positive cells were readily identified by immunofluorescence (Fig. 4D). Both glucagon and insulin staining appeared qualitatively stronger at 4 weeks post-transplantation in islets on ColIE scaffolds than on Coll scaffolds (Fig. 4D). Gross evaluation of the grafts upon retrieval revealed significantly more prominent vascularization on ColIE scaffolds compared with Coll scaffolds, both at 4 weeks and 12 weeks post-transplantation (Fig. 5, gross images, left panels). Histological evaluation by Masson's trichrome staining revealed the presence of an abundance of new blood vessels within the ColIE scaffolds (indicated by

white arrowheads) as early as 4 weeks post-transplantation, both in proximity to the islets at the edge (indicated with a *) and within the core of the CollE scaffolds, but only sparsely in Coll scaffolds (Fig. 5, top panels). At 12 weeks post-transplantation, blood vessels were more readily discernable in both Coll and CollE scaffolds, although they still appeared to be more abundant in the CollE scaffolds (Fig. 5, lower panels). The presence of fully formed blood vessels was further confirmed by the presence of red blood cells within their lumens (Supplementary figure S1–2).

The presence of blood vessels within the islet grafts was further assessed by immunofluorescent staining for CD31, a marker for vascular endothelial cells. The overall percentage (area fraction) of CD31 signal was significantly higher in CollE scaffolds than in Coll scaffolds at both 4 weeks ($p<0.0001$) and 12 weeks ($p<0.0001$) post-transplantation (Fig. 6A, 6B). Positive control for CD31 staining was provided by staining of a vascular network present in the epididymal fat pad (Supplementary figure S3).

Finally, the expression of genes associated with angiogenesis and *de novo* matrix deposition were evaluated in Coll and CollE scaffolds at both 4 and 12 weeks post-transplantation. Genes associated with angiogenesis (VEGFA, PDGFB, FGF1, and COL3A1) were found to express at significantly higher levels in CollE scaffolds than in Coll scaffolds at 4 weeks post-transplantation, and the differences were further accentuated at 12 weeks post-transplantation (Fig. 7A). We also measured expressions of genes associated with *de novo* islet-specific matrix deposition in islets on Coll or CollE scaffolds in comparison to levels present in normal murine pancreas. As expected, normal murine pancreatic tissue expressed high levels of such islet-specific matrix deposition genes, including COL6A1, COL4A1 and LAMA2 (Fig. 7B). At 4 weeks post-transplantation, expressions of COL6A1, COL4A1 and LAMA2 was higher in CollE scaffolds than in Coll scaffolds, although both were still significantly lower than those present in normal pancreas tissue. Furthermore, FN1 was significantly upregulated in CollE scaffolds compared to either Coll scaffolds or normal pancreas tissue (Fig. 7B), indicating early cellularization and remodeling in CollE scaffolds. Interestingly, at 12 weeks post-transplantation, expressions of COL6A1, COL4A1, LAMA2 and FN1 in CollE scaffolds dramatically increased, and were now comparable to those present in normal pancreas tissue and significantly higher than those detected in Coll scaffolds (Fig. 7B).

3.4. *In vitro* characterization of CollE scaffolds with human pancreatic islets

Finally, CollE scaffolds were evaluated for seeding human islets, in comparison to human islets seeded on Coll scaffolds or in 2D cultures. 500 IEQ of human islets were seeded on both Coll and CollE scaffolds and cultured *in vitro* for 7 days. CollE scaffolds preserved the morphology of human islets over the 7 days in culture (Fig. 8A), and the islets were retained with minimal loss as measured by area fractions on day 1 (~35%) and day 7 (~30%) (Fig. 8B). On the contrary, islets on Coll scaffolds were retained much less efficiently (day 1: ~15% and day 7: ~6%, **** $p<0.0001$). Viability of the cultured human islets was quantified by the Alamar Blue assay. Human islets cultured on CollE scaffolds showed a minimal decline of cell viability over the 7 days of culture (Fig. 8C). A faster decline of cell viability was observed of human islets cultured on Coll scaffolds, although the difference did not

reach statistical significance (Fig. 8C). Finally, islet function at day 0, 1, 3, and 7 of culture was assessed by insulin production in response to low (2.8 mM) and high (28 mM) glucose concentrations. Interestingly, human islets cultured on ColLE scaffolds showed a progressive increase of insulin production in response to high glucose concentration over the 7 days of culture (Fig. 8D). In contrast, this increase was much less profound by human islets cultured on Coll scaffolds, resulting in a statistically significant difference in their insulin production in response to high glucose concentration on day 7 of culture (Fig. 8D, $*p<0.05$). Consequently, the glucose stimulation index by islets cultured on ColLE scaffolds was significantly higher than that by islets cultured on Coll scaffolds on day 7 of culture (Fig. 8E, $*p<0.05$). These *in vitro* advantages of ColLE scaffolds now warrant future studies to examine their *in vivo* characteristics in appropriate transplantation models.

4. Discussion

The intraportal site is a sub-optimal site for islet transplantation due to a multitude of factors [1, 6]. Even in the absence of allo- or autoimmune-mediated injuries seen in allogeneic islet transplantation for autoimmune diabetic patients, autologous intraportal islet transplantation after total pancreatectomy still portend declining long-term islet function, pointing to the hostility of the intraportal site [1]. Furthermore, current organ shortage underscores an urgent need for innovative strategies for transplanting a marginal islet mass to achieve insulin independence, so that multiple pancreata are not needed for a single recipient. Thus, exploring extrahepatic sites for achieving normoglycemia by a marginal mass of islets is becoming increasingly appealing, especially at sites that can be accessed laparoscopically with ease [6].

In humans, the omentum carries several features that fulfill this need: it is highly vascularized; it can be manipulated laparoscopically; it has portal drainage; and it can accommodate islets and also allows for their easy retrieval. Additionally, studies have revealed that islets seeded in the omentum induce a reduced magnitude of immune responses despite enhanced islet vascularization, pointing to the superiority of the omentum over other alternative locations [44–46]. Remarkably, long-term survival of islets in nonhuman primates has been reported for islets seeded on biodegradable scaffolds and transplanted in the omentum [47]. Therefore, the omentum has a high potential to serve as an alternative site for clinical islet cell transplantation in humans, especially for single donor marginal islet mass transplantation [47]. The murine epididymal fat pad is the equivalent of the human omentum. Herein, using the overarching principles of biomimicry, we designed a biomimetic vasculogenic scaffold to enhance the engraftment of a marginal islet mass in the epididymal fat pad. We chose to use a mixture of collagen type I and elastin to fabricate our ColLE scaffolds because collagen and elastin are the most abundant components of the ECM in the body and provide essential cues for cell attachment, migration and organization [37]. Specifically, collagen is essential in maintaining intact tissue architecture, whereas elastin provides resilience and flexibility to tissues, a property essential to the function of certain tissues such as blood vessels [23]. Recent studies further suggest a novel role of elastin in promoting angiogenesis and thus tissue regeneration [37, 39, 48–50]. Although clinical adverse reactions to implants made of collagen of xenogeneic origin (e.g. bovine) is exceedingly rare, they do occur [51]. Thus, an understanding of mechanisms of such adverse

reactions is essential to the design and application of new collagen-based biomedical devices. Further blending of bovine elastin to the scaffolds adds potential additional xenogeneic antigens. Thus, host xenogeneic immune responses to the ColIE scaffolds, particularly the possibility of their potentiation by concurrent allomunity, is an important question that warrants thorough investigation in future studies.

Murine islets average ~100–250 μm in diameter [52]. To optimize islet loading as well as their engraftment, our ColIE scaffolds were engineered to have two sets of pore distributions: 200–400 μm pores to ensure efficient islet loading and retention, and 20–50 μm pores to support vascularization (see Materials and Methods). Published data suggest that synthetic scaffolds with interconnect porosity and a pore diameter of 30–40 μm vascularize more rapidly *in vivo* [53, 54]. Thus, our ColIE scaffolds were casted through an optimized multi-step freeze-drying process to achieve an interconnected porosity of these two pore size distributions. *In vitro*, we found that our ColIE scaffolds were superior, in comparison to Coll scaffolds or 2D culture conditions, in accommodating and retaining murine islet cells, preserving their 3D shape (Fig. 2) and preserving their viability and function over a one-week culture period (Fig. 3). These properties are advantageous as islet shape has been shown to be crucial for proper islet function [55], and the ability to culture islets for a prolonged period of time has been reported to enhance *in vivo* islet function [56, 57]. Physically, ColIE scaffolds swell rapidly in physiologic solutions and due to its interconnected porosity, it is possible to seed the scaffold with islets by applying the islet suspension on the top of the scaffold and aspirating the medium from the bottom. Multiple applying/aspirating cycles result in optimal islet loading and retention during surgery. Of note, our ColIE scaffolds also display favorable tactile features during islet loading, and during islet transplant surgery they promptly adhere to and are easily wrapped in the epididymal fat pad, likely due to their similar consistency to that of the epididymal fat pad. Therefore, our ColIE scaffolds display a set of physical characteristics conducive for minimally invasive laparoscopic surgeries for intra-abdominal fat pad islet transplantation.

Most remarkably, a marginal islet mass (70 murine islets) delivered by our ColIE scaffolds in the epididymal fat pad allowed rapid reversal of hyperglycemia within the first two weeks after transplantation in diabetic recipients (Fig. 4). This feature of ColIE scaffolds is extremely clinically relevant. It is estimated that 9.4% of the U.S. population has diabetes [58]; however the shortage of suitable deceased donor pancreata for islet manufacturing is a significant obstacle to the widespread use of pancreatic islet transplantation as a treatment for diabetes. Among all deceased donor pancreata, only a small percentage is suitable for extracting islets for transplantation, and an even smaller percentage is ultimately used for islet transplantation, mostly due to insufficient islet yield secondary to islet damage and/or loss during the isolation process. In the case of autologous islet transplantation after total pancreatectomy, the number of islets obtained is also often marginal due to the presence of chronic inflammation and sometimes fibrosis of such pancreata. For these reasons, there is a strong need for new ways to reduce islet mass necessary to revert normoglycemia. Our ColIE scaffolds demonstrate the remarkable ability for rapid diabetes reversal with a marginal islet mass, therefore presenting a promising platform for islet transplantation through an alternative extrahepatic site. Collagen-based scaffolds have been reported to be associated with reduced fibrotic capsular formation compared to synthetic polymer scaffolds

[30, 59]. Accordingly, histological evaluation at 4 and 12 weeks post-transplantation revealed that no fibrotic capsule was formed around our ColIE scaffolds (Fig. 5). In fact, islet morphology was fully preserved and islets were surrounded by an abundance of newly-formed vascular network as early as 4 weeks post-transplantation (Fig. 5A). Immunofluorescence further demonstrated the presence of numerous CD31⁺ cells corresponding to progenitor and/or mature endothelial cells [60–62] within the islets on ColIE scaffolds.

Further evidence of enhanced early vascularization of islets on ColIE scaffolds are provided by gene expression analysis (Fig. 7). As early as 4 weeks post-transplantation, all selected angiogenesis-associated genes (VEGFA, PDGFB, FGF1, Col3A1) are overexpressed in islets on ColIE scaffolds compared to those on Coll scaffolds; and these differences persist at 12 weeks post-transplantation. Published data support that formation of mature new vascular networks requires initiation by a pro-angiogenic growth factor such as VEGF followed by vessel stabilization by PDGF [63, 64]. VEGF and PDGF have been extensively used to enhance neovascularization of implants to improve their engraftment and overall tissue regeneration [64, 65]. It has been demonstrated that co-release of both VEGF [66] and PDGF [64] *in vivo* allows for not only formation of new blood vessels, but also ensures an overall higher degree of vessel maturity [63, 67]. In our study, we found that islets on ColIE scaffolds express a high level of PDGFB as early as 4 weeks post transplantation. This may indicate that vessel maturation is already underway as early as 4 weeks post-transplantation, which correlates with the enhanced early islet graft function (Fig. 4A) and a more abundant presence of CD31⁺ cells (Fig. 6A, 6B) seen in this group. At 12 weeks post-transplantation, the sustained expression of both genes (Fig. 7A) suggests a continued remodeling and maturation of the scaffold vasculature over time, as well as a persistent growth of vascular endothelial cells specifically promoted by VEGF signaling [68]. The ability of our ColIE scaffolds to induce the upregulation of both VEGFA and PDGFB genes endogenously without the need for any exogenous growth factor delivery represents a significant advantage in terms of safety and cost. Lastly, COL3A1 gene expression is also significantly enhanced at both time points in islets on ColIE scaffolds compared to those on Coll scaffolds. COL3A1 encodes for the pro- $\alpha 1$ chain of type III collagen found in blood vessels, and is frequently in association with type I collagen [69]. Between 4 and 12 weeks post-transplantation, a dramatic increase in its expression (~50 fold increase, Fig. 7A) correlates with an extensive new vessel formation detected within the ColIE graft (Fig. 6). These data from 4 and 12 weeks post-transplantation now indicate that it will be crucial to evaluate time points earlier than 4 weeks, when the difference of early islet graft function by Coll and ColIE scaffolds was more evident, to delineate the difference in kinetics of scaffold vascularization on Coll vs. ColIE scaffolds.

In addition to neovascularization, the formation of new islet-specific ECM is also crucial for islet function and survival [70]. In our study, we found that *de novo* matrix deposition-associated genes (COL6A1, COL4A1, LAMA2, FN1) are also significantly upregulated at both time points in islets on ColIE scaffolds compared to those on Coll scaffolds (Fig. 7B). These data further support that remodeling occurring on ColIE scaffolds is more enhanced in comparison to Coll scaffolds. Adult human islet ECM consists primarily of collagen IV and VI, laminin and fibronectin [5, 18]. These proteins engage integrins on the surface of islet

cells to mediate adhesion, provide structural support, and to activate intracellular signaling [71]. It has been hypothesized that early islet cell death after transplantation is due to a lack of integrin signaling, resulting in islet apoptosis [72]. Therefore, enhanced expressions of ECM components induced by ColLE scaffolds may function to support islet attachment and their intracellular integrin signaling, leading to superior islet viability and function after transplantation [71].

In examining ECM expression on our scaffolds, we used control murine pancreas tissue as a gold standard for the ideal islet ECM. At 4 weeks post-transplantation, the relative expression level of ECM genes in islets on both ColLE and Coll scaffolds is significantly lower than that in control pancreas tissue, with the exception of the gene FN1. FN1 encodes for fibronectin, a glycoprotein of the ECM involved in cell adhesion and migration, and is particularly important for tissue remodeling. Our finding suggests that ColLE scaffolds may be cellularized and remodeled at a significantly faster rate than Coll scaffolds, which correlates with a significantly higher number of cells as well as an enhanced neovascularization found throughout ColLE scaffolds at an earlier time point post-transplantation (Fig. 5). At 12 weeks post-transplantation, expression of all ECM genes in islets on ColLE scaffolds matches that of control pancreas tissue, demonstrating that ColLE scaffolds have significantly remodeled over time to allow for appropriate deposition of the islet-specific ECM. Coll scaffolds show an increased FN1 expression only at 12 weeks post-transplantation, indicating a delayed remodeling in comparison to ColLE scaffolds. Although ECM gene expressions in islets on Coll scaffolds never reach those seen in ColLE scaffolds or control pancreas tissue, at the terminal time point (i.e. 90 days post transplantation) islets transplanted on Coll scaffolds are also able to revert diabetes, suggesting that the level of neovascularization and islet ECM deposition on Coll scaffolds are nonetheless eventually sufficient to support the engraftment and function of the transplanted islets. However, it is worth pointing out that in this group normoglycemia is only slowly achieved after one month following transplantation.

Finally, given the promising results obtained with *murine* islets on ColLE scaffolds, we tested the scaffolds with *human* pancreatic islets (Fig. 8). We found that ColLE scaffolds is also able to retain human islets more efficiently than Coll scaffolds, and maintain their morphology, viability and function over an extended period of culture time (Fig. 8). More importantly, human islets cultured on ColLE scaffolds produce a higher amount of insulin upon glucose stimulation in comparison to islets cultured in 2D or on Coll scaffolds. Collectively, our data suggest that ColLE scaffolds can support the integrity and functionality of human islets, therefore may be a promising platform for clinical application for human islet transplantation.

5. Conclusions

In summary, our study demonstrates that ColLE scaffolds promote vascularization of an extrahepatic site for islet transplantation and allow for sufficient islet engraftment, survival, and function to restore euglycemia with a marginal islet mass in diabetic recipients. Our results suggest that the role of ColLE scaffolds in clinical settings in promoting insulin independence by marginal islet mass transplantation should be further explored, including in

autologous islet transplantation after pancreatectomy and/or in allogeneic islet transplantation using deceased donor pancreata.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Limitations in the current intrahepatic islet transplantation have restricted a widespread application of this therapy for the treatment of insulin-dependent diabetes;
- A new vasculogenic collagen/elastin scaffold (CollE) was engineered to enhance islet engraftment and functionality following extrahepatic islet transplantation;
- Islets seeded on CollE scaffolds transplanted in the epididymal fat pad allowed for diabetes reversal by a marginal islet mass within two weeks post transplantation.

Figure 1 –.

A representative stereomicroscope image of the CollE scaffold (A) and micrograph showing its anisotropic interconnected porous structure by confocal laser microscopy (B). Graphs showing scaffold pore diameters as measured from confocal laser microscopy images (C), overall porosity (D) and swelling properties (E), comparing CollE with Coll scaffolds. Data from (C)-(E) were averages of 5 samples from each experiment, and the experiment was repeated 2 times.

Figure 2 –.

Islet cells (red) seeded on Coll and ColIE scaffolds (blue) and cultured *in vitro* for 1 day and 7 days (A). At 7 days, an inset is provided to show the morphology of islet cells on the scaffolds (A). The graph shows the area fraction (%) of islets at 1 day and 7 days post-culture on Coll or ColIE scaffolds (B). Values are reported as mean $\pm$ standard deviation. A value of $p < 0.05$ was considered statistically significant: * $p < 0.05$ and **** $p < 0.0001$. Data from (B) were averages of 4 samples from each experiment, and the experiment was repeated 2 times.

Figure 3 –.

LIVE/DEAD[®] assay of murine islet cells cultured in 2D (2D CTRL) or on Coll and ColIE scaffolds (A). Images were acquired by confocal laser microscopy and live cells were shown in green whereas dead cells were shown in red. Viability was quantified by the LIVE/DEAD[®] staining and reported as percentage of live cells over total number of cells (B). Viability assessed by Alamar Blue assay over 7 days of culture (C). On day 7 of culture, insulin production at high (28 mM) and low (2.8mM) glucose concentrations (D) and stimulation index calculated as the ratio of insulin produced at high over low glucose

concentration (E). Values are reported as mean $\pm$ standard deviation. A value of $p < 0.05$ was considered statistically significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Data from (B) - (E) were averages of 4 samples from each experiment, and the experiment was repeated 2 times.

Figure 4 –.

Blood glucose levels 0 – 14 days (A) and 15 – 90 days (B) post marginal islet mass transplantation. Glucose tolerance test at 90 days post-transplantation (C).

Immunofluorescence images evaluating islet functionality transplanted via Coll and ColIE scaffolds at 4 weeks and 12 weeks post-transplantation. Sections were stained with DAPI (blue), glucagon (green) and insulin (red); magnification = 20X (D). Values are reported as mean $\pm$ standard deviation. A value of $p < 0.05$ was considered statistically significant: * $p < 0.05$, **** $p < 0.0001$. Data from (A)-(C) were obtained from a total of 8 mice from 2 independent experiments.

Figure 5 –.

Gross images and Masson's trichrome staining of islet grafts on Coll and CollE scaffolds at 4 and 12 weeks post-transplantation. The black dotted lines demarcate the Coll and CollE scaffolds within the retrieved epididymal fat pad. Histological evaluation by Masson's trichrome staining: islets were stained as bright pink and marked with "*" in 40X and 100X images. Blood vessels with red blood cells in the lumen were indicated by white arrowheads in 40X and 100X images.

Figure 6 -

Immunofluorescence images evaluating neovascularization of the islet grafts at 4 weeks and 12 weeks post-transplantation. Sections were stained with DAPI (blue), anti-CD31 (green) and anti-insulin antibody (red); magnification = 20X (A). Quantification of CD31 expression reported as the area fraction (%) (B). The area fraction (%) was defined as the area occupied by CD31 signal divided by the total insulin positive area, both within the acquired field of view. Values are reported as mean $\pm$ standard deviation. A value of $p < 0.05$ was considered statistically significant: **** $p < 0.0001$. Data from (B) were averages of 5 retrieved islet grafts from 2 independent experiments.

Figure 7 –

Gene expression analysis of angiogenesis -associated genes (A) and *de novo* islet-specific matrix deposition-associated genes (B) in Coll and CollE scaffolds at 4 weeks and 12 weeks post-transplantation. Results were first normalized to the expression of the housekeeping gene GAPDH, and then expressed as “relative-fold expression” in comparison to the expression level of the same gene from the control kidney subcapsular islet graft. Levels of expression of *de novo* islet-specific matrix deposition-associated genes in Coll and CollE scaffolds were further compared to the levels present in normal murine pancreas tissue. Data from (A)-(B) were averages of 3 retrieved islet grafts from a total of 2 experiments. Technical triplicates were performed for all qPCR reactions and the averages of the triplicates were used for statistical comparisons plotted in the bar graphs. Values are reported as mean $\pm$ standard deviation. A value of $p < 0.05$ was considered statistically significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Figure 8 -

Human islets seeded on Coll and CollE scaffolds (500 IEQ/scaffold), and imaged by stereomicroscopy (left panels) and confocal laser microscopy (right panels) on day 1 and day 7 of culture (A). Area fraction (%) for islets on day 1 and day 7 (B). The area fraction was defined as the area occupied by the islet over the area of signal at 461nm, corresponding to the scaffold surface (n=5) (B). Alamar Blue viability assay performed over the 7-day culture period, expressed as % of reduction (C). Insulin production to high (H, 28 mM) and low (L, 2.8 mM) glucose solutions over the 7-day culture period (D). Stimulation index (SI)

was calculated as the ratio of insulin production at high over low glucose concentrations (E). Values are reported as mean $\pm$ standard deviation. A value of $p < 0.05$ was considered statistically significant: * $p < 0.05$. Data from (B)-(E) were obtained from 1 batch of human islets. 5 replicates were used to obtain the average values presented in (B)-(E).

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