

# Bioengineering a highly vascularized matrix for the ectopic transplantation of islets

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**Abbreviations:** BSA, Bovine Serum Albumin; CC, collagen and chitosan; CCC, collagen, chitosan, and chondroitin; CCCL, collagen, chitosan, chondroitin and laminin; C, Collagen matrix; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FBS, Fetal Bovine Serum; HBSS, Hank's Balanced Salt Solution; IBMX, isobutylmethylxanthine; NHS, N-hydroxysuccinimide; NPI, neonatal porcine islets; RPMI, Roswell Park Memorial Institute medium; SDF-1, Stromal cell-derived factor-1; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor

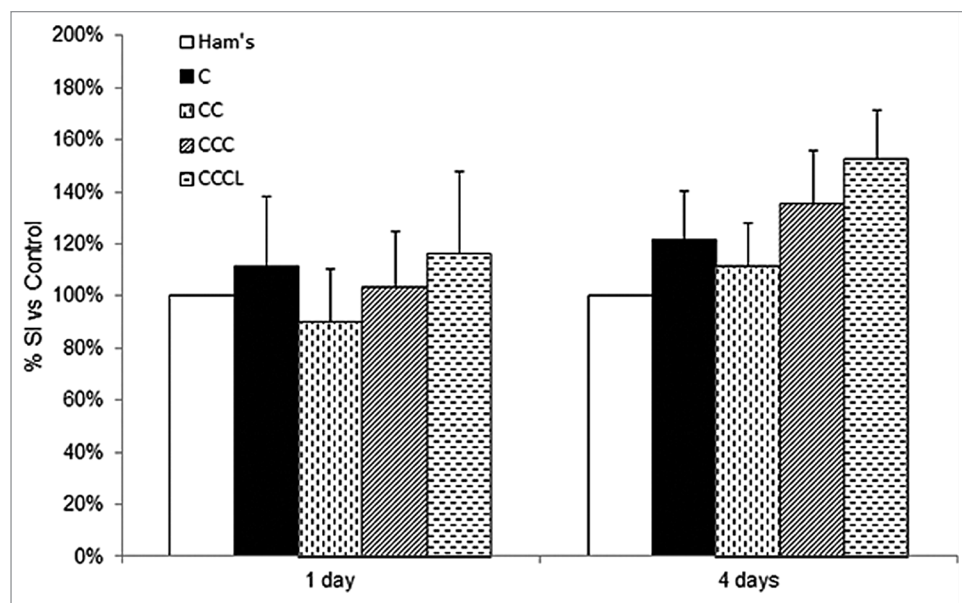
Islet transplantation is a promising treatment for Type 1 diabetes; however limitations of the intra-portal site and poor revascularization of islets must be overcome. We hypothesize that engineering a highly vascularized collagen-based construct will allow islet graft survival and function in alternative sites. In this study, we developed such a collagen-based biomaterial. Neonatal porcine islets (NPIs) were embedded in collagen matrices crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide containing combinations of chondroitin-6-sulfate, chitosan, and laminin, and compared with controls cultured in standard media. Islets were examined for insulin secretory activity after 24 h and 4 d and for apoptotic cell death and matrix integrity after 7 d *in vitro*. These same NPI/collagen constructs were transplanted subcutaneously in immunoincompetent B6.Rag<sup>-/-</sup> mice and then assessed for islet survival and vascularization. At all time points assessed during *in vitro* culture there were no significant differences in insulin secretory activity between control islets and those embedded in the collagen constructs, indicating that the collagen matrix had no adverse effect on islet function. Less cell death was observed in the matrix with all co-polymers compared with the other matrices tested. Immunohistochemical analysis of the grafts post-transplant confirmed the presence of intact insulin-positive islets; grafts were also shown to be vascularized by von Willebrand factor staining. This study demonstrates that a collagen, chondroitin-6-sulfate, chitosan, and laminin matrix supports islet function *in vitro* and moreover allows islet survival and vascularization post-transplantation; therefore, this bio-engineered vascularized construct is capable of supporting islet survival.

## Introduction

Since 2000, when the “Edmonton Protocol” achieved insulin independence in 7 patients by using islets from multiple donors and steroid-free anti-rejection therapy, islet transplantation has been seen as an increasingly promising treatment for patients with type 1 diabetes.<sup>1–4</sup> Despite remarkable progress in clinical islet transplantation, the liver implantation site remains far from ideal.<sup>5–9</sup> Although alternative sites have been suggested, including subcutaneous and intramuscular spaces, poor blood supply in these sites results in delayed graft revascularization and thereby loss of islet cell mass and function.<sup>6</sup> Our group has previously used collagen-based biomaterial matrices to facilitate cell delivery resulting in revascularization of ischemic tissue.<sup>10,11</sup> Such a collagen-based matrix could potentially be used to deliver islets to an alternate site, then support islet survival and function through rapid neovascularization.

Collagen is a highly biocompatible polymer and natural extracellular matrix that has been widely examined as a potential biomaterial for vascularization and islet delivery. Collagen and other polymers are commonly considered because they can be engineered to have similar properties to the extracellular matrix, allowing vascularization and cell support. Various approaches have been explored, including using a polyethylene terephthalate mesh bag around a collagen sponge to deliver Sprague-Dawley rat islets intramuscularly in Lewis rats;<sup>12</sup> a polytetrafluoroethylene “solid support” in collagen delivering isogenic rat islets in a peritoneal site;<sup>13</sup> and polyurethane foam filled with heparin and collagen delivering syngeneic rat islets in the subcutaneous space.<sup>14</sup> These devices utilize a combination of natural and synthetic polymers, which is advantageous because of the ease of manufacturing and the control of mechanical properties of the synthetic polymers. Polymers are widely used as biomaterials for cell

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**Figure 1.** Glucose stimulated insulin secretions of control and collagen embedded NPIs were measured following 1 and 4 d in culture. The stimulation indices (SI) were determined by dividing the amount of insulin released at high glucose (20.0 mM) by that at low glucose (2.8 mM). The stimulation indices were subsequently normalized to the non-embedded control values. The stimulation indices for the control Ham's F10 condition after 1 and 4 d in culture were  $1.42 \pm 0.48$  and  $1.05 \pm 0.18$ , respectively. Data are expressed as mean  $\pm$  SEM (n = 6 per time point).

delivery and inducing vascularization because of their potential to mimic the natural extracellular matrix. However, natural polymers, compared with synthetic polymers, have improved biocompatibility of both the biomaterial and the degradation byproducts, thereby being less immunogenic. The ideal device for islet delivery should then be based on natural polymers such as collagen but also have the mechanical properties of synthetic polymers.

The poor mechanical properties resulting from the purification process of collagen necessitate modifications in order to restore mechanical strength. Two common methods to achieve this goal are chemical crosslinking and the addition of copolymers. The crosslinker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) combined with N-hydroxysuccinimide (NHS) has been used to achieve this with minimal negative impact on cell viability.<sup>11,15,17</sup> The addition of copolymers such as chitosan, chondroitin and laminin to the matrix can also lead to improved mechanical strength and local angiogenesis.<sup>16,17</sup> Chitosan, prepared from the deacetylation of chitin, is a biocompatible polymer that has been shown to accelerate wound healing and vascularization.<sup>16</sup> Crosslinking chitosan to collagen may improve the resistance of the collagen-based matrix to enzymatic degradation. Chitosan also possesses unique tissue-adhesive and antimicrobial properties that could improve the properties of the matrix.<sup>16</sup> Collagen-chitosan constructs are successful cell delivery vehicles that promote neovascularization.<sup>16</sup> Chondroitin-6-sulfate is a sulfated glycosaminoglycan that interacts with functional proteins to bind growth factors into the biomaterial.<sup>17</sup> Chondroitin additionally ameliorates the matrix structure by regulating matrix elasticity, allowing collagen-chondroitin-6-sulfate gels to be utilized as potential islet delivery devices.<sup>16,18,19</sup> Laminin, an important basement membrane protein, may aid in

the retention of transplanted islets *in vivo* in addition to acting as a guidance molecule for vascularization and islet survival.<sup>20,21</sup>

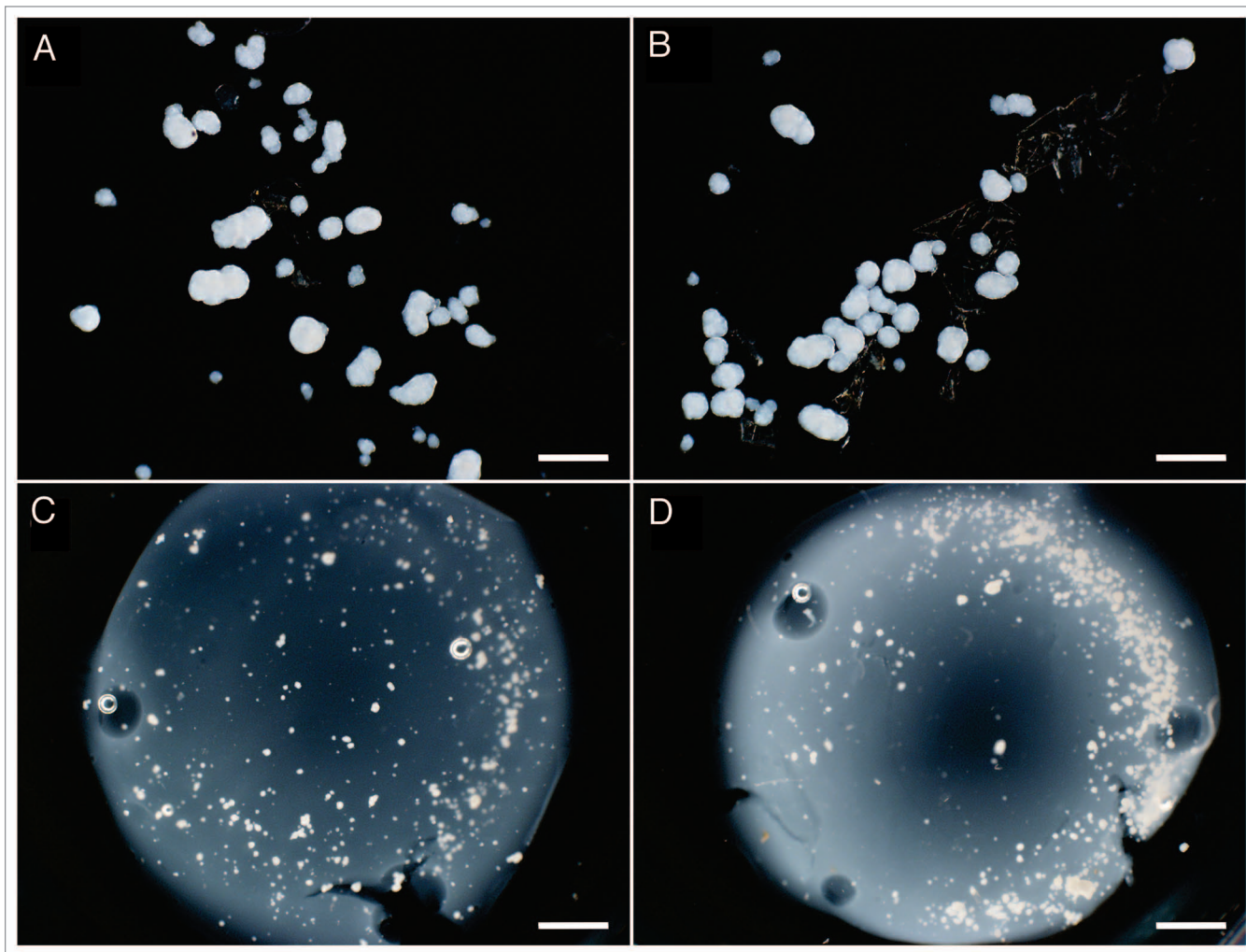
The objective of this study was to examine the effect of various collagen-based matrices on the *in vitro* function of NPIs and their survival following transplantation in the subcutaneous space of mice. Neonatal porcine islets (NPIs) were used because they are a potential clinical source of islets for treating patients with type 1 diabetes.<sup>22,23</sup>

## Results

### *In vitro* assessment of islet: Collagen matrices

Prior to conducting the transplant studies we assessed the glucose stimulated insulin secretion of collagen embedded NPIs in order to examine if the matrices had an adverse effect. The insulin secretory capacities of NPIs embedded in the different collagen-based matrices and cultured for 1 and 4 d were tested by comparing the amount of cellular insulin that was released at low glucose (2.8 mM) and high glucose (20.0 mM). No statistically significant differences were noted in the amounts of insulin secreted at low or high glucose when comparing all collagen conditions with control NPIs. Similarly, when the stimulation indices were calculated no significant differences were observed in any conditions at either 1 d or 4 d post culture (Fig. 1). The stimulation indices shown in Figure 1 are typical of immature NPIs.

Differences in the mechanical properties were noted between the various matrices. When NPIs were added to matrices containing collagen alone (C) or collagen and chitosan (CC), the matrices did not crosslink completely. Culture media added on top of these C and CC matrices accelerated the degradation of



**Figure 2.** NPIs embedded in C (A), CC (B), CCC (C) and CCCL (D) matrices were cultured for 7 d then dark field images were taken of the matrices in a 6 well plate. Scale bars are 400  $\mu\text{m}$  for (A) and (B) and 1.6 mm for (C and D).

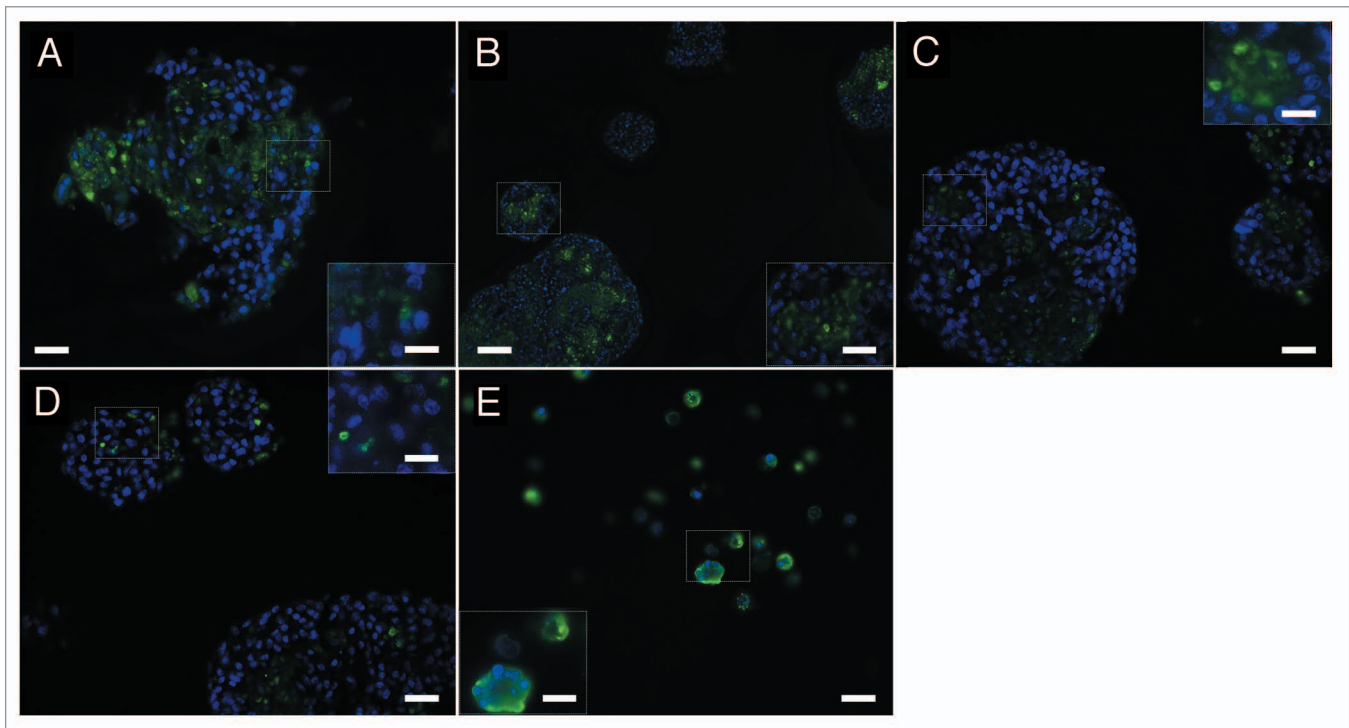
the matrices after 7 d, leaving small pieces of matrices and loose NPIs (Fig. 2A and B). The CCC matrices did crosslink with the addition of NPIs and appeared similar to the CCCL matrices after 7 d in culture (Fig. 2C and D).

To determine the effect of the matrices on islet survival, matrices containing NPIs were also cultured for 7 d then stained for insulin and using a TUNEL assay for apoptosis (Fig. 3). Since the C and CC matrices did not adequately crosslink, we embedded the NPIs with the remaining separated pieces of the matrix in agarose. While all matrices showed intact islets, the CCCL matrix (Fig. 3D) had significantly fewer apoptotic cells with  $4.3 \pm 0.2\%$  of the DAPI positive cells staining for TUNEL compared with the C matrix at  $9.9 \pm 0.4\%$  ( $P < 0.05$ ), the CC matrix at  $7.3 \pm 0.2\%$  ( $P < 0.05$ ), and the CCC matrix at  $7.2 \pm 0.2\%$  ( $P < 0.05$ ) (Fig. 3A, B, and C, respectively). Some of the cells stained positively for TUNEL but not for DAPI, likely indicating apoptotic cells with extremely fragmented DNA.<sup>24</sup>

#### In vivo assessment of islet: Collagen matrices

Based on the above data, the C and CC matrices were determined to be inferior and were no longer considered as potential

biomaterials for islet transplantation. The CCCL matrix exhibited no adverse effects on mechanical stability, islet survival, or islet function. Chondroitin-6-sulfate binds functional proteins,<sup>16</sup> chitosan improves vascularization, and laminin enhances vascularization<sup>20</sup> and islet survival;<sup>21</sup> therefore, the following in vivo transplant studies were conducted using the CCCL matrix. In a first series of experiments we implanted the CCCL matrices without NPIs subcutaneously into BALB/c mice in order to examine their potential to become vascularized. Upon retrieval, the matrices were adhered to the hypodermis and maintained their size and shape over the 28 d period. A thin, transparent membrane formed around the transplanted matrix by 4 d after transplantation. Retrieved matrices were fixed and paraffin embedded, and sections were stained for von Willebrand factor (vWF) to evaluate the potential for vascularization of the matrix. At 7 d, vWF positive endothelial cells began to penetrate the periphery of the matrix (Fig. 4A). Two weeks post-transplantation, small capillaries with a vWF positive lining were visible, migrating toward the interior of the matrix (Fig. 4B). After quantification of capillary size, these



**Figure 3.** NPIs embedded in C (A), CC (B), CCC (C) and CCCL (D) matrices following 7 d in culture were fixed and paraffin-embedded, then sections were stained for TUNEL and compared with TUNEL positive controls (E). Scale bars of A-E are 20  $\mu\text{m}$  and scale bars of insets are 10  $\mu\text{m}$ .

early capillaries were found to have a mean size of  $53.54 \pm 0.75 \mu\text{m}^2$ . At 3 weeks, the capillaries increased in size to a mean area of  $75.24 \pm 2.32 \mu\text{m}^2$  as well as in number, and some appeared to be combining into larger structures (Fig. 4C). After 4 weeks, the capillaries were widespread throughout the matrix (Fig. 4D) and had grown to a significantly larger mean size of  $221.68 \pm 5.63 \mu\text{m}^2$  ( $P < 0.05$ ). These data clearly demonstrate that the CCCL matrix can become vascularized following subcutaneous implantation.

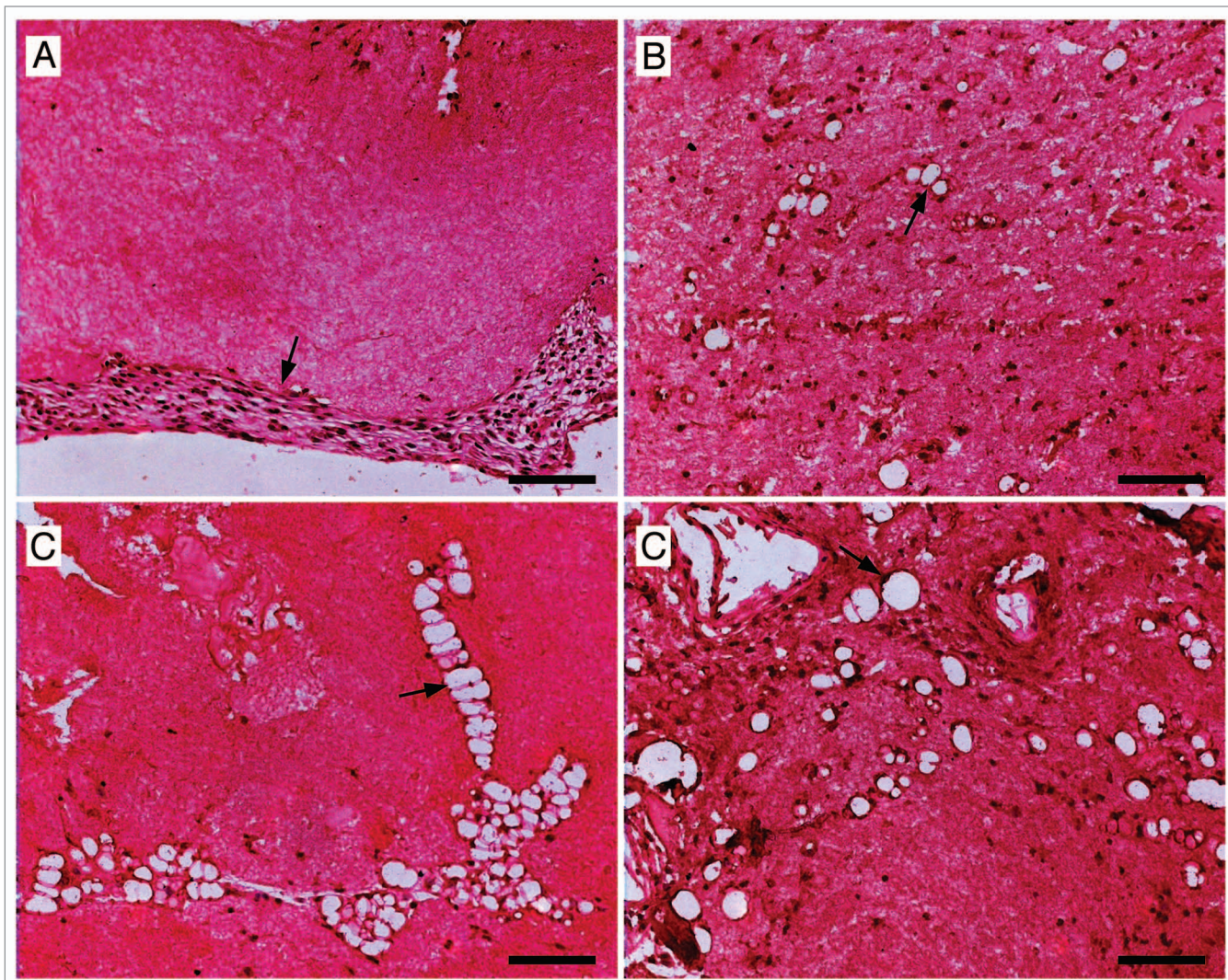
After confirming the ability of the CCCL matrix to become vascularized, C, CC, and CCCL matrices containing NPIs were transplanted subcutaneously in naïve immunoincompetent B6.Rag<sup>-/-</sup> mice and retrieved after 4 (CCCL only), 7, 14, 21, and 28 d and examined for gross morphology (Fig. 5). The CCCL matrices were transplanted using a microspatula, but because the C and CC matrices did not crosslink sufficiently to be manipulated, they were injected using a large orifice pipette. Prior to implantation, NPIs were distributed throughout the CCCL matrix (Fig. 5A). Following transplantation, blood vessels were observed in the periphery of the matrices as early as four days (Fig. 5B) as well as at 7 and 28 d (Figs. 5C and D). However, following implantation NPIs were only observed at 4 d since the matrices become overgrown with connective tissue making it difficult to detect the NPIs within the matrix. At 7 d and later, no C or CC matrices were found; it is likely that these matrices degraded quickly due to their lack of mechanical integrity at the time of transplantation.

We therefore conducted another series of CCCL:NPI transplants in non-diabetic B6.Rag<sup>-/-</sup> mice and collected the

grafts for histological assessment. It should be noted that since NPIs are not fully developed islets the number of insulin and glucagon positive cells are lower than that observed in adult islets. When examined for the presence of insulin and glucagon positive cells, the proportion of insulin and glucagon positive cells tended to increase at 4 d (Fig. 6C and D) and 7 d (Fig. 6E and F) post-transplantation, as compared with those prior to transplantation.

## Discussion

This study demonstrates that our collagen-based matrix has the potential to create a site for islet transplantation. The insulin secretory capacity of NPIs seeded into the CCCL matrices was not adversely affected and when transplanted subcutaneously the NPIs were shown to survive as the matrix became more vascularized. When compared with controls cultured in HAM'S F10, insulin secretion of NPIs in all tested collagen matrices was comparable; the stimulation indices of the collagen embedded NPIs tended to be higher than controls most notably in CCCL, however this was not significantly different. Nonetheless these data demonstrate that the matrices did not have an adverse effect on NPI viability as indicated by comparable stimulation indices. The transplanted CCCL matrices were also shown to support NPI survival in vivo for up to 28 d as evidenced by the positive insulin and glucagon staining at each time point. There were no signs of inflammation or adverse reaction to the matrices, and the matrix maintained its size and shape over the 28 d period indicating a slow biodegradation



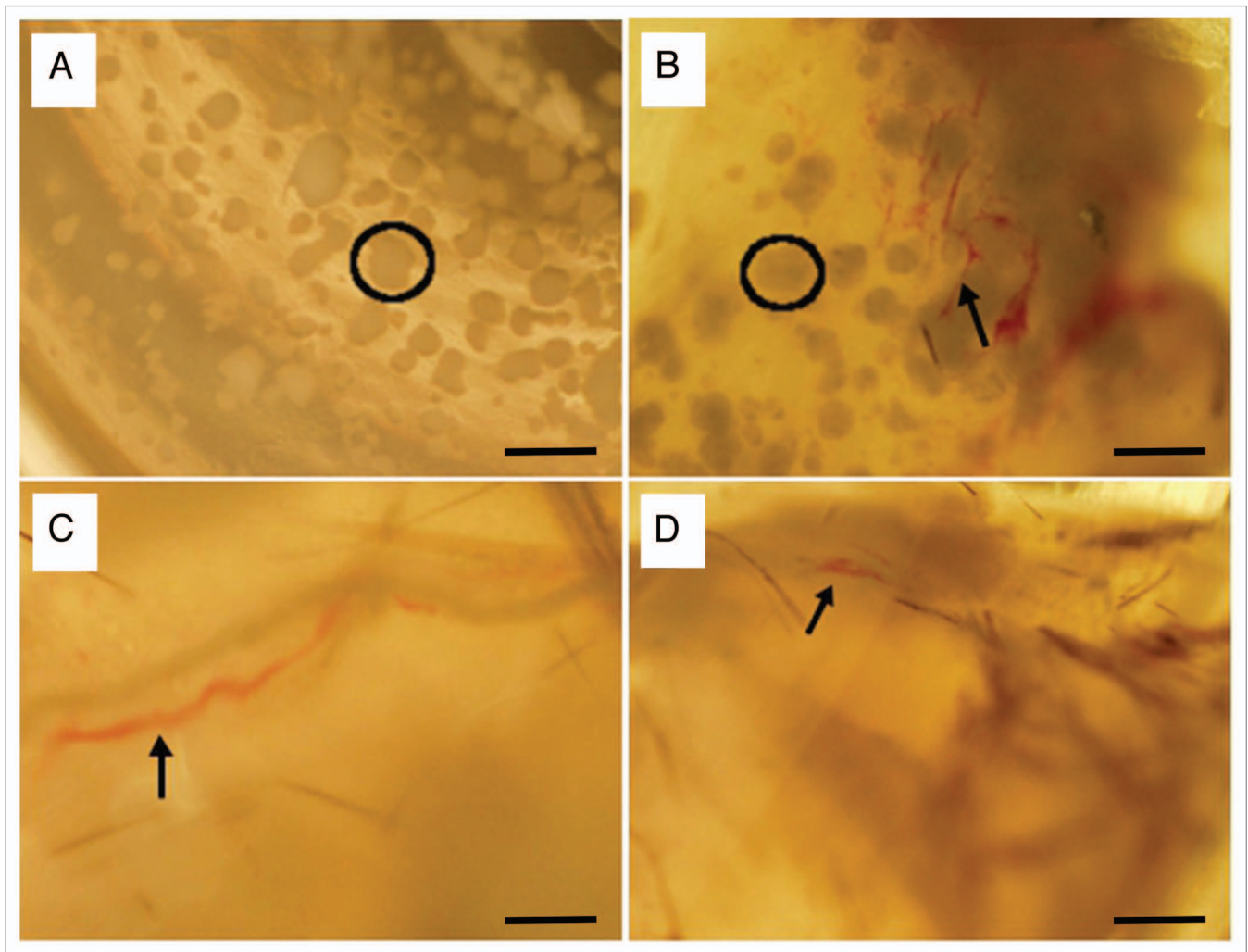
**Figure 4.** In order to examine whether CCCL matrices without islets can be vascularized *in vivo* the CCCL matrices were implanted subcutaneously in immunoincompetent BALB/c mice and retrieved after 7 (A), 14 (B), 21 (C) and 28 d (D). The grafts were fixed and paraffin-embedded; sections were stained for von Willebrand Factor (vWF), hematoxylin, and eosin. All scale bars are 100  $\mu$ m. Arrows indicate vWF positive cells (A) and capillaries (B–D)

rate. Islet survival over a longer term must still be investigated further.

A critical feature of using a collagen-based matrix for islet transplantation is being able to maintain mechanical stability. Mechanical stability is essential for physical manipulation of the matrix in order to successfully place it into the transplant site. Although this collagen based matrix is similar to that described previously by Suuronen et al.<sup>20,26</sup> to recruit and deliver endothelial progenitor cells, our matrix required alteration to support NPIs. In this study we observed that collagen alone and collagen with chitosan matrices degraded quickly during culture upon the addition of NPIs before crosslinking and thereby lost their mechanical stability. Therefore, we had to improve the integrity of the CCCL matrix and its ability to crosslink upon the addition of NPIs. Differences from the previously described matrix<sup>20,26</sup> include using pure rat tail collagen instead of porcine collagen; using a higher concentration of chondroitin-6-sulfate; using 20  $\mu$ g/mL of laminin instead of 40  $\mu$ g/mL; and using

10x DMEM and 10x HEPES as a “collagen buffer” instead of 2-(N-morpholino) ethanesulfonic acid buffer to dissolve the crosslinking chemicals.

When the co-polymer chondroitin-6-sulfate was added these matrices had improved mechanical stability, thus allowing for ease of implantation into the subcutaneous space. Additionally, the CCCL matrix better supported islet viability, as shown by fewer apoptotic cells compared with the C, CC, and CCC matrices. Because chitosan, chondroitin-6-sulfate, and laminin have beneficial properties such as binding functional proteins (chondroitin-6-sulfate) and enhancement of vascularization and islet survival (laminin), we decided to use the CCCL matrix for the subsequent *in vivo* transplant studies. To confirm this result, C and CC matrices were transplanted subcutaneously; as early as 7 d, no C matrices were found in any of the mice and only one very small CC matrix was found with no visible islets. This indicates that the C and CC matrices, in addition to being much more difficult to transplant, biodegrade more quickly than the



**Figure 5.** Gross morphology of NPIs in the CCCL gels and transplanted subcutaneously in immunocompromised B6.Rag<sup>-/-</sup> mice. Grafts were analyzed pre-transplant (A) and after 4 (B), 21 (C) and 28 (D) days. Circles indicate visible islets; arrows indicate visible vasculature. Scale bars are 400  $\mu$ m.

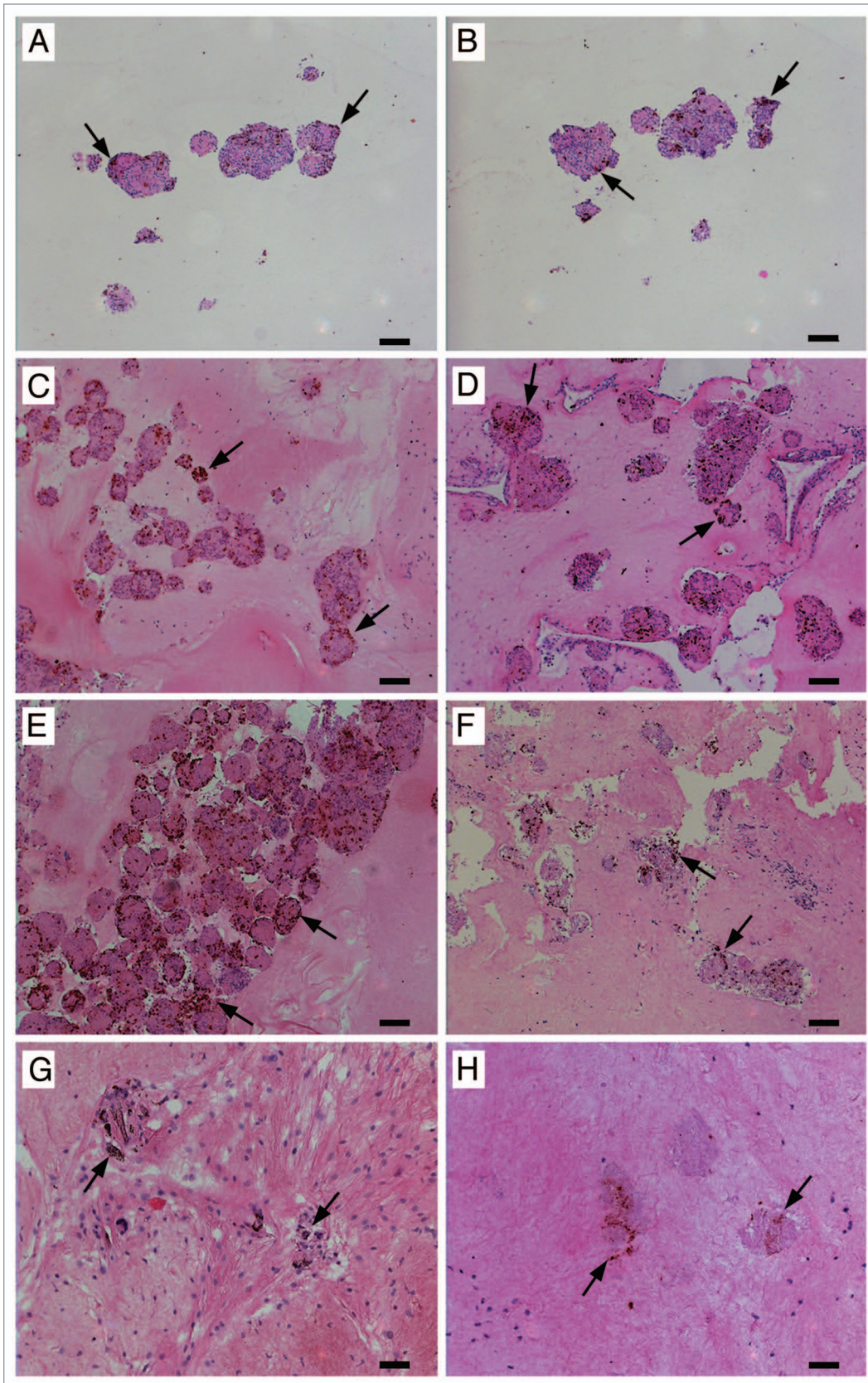
CCCL matrix and are therefore not suitable as biomaterials for NPI transplantation.

Our data demonstrate that at 4 d post-transplant vWF positive endothelial cells begin to penetrate the periphery of the CCCL matrix and as time progresses capillaries begin to form then subsequently increase in size and number. These data clearly demonstrate that the CCCL matrices can become highly vascularized, allowing for NPI survival. Although it is unknown if these capillaries are sufficient to support longer-term islet survival, other components may be added to the matrix such as vascular endothelial growth factor (VEGF) to further promote the growth of larger blood vessels. VEGF has previously been shown to increase vascularization in collagen-based matrices, and it is known to bind to chondroitin-6-sulfate.<sup>14,24</sup> Stromal cell-derived factor-1 (SDF-1) is an alternative growth factor that has also been shown to improve recruitment of angiogenic cells in a collagen-based matrix *in vivo*.<sup>25</sup>

Another potential benefit of a CCCL matrix is that it could theoretically allow for the co-localization of ancillary cells such as

mesenchymal stem cells (MSCs) with the islets. MSCs have been shown to promote vascularization in various models, including in a collagen-glycosaminoglycan scaffold wherein the mesenchymal stem cells were observed to adopt an endothelial phenotype, thereby enhancing vascularization.<sup>27</sup> Moreover, MSCs have been also shown to have beneficial effects when co-transplanted with islets, such as promoting graft revascularization, prevention of islet allograft rejection and protection of human islets from pro-inflammatory cytokines.<sup>28-31</sup> Because the kidney is not an applicable site for clinical islet transplantation and the liver will not ensure close association of the co-grafted cells, an alternative site must be developed. CCCL matrices may be the solution because they would greatly improve the likelihood that the cells are co-localized.

Although islets can be supported in a collagen-based matrix as it becomes vascularized over time, it would be ideal to transplant the islets into an environment with an already established vascular system. Two ways to create this environment would be to transplant islets into a prevascularized collagen-based matrix,



**Figure 6.** Neonatal porcine islets in the CCCL gels were transplanted subcutaneously and retrieved after 4 (**C** and **D**), 7 (**E** and **F**), and 28 (**G** and **H**) days. Pre-transplantation grafts were also collected (**A** and **B**). The grafts were fixed and paraffin-embedded; sections were stained for insulin, hemotoxylin and eosin (**A**, **C**, **E**, and **G**) and glucagon, hemotoxylin and eosin (**B**, **D**, **F**, and **H**). Arrows indicated insulin (**A**, **C**, **E**, and **G**) or glucagon (**B**, **D**, **F**, and **H**) positive cells. Scale bars for (**A**–**F**) are 100  $\mu$ m and for (**G** and **H**) are 50  $\mu$ m.

**Table 1.** Composition of matrices tested on neonatal porcine islets.

Material	Matrix composition			
	C	CC	CCC	CCCL
Collagen (mg/mL)	6.1	6.0	5.3	5.2
Chitosan (mg/mL)	-	0.2	0.2	0.2
Chondroitin(mg/mL)	-	-	1.1	1.0
Laminin (mg/mL)	-	-	-	0.1
EDC (mM)	31	30	26	26
NHS (mM)	35	34	30	30

A summary of the composition of matrix types tested and their respective polymer concentration. C, collagen; CC, collagen and chitosan; CCC, collagen, chitosan, and chondroitin, and CCCL, collagen, chitosan, chondroitin, and laminin.

or to increase vascularization in the surrounding site so that there are more vessels available to grow into the matrix. Both these approaches have successfully been used in other bioengineered devices, with both natural and synthetic polymers, with pre-vascularization periods ranging from 14 to 60 d.<sup>12,13,24,32-34</sup> Both approaches have also been combined with growth factors such as fibroblast growth factor (FGF)<sup>12,13,33</sup> and vascular endothelial growth factor (VEGF).<sup>24</sup> Prevascularizing the matrix could also be achieved by transplanting a stem cell population to promote angiogenesis. One or a combination of these approaches will be investigated further in order to optimize the conditions for islet survival and function.

Although the subcutaneous space may not be a practical site for clinical islet transplantation, it was used in this study to examine the feasibility of using the collagen matrix. A more practical site would be the omental pouch; however, the size of the collagen matrices did not permit us to use this site in mice. The ideal site for clinical islet transplantation should be optimal for islet survival and function. It should allow for portal drainage of secreted hormones without direct exposure to high concentrations of immunosuppressive drugs, host platelets and complement; accommodate a large tissue volume; be easily accessible; and allow for retrieval of the grafted tissue. The omental pouch satisfies all of these criteria and has been used to successfully cure diabetes in both rats and dogs.<sup>35-38</sup> This site will be investigated in the future in a larger rodent model, such as the rat.

We have shown that embedding NPIs in a collagen-based matrix containing the co-polymers chitosan, chondroitin-6-sulfate and laminin does not adversely affect NPI function in vitro; when these matrices are implanted subcutaneously in mice they induced significant vascularization and they supported the survival of the NPIs. This suggests that the CCCL matrix may be used to create a site for islet transplantation, which could include several benefits including prevention of tissue loss and cellular co-transplantation. Further study in diabetic recipients is warranted.

## Materials and Methods

### Preparation of neonatal porcine islets

Porcine pancreases were obtained from 1 to 3 d old Duroc neonatal piglets from the University of Alberta Swine Research

Centre (1.5–2.0 kg body weight), and the islets were isolated and cultured for 5–7 d as described previously.<sup>22</sup> Briefly, the retrieved pancreases were cut into 1 to 3 mm tissue fragments, then exposed to 2.5 mg/mL collagenase (type XI; Sigma, C7657), filtered through a 500 µm nylon screen, washed in Hank's Basic Salt Solution (HBSS) (Gibco, H6136) supplemented with 0.25% BSA (fraction V; Sigma-Aldrich, A9543), 10 mM HEPES (Sigma-Aldrich, H4034), 100 U/mL penicillin, and 0.1 mg/mL

streptomycin (Lonza Walkersville, Inc., 09-757F). NPI were then cultured in non-tissue culture treated petri dishes containing HAM'S F10 tissue culture media purchased from Sigma-Aldrich (N6635), and supplemented with 14.3 mM sodium bicarbonate (Fisher, S233), 10 mM D-glucose (EM Science, DX0145-3), 2 mM L-glutamine (Sigma-Aldrich, G8540), 0.25% BSA (fraction V; Sigma-Aldrich, A9543), 50 µM isobutylmethylxanthine (IBMX) (Sigma-Aldrich, I5879), 10 mM nicotinamide (Sigma-Aldrich, N0636), 1.6 mM calcium chloride dihydrate (Sigma-Aldrich, C7902), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Walkersville, Inc., 09-757F). The islets were cultured at 37 °C for 5–7 d, with the medium changed the first, third and fifth days after isolation.

### Preparation of collagen matrices

Neonatal porcine islets (NPIs) were embedded in collagen-based matrices utilizing rat tail type one collagen (BD Biosciences, Inc., 354249) crosslinked with 30 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma-Aldrich, E6383) and 30 mM N-hydroxysuccinimide (NHS, Sigma-Aldrich, 56480) containing combinations of chondroitin-6-sulfate (Wako Pure Chemical Industries, 034-14612), chitosan (Sigma-Aldrich, C3646), and mouse laminin (BD Biosciences, Inc., 354232), as outlined in Table 1. In Table 1, the acronym C refers to gels with collagen, CC collagen with 5% v/v chitosan, CCC collagen with 5% v/v chitosan and 40% w/v chondroitin, and CCCL collagen, chitosan, chondroitin and 20 µg/mL laminin. Following embedding in the various collagen matrices, islets were cultured overnight in supplemented HAM'S F10. Controls included non-embedded islets cultured in supplemented HAM'S F10 (NPIs).

### In vitro assessment of islets and matrices

A static incubation assay<sup>22</sup> was used to assess glucose stimulated insulin secretion of collagen embedded and control NPIs following 1 and 4 d culture. Each condition was incubated in duplicate at 37 °C for 2 h in 1.5 mL RPMI (Roswell Park Memorial Institute medium) supplemented with 2.0 mM L-glutamine, 0.5% w/v BSA and either 2.8 mM (low) or 20.0 mM (high) glucose. The media was then separately collected and assayed for respective insulin contents by a rat insulin immunoassay that quantitatively detects porcine insulin (Meso Scale Discovery, K152BZC). Stimulation indices (SI) were calculated by dividing the amount of insulin released at 20.0 mM glucose



by that released at 2.8 mM glucose. The stimulation indices were subsequently normalized to the non-embedded control values and expressed as a percentage of the control SI.

Matrices containing NPIs were cultured for 7 d in HAM'S F10 culture media supplemented as above, then dark field images were taken to identify changes in mechanical integrity. The matrices were then collected and fixed in Shandon Zinc Formal-Fixx (Thermo Fisher Scientific, 6764255) if possible; if the matrices did not have sufficient integrity to be collected on their own, the liquid matrix and NPIs were collected, fixed for 24 h in 1% formalin in PBS, and then embedded in agarose plugs. Apoptosis of the NPIs in the matrices was assessed using a commercial TUNEL assay kit (Invitrogen Molecular Probes A23210). After rehydration, antigen retrieval was performed in sodium citrate buffer (pH 6.0), then slides were incubated according to the kit instructions. The slides were mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen Molecular Probes P36935). To quantify the percentage of TUNEL positive cells, five images of TUNEL and DAPI staining were taken then the images were combined in ImageJ (National Institutes of Health). Separate images of the TUNEL and DAPI positive channels were altered to black and white, then the number of particles counted. Each particle was confirmed to be TUNEL or DAPI positive visually and the brightness of the color threshold adjusted to only include positive cells. This process was repeated by 2 independent viewers.

#### In vivo assessment of matrices and islets with matrices

Based on the in vitro data only the CCCL matrices were assessed in subsequent in vivo experiments. First, in order to examine whether CCCL matrices alone can be vascularized in vivo CCCL matrices were prepared without islets and transplanted subcutaneously in naïve BALB/c mice. Weekly up to 28 d these grafts were retrieved and examined for evidence of vascularization. Second, C, CC, and CCCL embedded NPI were cultured for 24 h with supplemented HAM'S F10 culture media to ensure no excess crosslinker remained in the matrices that could cause a negative reaction upon transplantation. These matrices were then transplanted subcutaneously in naïve immunoincompetent B6.Rag<sup>-/-</sup> mice. Pre-transplantation, at 4 d, and then weekly up to 28 d these grafts were retrieved and assessed for gross morphology, evidence of vascularization and islet survival. For histological assessment, retrieved collagen-based matrices were fixed in Shandon Zinc Formal-Fixx (Thermo Fisher Scientific, 6764255) then embedded in paraffin and 5 µm sections were prepared. To assess vascularization, von Willebrand Factor (vWF) staining was utilized to visualize arterioles and capillaries and islet survival, and sections of the grafts were also stained

for insulin, hemotoxylin and eosin. After rehydration, antigen retrieval for vWF was performed in sodium citrate buffer (pH 6.0). All immunohistochemical samples were blocked with 20% normal goat serum for 20 min (NGS, Jackson ImmunoResearch Laboratories Inc., 0005-000-121). Slides were also stained with a guinea pig anti-insulin antibody (Dako, A564) diluted at 1:1000; a mouse anti-glucagon antibody (Sigma G2654) or a rabbit anti-vWF antibody (abcam, ab6994) diluted at 1:500. Slides were visualized with an Axioscope II microscope equipped with an AxioCam MRC and analyzed with Axiovision 4.6 software (Carl Zeiss, Gottingen, Germany). To quantify the degree of vascularization five images of vWF staining were taken from each of two retrieved matrices and the images were combined for particle analysis in ImageJ (National Institutes of Health). Particles were counted if they were between 50 and 10000 µm<sup>2</sup> in size, with a circularity greater than 0.2. Each particle was confirmed to be a capillary visually and excluded by hand if mistakenly included by the software.

#### Statistical analysis

All statistics were performed using the Kruskal-Wallis 1-way analysis of variance with the Šidák correction; the level of significance was considered to be  $\alpha = 0.05$ . All statistical analyses were performed with STATA 11 (StataCorp LP). Results are presented as mean  $\pm$  SEM.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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