

# Reversal of insulin-dependent diabetes using islets generated *in vitro* from pancreatic stem cells

VIJAYAKUMAR K. RAMIYA<sup>1</sup>, MICHAEL MARAIST<sup>1</sup>, KARL E. ARFORS<sup>2</sup>, DESMOND A. SCHATZ<sup>3</sup>,  
AMMON B. PECK<sup>1,4</sup> & JANET G. CORNELIUS<sup>4</sup>

<sup>1</sup>Ixion Biotechnology, 13709 Progress Blvd., Box 13, Alachua, Florida 32615, USA

<sup>2</sup>Q-Med of Scandinavia, San Diego, California, USA

<sup>3</sup>Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida 32610, USA

<sup>4</sup>Department of Pathology, Immunology & Laboratory Medicine, University of Florida College of Medicine, Gainesville, Florida 32610, USA

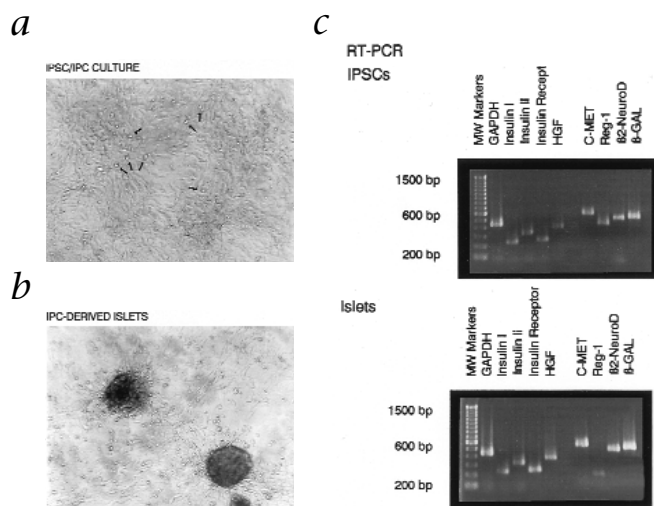
Correspondence should be addressed to A.B.P.; email: peck.pathology@mail.health.ufl.edu

**Ductal structures of the adult pancreas contain stem cells that differentiate into islets of Langerhans. Here, we grew pancreatic ductal epithelial cells isolated from prediabetic adult non-obese diabetic mice in long-term cultures, where they were induced to produce functioning islets containing  $\alpha$ ,  $\beta$  and  $\delta$  cells. These *in vitro*-generated islets showed temporal changes in mRNA transcripts for islet cell-associated differentiation markers, responded *in vitro* to glucose challenge, and reversed insulin-dependent diabetes after being implanted into diabetic non-obese diabetic mice. The ability to control growth and differentiation of islet stem cells provides an abundant islet source for  $\beta$ -cell reconstitution in type I diabetes.**

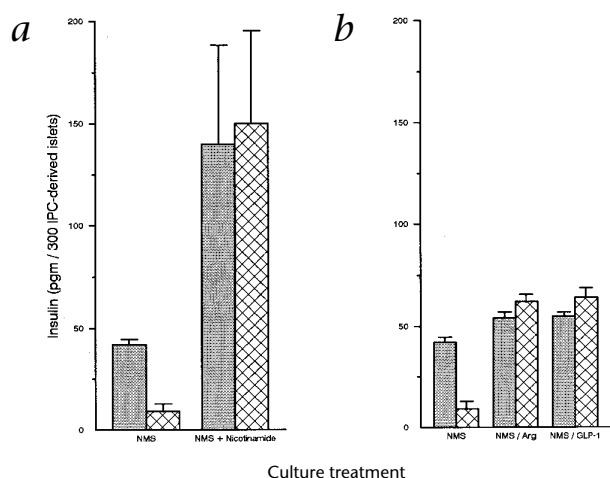
Islets of Langerhans have a cellular organization ideal for rapid, yet finely controlled, responses to changes in blood glucose levels<sup>1–6</sup>. During embryogenesis, islet development seems to be initiated from stem cells associated with the pancreatic ductal epithelium that differentiate into the various islet-associated endocrine cell populations<sup>7–11</sup>. Thus, it is generally accepted that all endocrine cell types of the pancreatic islets (glucagon-producing  $\alpha$  cells, insulin-producing  $\beta$  cells, pancreatic polypeptide-producing  $\gamma$  cells, and somatostatin-producing  $\delta$  cells) arise from the same ductal epithelial stem cell through sequential differentiation<sup>12–16</sup>. Pancreatic islets are organized into spheroid structures in which  $\beta$  cells form a core surrounded by a mantle of  $\alpha$  (or  $\gamma$ ) cells and interdigitating  $\delta$  cells<sup>6,12,17,18</sup>. Immature spheroid islet structures bud from the ductal epithelium and migrate short distances into the sur-

rounding acinar tissue, where angiogenesis occurs, permitting direct arteriolar blood flow to mature islets<sup>19–21</sup>. Unfortunately, the cellular organization of islets is destroyed in autoimmune diabetes, a disease in which a progressive autoimmune reaction results in the selective destruction of the insulin-producing  $\beta$  cells<sup>17,22–24</sup>. Thus, reversal of type I, insulin-dependent diabetes requires successful transplantation of whole pancreas or pancreatic islets.

Recently, attention has focused on the possible use of controlled differentiation of stem cells to obtain specialized cells useful in treating many diseases<sup>25</sup>. Pluripotent epidermal<sup>26–28</sup>, hematopoietic<sup>29</sup>, pancreatic islet<sup>30</sup> and mesenchymal<sup>31</sup> stem cell populations capable of both self-renewal and differentiation along multiple pathways of descent have been isolated from fetal and/or adult tissues and propagated *in vitro*. Here, we show how islets generated *in vitro* from pluripotent stem cells isolated from the pancreatic ducts of adult prediabetic non-obese diabetic (NOD) mice differentiate to form glucose-responsive islets that can reverse insulin-dependent diabetes after being implanted, with or without encapsulation, into diabetic NOD mice.



**Fig. 1** Cultures of epithelioid IPSCs, IPCs and IPC-derived islets. Single-cell suspensions of adult pancreatic ducts were cultured in glucose-depleted medium. After foci of epithelioid cells appeared, the cultures were 're-fed' every 5–6 d with medium containing 2.5 mM glucose and 0.5% normal mouse serum. Epithelial cells gave rise to small, rounded cells (IPCs; arrows in **a**), which underwent rapid proliferation to form organized cell clusters (IPC-derived islets; **b**). Relatively mature IPC-derived islets appear as smooth spheroids (100–150  $\mu$ m in diameter) composed of tightly-clustered cells. **c**, RNA from IPSCs/IPC or IPC-derived islets was used to generate islet-associated differentiation marker mRNA profiles by RT-PCR. The specificity for each amplified PCR product was determined by Southern blot analyses, with hybridization to internal sequence probes specific for each marker (above blots). Left margin, molecular weight (MW) marker sizes.



**Fig. 2** Increased production and release of insulin by nicotinamide and other secretagogues. IPC-derived islets were cultured for 5 d with or without 10 mM nicotinamide (**a**) or were stimulated for 3 h on day 5 with 10 mM arginine (Arg) or 1 mM glucagon-like peptide-1 (GLP-1) (**b**). Insulin levels (pgm, picogram) in the supernatants and cell extracts after glucose stimulation were measured by ELISA: shaded bars, intracellular; cross-hatched bars, secreted. NMS, normal mouse serum.

### *In vitro* growth of islets in stem cell cultures

We established cultures of islet-producing stem cells (IPSCs) from digested pancreatic tissue freshly explanted from prediabetic NOD/Uf mice, as described<sup>30</sup>. Single-cell suspensions of tissue digests cultured in Earle's high-amino-acid medium with normal mouse serum produced a monolayer of epithelioid-like IPSCs from which islet progenitor cells (IPCs) budded (Fig. 1a). These IPCs proliferated into well-organized islet-like structures (Fig. 1b) that generally grew to a constant diameter (100–150  $\mu\text{m}$ ) with the center containing differentiating cells that stained weakly for insulin or insulin plus glucagon, and more differentiated cells staining strongly for glucagon apparent at the periphery. Many of the cells expressed both glucagon and insulin simultaneously, a phenotype reported for immature islet cells on their path to end-stage differentiation<sup>18</sup>, indicating that these IPC-derived islets remain immature in the absence of further manipulations. IPSCs have been maintained in long-term cultures (more than 3 years) by serial transfer (usually through 1:2 'splits' in flasks 25  $\text{cm}^2$  in area), retaining their ability to produce islets in more than 200 culture flasks. The number of IPC-derived islets obtained per culture in flasks 25  $\text{cm}^2$  in area has ranged from 1,400 to 2,700 per collection, and cells have been collected from each flask many times, resulting in nearly an increase of 10,000-fold in the number of available islets per pancreas. However, rapid serial passages can result in the selection of proliferative, non-productive lines. Aliquots from different passages containing IPSCs/IPCs have been shown to survive storage at  $-80^\circ\text{C}$ .

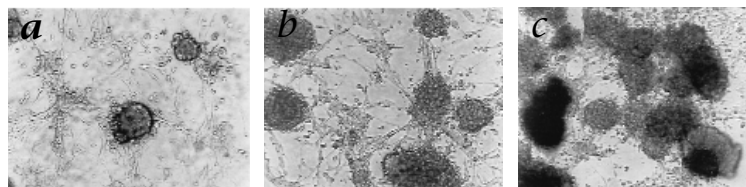
### Characteristics of IPSCs and *in vitro*-grown islets

We determined the expression of endocrine hormones and islet cell-associated differentiation markers by IPSCs and IPC-derived islets by detecting mRNA transcripts after RT-PCR. We detected transcripts for insulin I, insulin II, insulin receptor, and hepatocyte growth factor and its receptor, C-MET (Fig. 1c). In addition, both cell populations expressed glucagon, so-

matostatin, glucose transporter-2 receptor, glutamic acid decarboxylase-67 (but not -65) and insulin-like growth factors -I and -II (data not shown). We also analyzed expression of mRNA transcripts of several genes related to islet development and differentiation, including regenerating gene-1, insulin-promoting factor-1 (PDX-1),  $\beta$ -galactosidase, tyrosine hydroxylase and beta2/neuroD. IPSCs/IPCs expressed larger amounts of insulin-promoting factor-1 and tyrosine hydroxylase mRNA transcripts than did IPC-derived islets, whereas amounts of regenerating gene-1,  $\beta$ -galactosidase and beta2/neuroD were equivalent in both populations. Other factors expressed by IPSC/IPC lines included paired box genes 4 and 6, insulin-related protein-1 and Nkx6.1 (Drosophila NK transcription factor-related, gene family 6, locus 1), whereas neither IPSC/IPC nor islet cell populations expressed transcripts of Nkx2.2 or the hematopoietic stem cell markers erythropoietin and CD34 (data not shown). These results indicate the subtle changes that occur during the formation of IPC-derived islets from their epithelioid progenitors. As individual IPC-derived islets can dissociate into single cells capable of giving rise to more islets, expression of some precursor markers, such as  $\beta$ -galactosidase, by the islet clusters is not unexpected.

### Glucose stimulation of insulin release

To determine if IPC-derived islets were capable of secreting insulin, we cultured 300 islets in Earle's high-amino-acid medium containing either 0.5% normal mouse serum alone or in combination with 10 mM nicotinamide. Nicotinamide is a poly (ADP-ribose) synthetase inhibitor known to differentiate and increase  $\beta$ -cell mass in cultured human fetal pancreatic cells<sup>32</sup>, as well as to protect  $\beta$  cells from desensitization induced by prolonged exposure to large amounts of glucose<sup>33</sup>. After a 5-day incubation, cells were washed and then stimulated with 17.5 mM glucose. Nicotinamide-treated islets had more insulin and secreted significantly more insulin than did cultures treated with glucose alone (Fig. 2a;  $P < 0.05$ ). Secretagogues such as arginine, which stimulates islet  $\beta$  cells through voltage-dependent calcium channels<sup>34</sup>, and glucagon-like peptide-1, which stimulates  $\beta$  cells through an increase in cAMP through the protein kinase A pathway<sup>35</sup>, in conjunction with 17.5 mM glucose, also induced insulin release from the IPC-derived islets, but to a lesser degree than nicotinamide (Fig. 2b). Nicotinamide, in combination with various growth factors, also induced the differentiation of IPCs to islets. The presence of increasing concentrations of nicotinamide in the presence of epidermal growth factor and hepatocyte growth factor resulted in a 500–1,000% increase in the numbers of islets produced per culture (Fig. 3b and e) compared with that in cultures not containing nicotinamide (Figs. 3a and 1b).



**Fig. 3** Increased differentiation of IPCs to islets in the presence of specific growth factors. IPSC/IPC cultures were established and permitted to differentiate, which, in normal conditions, leads to 50–75 islets/ $\text{mm}^2$  (**a**). Increased islet formation occurred when the IPSC/IPC cultures were treated with a combination of 10 ng/ml epidermal growth factor, 10 ng/ml hepatocyte growth factor and nicotinamide at a concentration of 5 mM (**b**) or 10 mM (**c**).



**Fig. 4** Anatomical and histological characteristics of the kidney subcapsular region of IPC-derived islet implantation. **a**, Distention of the kidney capsule (right), showing the site of the IPC-derived islet implant. Top left, injection site. **b**, Histological section of the implant site, showing the general loss of islet structure and the formation of a contiguous cell mass, although remnants of the islets are visible. The implant site shows intense punctate staining with antibodies against insulin.

These results indicate the potential of growth factors to regulate differentiation and maturation of IPC-derived islets.

#### Reversal of diabetes after implantation of *in vitro*-grown islets

Human fetal pancreatic precursor cells in an *in vivo* environment develop the ability to secrete hormones<sup>36</sup>. This led us to investigate whether IPC-derived islets could be stimulated to differentiate into functional islets if placed in an *in vivo* environment. We maintained female, diabetic NOD mice ( $n = 8$ ) for at least 3 weeks on insulin, then implanted 300 IPC-derived islets into the subcapsular region of one kidney. Then, after 5 days, we weaned the mice from insulin injections. Within 1 week after being weaned from insulin, these implanted diabetic mice showed a decrease in blood glucose, from approximately 400 mg/dl to 180–220 mg/dl. We killed the implanted mice at various times (up to 55 days) after the implantation to assess the implant histologically. All implanted mice remained healthy and insulin-independent until being killed, whereas gross morphology of the implant site (Fig. 4a) showed single masses of endocrine cells that stained strongly with antibodies against insulin (Fig. 4b). In contrast, non-implanted control mice ( $n = 8$ ) had increasingly high levels of blood glucose (400–800 mg/dl), wasting syndrome and died prematurely from complications of diabetes. A comparison of blood glucose levels between an implanted and non-implanted mouse (Fig. 5a) was consistent with the idea that the implanted, IPC-derived islets could provide adequate insulin to maintain stable blood glucose levels over the time of the experiment.

Transplantation presents several problems, including immune rejection of the graft and finding a site suitable to accommodate adequate numbers of implanted islets. The subcapsular region of the kidney limits the size of the implanted graft even without immune-protective encapsulation. To address this, we maintained diabetic NOD mice ( $n = 3$ ) on daily insulin therapy and implanted 5,000 IPC-derived islets subcutaneously, either alone ( $n = 1$ ) or encapsulated ( $n = 2$ ) in an immunologically inactive cross-linked hyaluronic acid-based gel. Although we used this gel to protect the implanted islets from possible autoimmune reactivity, it is also well-suited for protection of allogeneic islets from an allograft response. Implanted mice were weaned from insulin 2 days after the implantation, but on day 26 after the implantation, one recipient of encapsulated islets became hypoglycemic, presumably from excessive insulin secretion by the islets, and died (Fig. 5b) when no glucose therapy was initiated. Blood glucose levels of the remaining two mice

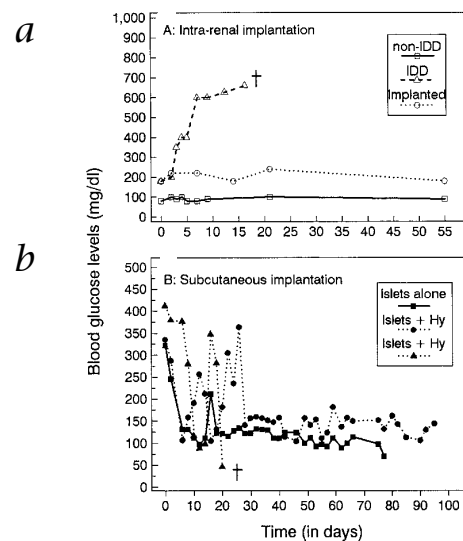
stabilized at near-normal levels (100–150 mg/dl) and remained normal for more than 3 months with no evidence of autoimmune graft rejection. Although the absence of autoimmune destruction of these implants indicates that *in vitro* generation of IPC-derived islets reduces antigenicity, the exact mechanism is still being investigated.

#### Islet induction of angiogenesis

Long-term survival of islets requires neovascularization of the graft in the host animal. The prolonged stabilization of blood glucose (for more than 3 months) in the two recipients of IPC-derived islets here indicates the potential of these clusters to induce angiogenesis. To visualize neovascularization, we placed four IPC-derived islets in a dorsal skin-fold chamber in an NOD-severe combined immunodeficiency mouse. One week later, we injected rhodamine-conjugated dextran intravenously into the mouse to allow us to visualize vascularization. A rich, newly formed glomerulus-like network of microvessels surrounding the islets had developed (Fig. 6). In addition, there was an increase in islet mass with the increased blood flow to the implanted islets.

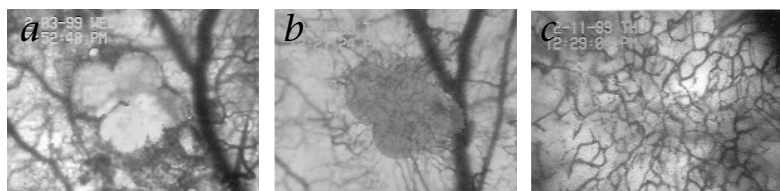
#### Discussion

Our work here has focused on the differentiation of pancreatic ductal epithelial IPSCs to functional IPC-derived islets, maturation of these islets to respond to glucose challenge, and the implantation of these functional islets to reverse diabetes in NOD mice. It is evident from our experiments that *in vitro* neo-



**Fig. 5** Reversal of insulin dependent diabetes in NOD mice with subcapsular kidney or subcutaneous implantation of IPC-derived islets. **a**, Approximately 300 IPC-derived islets from IPSC cultures that had been producing islets for 6–8 months were injected into the kidney subcapsular regions of eight female diabetic NOD/Uf mice. Animals were weaned from insulin over 5–7 d, and blood glucose levels were determined. Data represent mice monitored for the 55 days of the experiment. Control diabetic female mice did not receive implants but were weaned from insulin using the same protocol ( $-\Delta-$ ). **b**, Approximately 5,000 IPC-derived islets from IPSC cultures that had been producing islets for 18–21 months were implanted subcutaneously into three female diabetic NOD/Uf mice. In two mice, hyaluronic acid-based gel (Hy) was first injected into the pocket, followed by the islet clusters, to encapsulate the implants. Then, 2 d after the implantation, the animals were weaned from insulin. Blood glucose levels were determined at the same time every second day throughout the experiment.

**Fig. 6** Induction of angiogenesis *in vivo* by IPC-derived islets. Four IPC-derived islets were placed in a dorsal skinfold chamber on a NOD-severe combined immunodeficiency mouse and the skinfold was attached to the stage of an intravital microscope. At time 0 (**a**) and day 8 (**b**), rhodamine-conjugated dextran was injected intravenously into the mouse to obtain enhanced contrast. **c**, Magnification of the implanted islets on day 8 shows the extent of micro-vascularization.



genesis of islets from isolated pluripotent progenitor cells is possible. Despite our ability to induce IPC-derived islets to secrete detectable levels of insulin *in vitro* in the presence of specific factors, it is essential to understand what the *in vivo* environment provides to allow these islets to further mature/differentiate into fully functional islets with maximal insulin production capacity. Several growth factors are mitogenic to ductal epithelial cells that give rise to the islet endocrine cells. These include vascular endothelial growth factor<sup>37</sup>, hepatocyte growth factor<sup>38,39</sup>, regenerating gene-1 (ref. 40), transforming growth factor- $\alpha$  (ref. 41) and islet neogenesis-associated protein<sup>42</sup>. In addition, factors such as hepatocyte growth factor<sup>43</sup>, beta-cellulin and activin A (ref. 44) differentiate acinar cells into insulin-secreting cells. Creating an *in vitro* environment using combinations of these and other factors will enhance even more the potential of generating increasing numbers of transplantable, IPC-derived islets. Although much emphasis has been placed on the importance of  $\beta$  cells, hormones produced by other endocrine cells of the islet are also essential for strict glucose homeostasis. Thus, the presence of all islet cell types within the *in vitro*-generated islets represents an ideal situation.

This IPSC/IPC technology has the potential to circumvent the need for fetal, allogeneic or xenogeneic tissue for transplantation of  $\beta$  cells into insulin-dependent diabetic patients. Furthermore, it could provide a source for generating functionally useful islets easily manipulated or genetically engineered. Perhaps most unexpected, however, is that *in vitro*-generated syngeneic islets were not rejected by the autoimmune hosts even in the absence of protective encapsulation. Understanding the mechanism underlying this phenomenon could prove most important, especially if the implantation of *in vitro*-generated islets results in peripheral tolerance. On the other hand, an encapsulating agent, such as the gel we used here, may provide for reduced antigenicity for freshly explanted or even *in vitro*-generated allogeneic islets, alleviating the current requirements for intensive immunosuppressive drug therapy after islet implantation.

## Methods

***In vitro* growth of islets from stem cells.** Adult islet ductal structures were individually isolated from a collagenase (Sigma) digest and cultured in T-25 flasks (Corning Costar, Cambridge, Massachusetts) in glucose-depleted Earle's high-amino-acid medium supplemented with 2% normal mouse serum until foci of epithelioid cells formed, usually within 4–5 weeks. These cells were then 're-fed' every 5–6 d with Earle's high-amino-acid medium containing 2.5 mM glucose and normal mouse serum at a final concentration of 0.5%, resulting in a confluent layer of epithelioid cells (IPSCs) from which IPCs budded. The IPCs underwent rapid proliferation with daughter cells remaining aggregated, thereby forming organized cell clusters (IPC-derived islets).

Detection of gene expression by RT-PCR. Total RNA was prepared from cultures of either IPSCs/IPC or IPC-derived islets using Trizol<sup>TM</sup> (Life Technologies). MAPPING (message amplification phenotyping) of the mRNA profiles for IPSC/IPC cultures or IPC-derived islets using RT-PCR was

done according to published protocols<sup>45</sup>. Each primer set was designed based on sequences of open reading frames obtained from GENBANK and synthesized by Life Technologies. PCR products were separated by electrophoresis in 1.2% agarose gels and transferred to nylon membranes for Southern blot analysis with UV cross-linking. The specificity of each amplicon was determined by hybridization with a digoxigenin-labeled internal sequence probe, then detected using the Genius colorimetric detection system (Roche Molecular Biochemicals, Indianapolis, Indiana). mRNA expression for specific factors during differentiation was quantified by estimating the densities of bands of the PCR products for IPSC/IPC cultures and those of IPC-derived islet cultures relative to the density of the band for glyceraldehyde phosphodehydrogenase.

**Measurement of insulin protein by enzyme-linked immunosorbent assay (ELISA).** Insulin secretion by and the insulin content of IPC-derived clusters were determined by ELISA (Crystal Chemical, Chicago, Illinois). Cell cultures, washed twice with Krebs' Ringer's buffer, were incubated 3 h at 37 °C in 17.5 mM glucose. Culture supernatants were collected and stored at -70 °C until being assayed for the presence of insulin. The remaining islets were then subjected to an overnight acid-ethanol extraction at 4 °C. The cell-free extracts were collected, neutralized with 0.4 M Tris base and stored at -70 °C until being assayed for insulin. Insulin content in supernatants and cell extracts was determined by ELISA, using rat insulin as the standard for quantification. Determinations used a minimum of four cultures. Comparison between groups was done using a one-tailed *t*-test.

**Islet implantations.** All animal studies were in accordance with University of Florida institutional guidelines. Female diabetic NOD/Uf (the University of Florida substrain of the NOD mouse) mice maintained on daily insulin injections for 4 weeks were anesthetized using metofane (Pitman-Moore, Mundelin, Illinois). The left kidney was exposed through a small incision in the flank. A small channel was made under the kidney capsule with a 27-gauge needle and approximately 300 IPC-derived islets were injected into the subcapsular region through a small catheter. The opening in the kidney capsule was cauterized and the skin incision was closed with surgical clips. Animals received a full dose of insulin daily for 3–4 d after the implantation, then a half-dose of insulin daily for an additional 2 d before being completely weaned from further insulin injections. Control diabetic female mice did not receive implants but were weaned from insulin using the same protocol. Alternatively, female diabetic NOD/Uf mice were anesthetized with metofane and a small incision was made in the skin of the shoulder. Using a hemostat, a pocket was opened subcutaneously, into which an estimated 5,000 IPC-derived islets were implanted in 20  $\mu$ l of HBSS without encapsulation. To encapsulate islets, 100  $\mu$ l of the hyaluronic acid-based gel Restalyne<sup>TM</sup> (provided by Q-Med Sweden, Uppsala, Sweden) was first injected into the pocket, followed by injection of 20  $\mu$ l of islet cluster suspension into the gel. The pocket was closed with surgical clips. Two days after implantation, the animals were weaned from insulin. Blood glucose levels were determined at the same time every second day using an AccuChek-EZ glucose monitor (Roche Molecular Biochemicals, Indianapolis, Indiana).

**Intravital microscopy to observe angiogenesis.** IPC-derived islets were placed in a dorsal skinfold chamber on individual NOD-severe combined immunodeficiency mice, as described<sup>21</sup>. Each mouse was immobilized in a plexiglass tube, without anesthesia, and the skinfold attached to the microscope stage. Intravital microscopy used a Leitz Ploemopak epi-illuminator equipped with I2 and N2 filter blocks and video-triggered stroboscopic illumination from a xenon arc (Strobex 236; Chadwick Helmuth, Mountain

View, California). At appropriate times after implantation, 0.1 ml rhodamine-conjugated dextran 500,000 (Sigma) was injected intravenously into the mouse to obtain enhanced contrast. Samples were viewed using either Nikon X4 (numerical aperture (NA), 0.10), Nikon X10 (NA, 0.30) or Leitz X25 (NA, 0.60) objectives. A silicon intensified target camera (SIT68; Dage-MTI, Michigan City, Indiana) was attached to the microscope and connected to a monitor (Panasonic TR-930).

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