

Markedly Decreased Blood Perfusion of Pancreatic Islets Transplanted Intraportally Into the Liver

Disruption of Islet Integrity Necessary for Islet Revascularization

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Experimental studies indicate low revascularization of intraportally transplanted islets. This study aimed to quantify, for the first time, the blood perfusion of intrahepatically transplanted islets and elucidate necessary factors for proper islet graft revascularization at this site. Yellow chameleon protein 3.0 islets expressing fluorescent protein in all cells were transplanted. Graft blood perfusion was determined by microspheres. The vascular density and relative contribution of donor blood vessels in revascularization was evaluated using islets expressing green fluorescent protein under the Tie-2 promoter. Blood perfusion of intrahepatic islets was as a mean only 5% of that of native islets at 1-month posttransplantation. However, there was a marked heterogeneity where blood perfusion was less decreased in islets transplanted without prior culture and in many cases restored in islets with disrupted integrity. Analysis of vascular density showed that distorted islets were well revascularized, whereas islets still intact at 1-month posttransplantation were almost avascular. Few donor endothelial cells were observed in the new islet vasculature. The very low blood perfusion of intraportally transplanted islets is likely to predispose for ischemia and hamper islet function. Since donor endothelial cells do not expand posttransplantation, disruption of islet integrity is necessary for revascularization to occur by recipient blood vessels. *Diabetes* 61:665–673, 2012

Transplantation of pancreatic islets has, in recent years, become a treatment for selected patients with type 1 diabetes (1). However, at least two donor pancreata are needed to reverse hyperglycemia, which is far more than the alleged 10–20% of the total islet volume suggested to be enough to maintain normoglycemia in humans (2). Moreover, in contrast to whole-organ transplantation, there seems to be a progressive decline in function of islet transplants (3). Several factors may contribute to the decline in islet graft function, including implantation of a suboptimal islet mass

due to instant cell death (4), lipotoxicity (5), and insufficient vascular engraftment (6) and amyloid deposition (7).

Native pancreatic islets are richly vascularized (8). The high blood perfusion of islets is important for β -cells to maintain their oxygen-dependent nutrient metabolism and insulin release at different demands (9,10). The rich vascularization and high blood perfusion also facilitate glucose sensing by the β -cells, distribution of islet hormones to their target organs, and paracrine interactions between islet endothelial and endocrine cells (11–14).

Intraportally transplanted islets embolize the liver and may cause local hepatic necrosis or infarction in their surroundings, further limiting their oxygen supply besides their being avascular early after transplantation (15). Despite being implanted intraportally, the islets are then revascularized by branches of the hepatic artery (16,17). We previously have reported decreased vascular density of intraportally transplanted mouse and human islets when compared with native islets (18,19). To determine the functional consequences of this on islet blood perfusion, in the current study, we combined fluorescent islets for transplantation with a fluorescent microsphere technique. This new method enabled us, for the first time, to measure islet graft blood perfusion, which could be quantified without histological sectioning. The vascular density and relative contribution of donor blood vessels in the revascularization of islets of different size, shape, and location in the liver were evaluated in parallel using islets expressing green fluorescent protein (GFP) under the Tie-2 promoter for transplantation.

RESEARCH DESIGN AND METHODS

Adult, male C57Bl/6 nu/nu mice were purchased from Taconic (Ry, Denmark), whereas transgenic YC-3.0 mice (20) were donated by Roger Tsien (University of California, San Diego, CA). Transgenic YC-3.0 mice express the yellow chameleon protein 3.0 (YC-3.0) under the regulation of the β -actin and cytomegalovirus promoters. Transgenic Tie-2 GFP mice expressing GFP under the Tie-2 promoter were purchased from The Jackson Laboratory (Bar Harbor, ME). The animal experiments were approved by the Swedish laboratory animal ethical committees in Uppsala and Stockholm.

Islet isolation and culture. Pancreatic islets of adult male YC-3.0 or Tie-2 GFP mice were isolated using collagenase digestion and density gradient purification. Under deep anesthesia with sodium pentobarbital (200 mg/kg i.p.), a laparotomy was performed and the pancreas exposed. Collagenase A (1.5 mg; from *Clostridium histolyticum*; Roche Diagnostics, Mannheim, Germany) dissolved in 4 mL Hanks' buffer (National Bacteriological Laboratory, Stockholm, Sweden) was injected into the pancreas via the common bile duct. The animal was killed and the pancreas dissected from surrounding tissues, removed, and incubated in a water bath for ~12 min at 37°C. Intact islets were separated by a density gradient (Histopaque-1077) centrifugation at 900g for 22 min. Fragmentation of islets was performed by vigorous shaking of the tissue for

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20 min in a water bath at 37°C. After washing, islets were handpicked and then transplanted after overnight incubation or cultured free floating at 37°C (95% air and 5% CO₂) in groups of 150 for 4 days before transplantation (21). The culture medium was changed every 2nd day.

Islet characterization. Groups of islets for transplantation were characterized for exocrine contamination by dithizone staining and functionally for glucose-stimulated insulin release in duplicate batch-type experiments (22). Duplicate samples of overnight-incubated 60 intact or fragmented islets were investigated for release of matrix metalloproteinase (MMP)-9 and vascular endothelial growth factor (VEGF)-A during 4 h in 1 mL Krebs-Ringer bicarbonate HEPES buffer supplemented with 5.6 mmol/L glucose at 37°C (95% air and 5% CO₂). Contents of insulin, MMP-9, and VEGF-A were measured by a mouse insulin ELISA (Mercodia, Uppsala, Sweden) and mouse VEGF and total MMP-9 ELISAs (R&D Systems, Abingdon, U.K.), respectively. Duplicate samples of overnight-incubated or 4-day-cultured islets were stained with 20 µg/mL propidium iodide (PI; Sigma-Aldrich) and 10 µg/mL bisbenzimidazole (Hoechst) for 30 min at 37°C followed by fluorescence microscopy. Fluorescent images were analyzed with IMARIS 7.1.1 (Bitplane, South Windsor, CT).

Islet gene expression. After overnight or 4 days of culture, isolation of total RNA from duplicate samples of Tie-2 GFP islets was performed with RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). All of the samples were run through gDNA Eliminator spin columns to eliminate genomic DNA (Qiagen). Conversion of RNA to cDNA was performed with a reverse-transcription system (Promega Biotech, Stockholm, Sweden) using Oligo (dT)₁₅ primers. We used custom-made TaqMan Array Micro Fluidic Cards with Applied Biosystems 7900HT for profiling gene expression using the comparative cycle threshold (C_T) method of relative quantification. A total of 96 genes were run and analyzed according to the manufacturer's instructions (18S rRNA as housekeeping gene) (Supplementary Data 1).

Islet transplantation. Male C57Bl/6 nu/nu mice were used as recipients. Islets were transplanted selectively into the right liver lobe. In brief, ~200 intact islets, or 200 islets with disrupted integrity obtained by prolonged collagenase treatment the day before transplantation, were packed in a butterfly needle (25G) in a volume of 0.1–0.2 mL. The mice were anesthetized with intraperitoneal administration of 0.02 mL/g body wt Avertin (2.5% v/v solution of 10 g 97% v/v 2,2,2-tribromo-ethanol [Sigma-Aldrich] in 10 mL 2-methyl-2-butanol [BDH Merck Ltd., Poole, U.K.]). An abdominal incision was made and the islets were infused into the portal vein. The left portal vein was temporarily clamped during the infusion of the islets.

Blood flow measurements. Control YC-3.0 mice or nude mice with 1-month-old islet grafts were anesthetized with Avertin (see above). Polyethylene catheters were inserted into the ascending aorta via the left carotid artery and into the left femoral artery. The carotid catheter was connected to a pressure transducer. Body temperature was maintained at 37–38°C.

Regional blood flow measurements were performed as previously described (10), although using fluorescent (10 µm; FluoSpheres; Invitrogen, Eugene, OR) instead of black microspheres. The animals were killed and graft-bearing liver lobes, pancreas, and adrenal glands were removed. The tissues were then placed between two object slides before counting the microspheres in a fluorescence microscope (Nikon Eclipse, TE2000-U). The pancreas was treated with a freeze-thawing technique to visualize the islets before study (23). The blood flow to the endocrine pancreas could thereby be evaluated separately.

All islets identified in graft-bearing livers were photographed, and presence of microspheres within the islet tissue or within a radius of 100 µm from an islet was counted, respectively. Also, all microspheres in the arterial blood reference sample were counted. Islet tissue that was spheroid-, elliptic- or disc-shaped with round boundaries was by analysis classified as intact islets, whereas irregularly shaped islets with uneven boundaries were classified as fragmented/disrupted islets. Blood flow values based on the microsphere content of the adrenal glands were used to confirm that the microspheres were adequately mixed in the circulation.

The fraction of islets within the pancreas was estimated by a point-counting method, where the number of intersections overlapping islets was counted in a light microscope. The total islet weight was estimated by multiplying the pancreatic weight with the islet volume fraction of the whole pancreas. The volume of the transplanted islets was estimated by measuring two diameters perpendicular to each other of the photographed islets. A mean value of the two diameters was used to calculate a volume of an estimated sphere. The photographed transplanted islets were analyzed with ImageJ 1.37v (Wayne Rasband, National Institutes of Health).

Immunohistochemistry. Control Tie-2 GFP mice or nude mice with 1-month-old Tie-2 GFP islet grafts were killed, and control pancreas or islet grafts were removed, fixed in 4% paraformaldehyde, and prepared for immunohistochemistry of cryosections. Sections were incubated for 1 h with a cocktail of primary antibodies; polyclonal guinea pig anti-insulin antibody (DAKO) at 1:50 dilution and a monoclonal rat anti-mouse CD31 antibody (BD Biosciences Pharmingen, San Diego, CA) at 1:300 dilution. Secondary antibodies, anti-guinea

pig Alexa Fluor 633 and anti-rat Alexa Fluor 555 (Molecular Probes), were applied for 20 min at 1:200 dilutions. The sections were mounted with coverslips using ProLong Gold Antifade reagent with DAPI (Molecular Probes).

Analysis of vascular density. The control pancreas and islet graft sections were scanned with a Laser Scanning Microscope ZEISS LSM510-Meta. All shown fluorescence images have been subjected to a median filter and changes in brightness and contrast for optimal visualization.

The scanned images were analyzed with IMARIS 7.0.0 (Bitplane). The vascular density was defined as an area of stained CD31 blood vessels per islet or liver tissue area. Likewise, the Tie-2 GFP vascular density was calculated using the area from native GFP fluorescence divided by islet or surrounding liver tissue area. The ratio of Tie-2 GFP vascular density and CD31 vascular density in islets was used to estimate the fractional contribution of donor endothelial cells in the islet graft vascular density. Islet tissue that was spheroid-, elliptic-, or disc-shaped with round boundaries was by analysis of consecutive sections classified as intact islets, whereas irregularly shaped islets with uneven boundaries were classified as fragmented/disrupted islets.

Assessment of hepatic steatosis. Oil Red O staining was performed on cryosections to evaluate accumulation of fat droplets in hepatocytes close to transplanted islets and in the rest of the liver (5). As positive control, liver sections from 4-month high-fat diet-treated (D12492; Research Diets, New Brunswick, NJ) C57Bl/6 mice were used.

Statistical analysis. Values are expressed as means ± SEM. Student *t* test was used for comparisons between parametric data with two experimental groups, whereas multiple comparisons between parametric data were performed by using ANOVA and Bonferroni post hoc test. Nonparametric data were compared using Mann-Whitney *U* test for two experimental groups and nonparametric ANOVA with Dunn post hoc test for multiple comparisons. For all comparisons, *P* < 0.05 was considered statistically significant.

RESULTS

All animals allocated to measurements of blood perfusion or vascular density weighed ~30 g and were normoglycemic. Mean arterial blood pressure in the animals was ~70 mmHg.

Blood flow measurements. The blood perfusion of native islets was 3.5–4 mL · min⁻¹ · g islet tissue⁻¹ and did not differ between control YC-3.0 mice and transplanted C57Bl/6 nude mice. In contrast, the blood perfusion of the 1-month-old intrahepatic islet grafts was as a mean only ~0.2 mL · min⁻¹ · g islet tissue⁻¹ (Fig. 1A). However, islets transplanted after overnight incubation had several-fold higher blood perfusion than islets cultured for 4 days before transplantation at 1-month follow-up (Fig. 1B). A marked heterogeneity existed between individual transplanted islets in an animal. Noteworthy, the blood perfusion of still-intact islets was in all cases beneath the detection level (i.e., such islets contained no microspheres), whereas some of the islets that had become disrupted and fragmented after transplantation had an even, fully restored blood perfusion (Fig. 1F). The arterial blood perfusion of liver parenchyma within a radius of 100 µm from the transplanted islets was ~3 mL · min⁻¹ · g islet tissue⁻¹ and thereby 10 times increased when compared with the rest of the liver (Fig. 1A). Islets transplanted after overnight incubation had higher arterial blood perfusion in the surrounding liver tissue at 1-month follow-up than corresponding islets transplanted after 4 days of culture (Fig. 1B). As a mean, 31 ± 4% (*n* = 10) of transplanted islets was identified and investigated in each animal.

Measurements of vascular density. Morphological assessment of graft-bearing livers showed that ~50% of transplanted islets were found within portal vein tributaries, whereas the rest had migrated deeper into the vessel walls at 1-month posttransplantation (Fig. 2). Despite the transplantation of seemingly intact islets, disrupted and fragmented islets were common and constituted 57% of the islet tissue, whereas the rest occurred as still-intact islets at 1-month posttransplantation. Both islets classified as

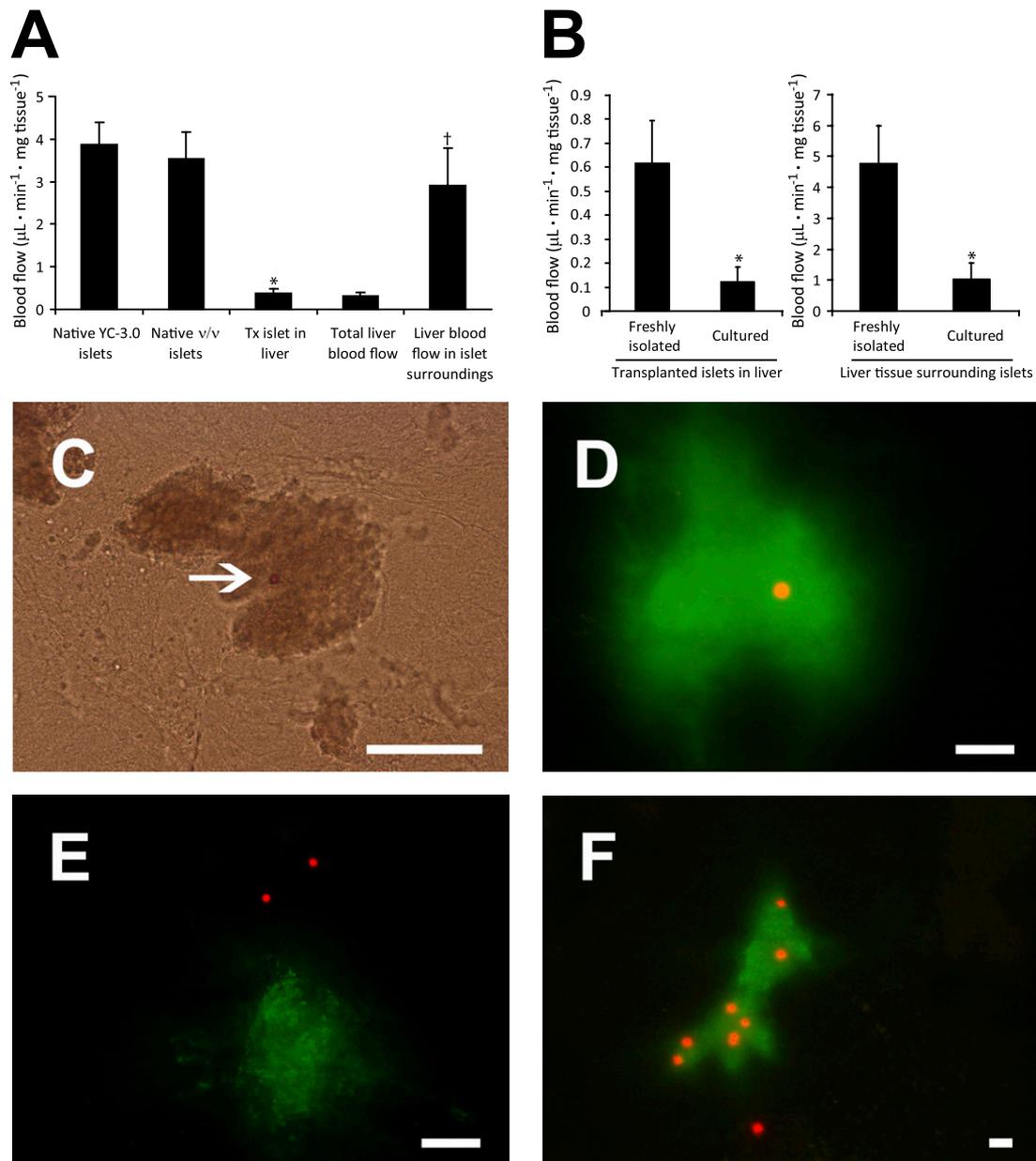


FIG. 1. Blood flow in native islets and islets 1 month after intraportal transplantation into the liver of mice. Calculated arterial blood flow values in native islets of YC-3.0 mice and graft-bearing nude mice and in transplanted (Tx) islets in the liver and their surroundings (A). * $P < 0.05$ compared with native YC-3.0 islets; † $P < 0.05$ compared with total liver artery blood flow. Subanalysis of blood flow values in intrahepatic islet grafts composed of freshly isolated islets or islets cultured for 4 days before transplantation (B). * $P < 0.05$ compared with corresponding group with freshly isolated islets. Images showing microsphere(s) in a native islet (C), in a transplanted islet (D), and in the surroundings of a transplanted islet (E). Blood flow in transplanted islets differed markedly between individual islets, where some distorted islets had high blood perfusion containing several microspheres (F). Data are means \pm SEM for 6 control YC-3.0 animals and 10 transplanted nude mice in A and 5 animals in each group in B. Scale bars in C–F are 100 μm . (A high-quality digital representation of this figure is available in the online issue.)

intact islets or with disrupted integrity at 1-month post-transplantation contained $\sim 80\%$ insulin-producing cells. The vascular density in the 1-month-old intrahepatically transplanted islets was as a mean 24% of that in native islets (Fig. 2A). Although mean values of vascular density were 70% higher in islets incubated only overnight before transplantation, there was no statistically significant difference when compared with islets cultured for 4 days before transplantation (Fig. 2A). A marked heterogeneity existed between individual transplanted islets. No differences in vascular density with regard to islet size or location were observed. However, whereas islets that remained intact were more or less avascular, disrupted and fragmented

islets contained as a mean 10 times more blood vessels (Fig. 2B). These new blood vessels were tortuous and similar to those observed in native islets. To discriminate between the importance of disruption of islet integrity for revascularization of transplanted islets and changes in islet structure induced by the revascularization procedure per se, we also transplanted islets with disrupted integrity. When compared with the normally intact islets for transplantation, these implanted fragments were much more revascularized (Fig. 2B). A large number of blood vessels were observed in the immediate surroundings of transplanted islets. Indeed, blood vessel numbers were increased within a radius of 100 μm from the transplanted

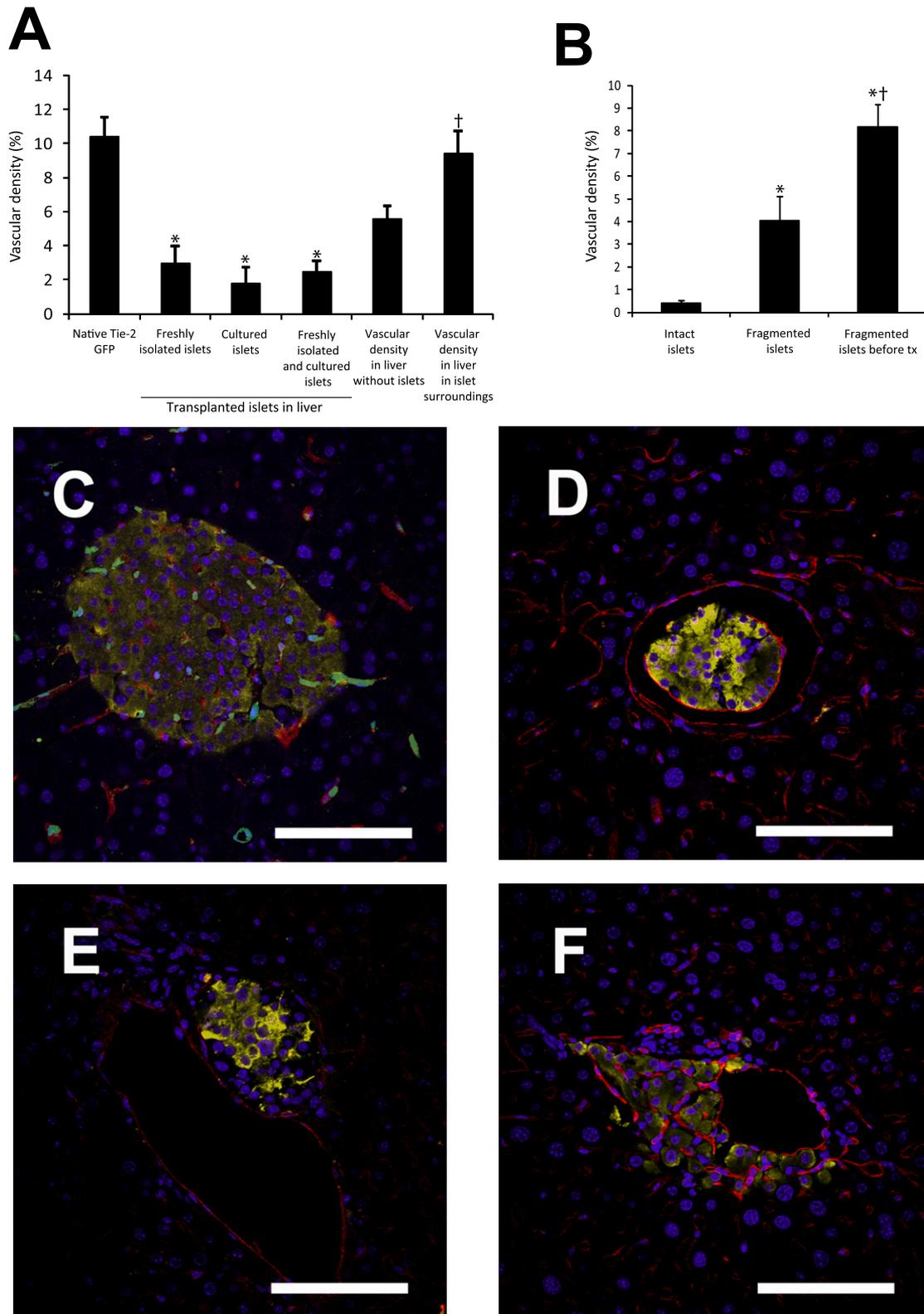


FIG. 2. Vascular density in native islets and islets 1 month after intraportal transplantation into the liver of mice. Calculated values of vascular density (CD31) in native Tie-2 GFP islets and in intrahepatic islets composed of freshly isolated islets and/or islets cultured for 4 days before transplantation, as well as the vascular density in livers within a radius of 100 μm of transplanted islets and in corresponding liver parts without islets (**A**). * $P < 0.05$ compared with native Tie-2 GFP islets; $\dagger P < 0.05$ compared with corresponding liver tissue without islets. Subanalysis of the vascular density in transplanted (Tx) islets still intact or subjected to fragmentation before or after transplantation (**B**). * $P < 0.05$ compared with intact islets; $\dagger P < 0.05$ compared with fragmented islets. Representative images showing vascular density (CD31, red) in a native islet (**C**) and in intact (**D** and **E**) or fragmented (**F**) transplanted islets. Tie-2 GFP positive cells (green) are richly present in native islets but scarce in transplanted islets. Sections are also stained for cell nuclei (DAPI, blue) and for insulin (yellow). Data are means \pm SEM for four to nine animals in each group in **A** and **B**. Scale bars in **C–F** are 100 μm . (A high-quality digital representation of this figure is available in the online issue.)

islets when compared with corresponding parts of liver without islets (Fig. 2A). Very few Tie-2 GFP positive endothelial cells (<10%) were observed in the intrahepatically transplanted islets, irrespective of whether the islets were incubated overnight or cultured for 4 days before transplantation (Fig. 3). Noteworthy, some Tie-2 GFP positive cells were also found to have migrated from the transplanted islets into the surrounding liver parenchyma and were of similar numbers ($\sim 0.13\%$ of tissue area) in the surroundings of transplanted freshly isolated and cultured islets (Fig. 3).

Characterization of islets for transplantation. Dithizone staining showed 95–100% purity of intact overnight-incubated and 4-day-cultured islets, whereas the purity of fragmented islets was slightly less (85–90%) (Supplementary Data 2). Insulin release from intact islets was similar 1 and 4 days after isolation, whereas the fragmentation caused increased insulin release both at low and high glucose, indicating leaking from β -cells damaged by the procedure (Supplementary Data 2). The release of VEGF-A and MMP-9 was approximately doubled from fragmented compared with intact islets 1 day after isolation (Fig. 4). A higher rate of cell death occurred in overnight-incubated intact islets when compared with corresponding 4-day-cultured islets (Fig. 5).

Angiogenesis gene array. The expression of 96 genes involved in angiogenesis was evaluated for overnight- and 4-day-cultured intact islets. The expression of 12 genes differed between these groups of islets, and most of the differences (9 of 12) favored blood vessel formation in freshly isolated islets (Fig. 6). Cadherin 5, endoglin, sphingosine-1-phosphate receptor 1, and platelet/endothelial cell adhesion molecule 1 (Pecam 1), which all mainly exert proangiogenic effects, were decreased in 4-day-cultured islets. Moreover, the antiangiogenic factors tissue inhibitor of metalloproteinase (TIMP)1, TIMP2, MMP-19, Ser (or Cys) peptidase inhibitor, clade F, member 1, and thrombospondin-2 were all increased in 4-day-cultured islets. In contrast, the proangiogenic factors fibroblast growth factor 1, hepatocyte growth factor, and MMP-2 increased during culture.

Liver lipid accumulation. No or very little lipid accumulation occurred in liver parts close to islets and in the rest of the liver (Supplementary Data 3).

DISCUSSION

In the current study, we developed a technique to quantify the blood perfusion of pancreatic islets transplanted intra-portal into the liver. By the combined use of fluorescent islets for transplantation and fluorescent microspheres for

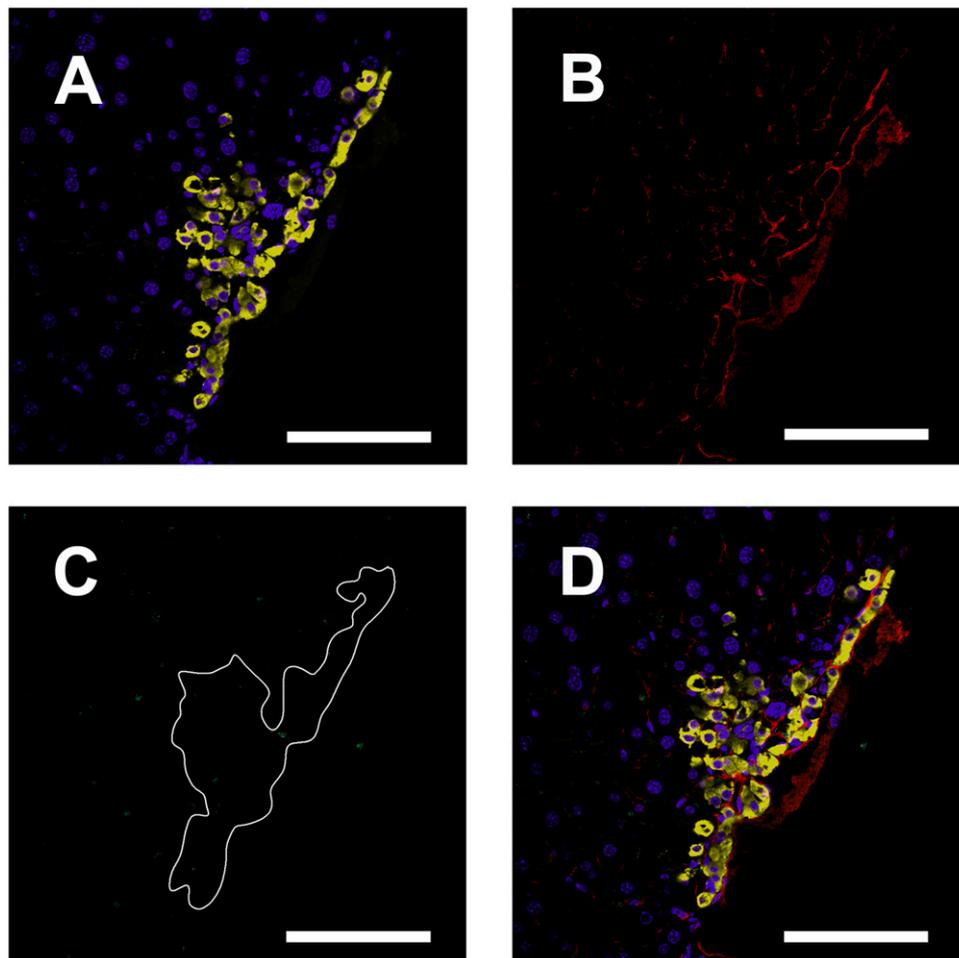


FIG. 3. Contribution of donor endothelial cells to the new vascularity within and in the surroundings of transplanted islets. Images of an intrahepatically transplanted Tie-2 GFP islet showing nuclei (DAPI, blue) and insulin (yellow) staining (A), CD31 (red) staining (B), and presence of Tie-2 GFP cells (green) reporting donor endothelial cells (C). Overlay image of A–C is shown in D. Scale bars are 100 μm . (A high-quality digital representation of this figure is available in the online issue.)

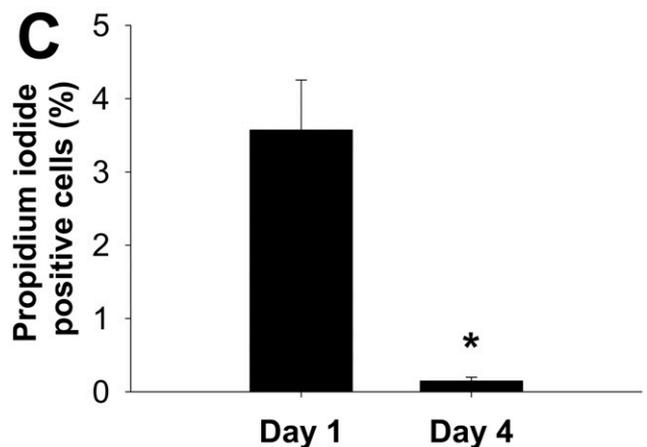
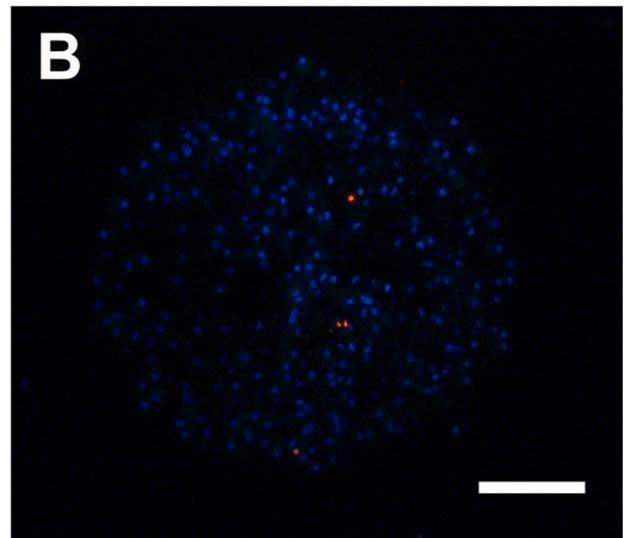
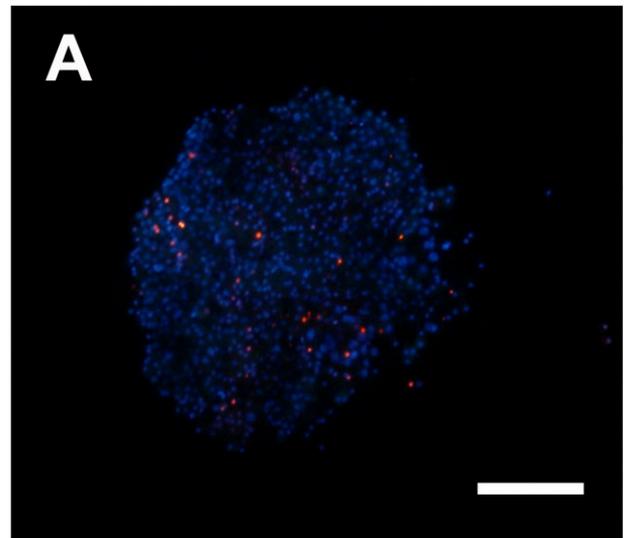
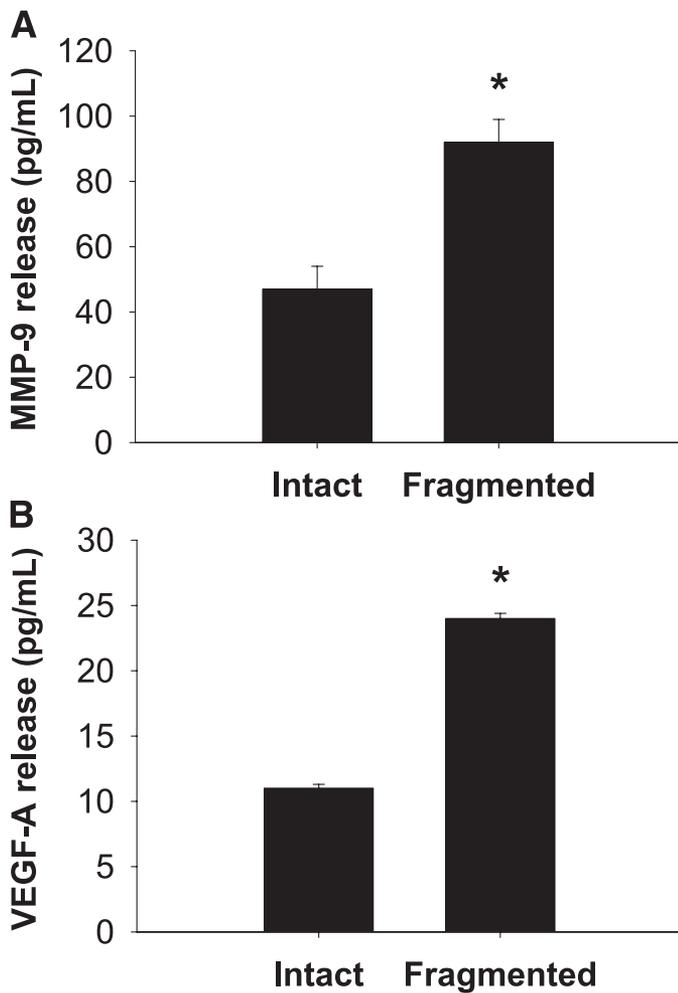


FIG. 5. Cell death in overnight-incubated and 4-day-cultured islets as assessed by PI staining. Representative images of PI staining (red) in overnight-incubated (A) and 4-day-cultured islets (B). Islets were also stained for cell nuclei (Hoechst; blue). Scale bars in A and B are 100 μ m. Calculated values of PI positive cells (C). Data are means \pm SEM for four animals. * $P < 0.05$ compared with overnight-incubated islets. (A high-quality digital representation of this figure is available in the online issue.)

measurements of regional blood flow, we could record the blood flow in native and transplanted islets. We have previously investigated islet physiology in YC-3.0 mice and observed no differences when compared with C57Bl/6 mice (24). In a similar manner, in the current study, we could observe no differences in blood perfusion between native YC-3.0 and C57Bl/6 nu/nu islets. In contrast, the blood perfusion of the intrahepatically transplanted islets was found to be only 5% of that in native islets, although there was a great heterogeneity in blood flow between individual transplanted islets. This was also reflected in a highly variable revascularization of the transplanted islets. A more thorough morphological assessment showed that this heterogeneity mainly reflected preferential revascularization of islets with disrupted integrity after transplantation, whereas islets that were maintained intact were avascular and without detectable blood perfusion. To further investigate the importance of disruption of islet integrity for islet graft revascularization, we transplanted not only seemingly intact islets but also islets with disrupted integrity. Indeed, these islet fragments also became well revascularized. Of interest, recent studies of angiogenic mechanisms show that angiogenesis does not merely occur by endothelial sprouting but, rather, recruitment of blood vessels into tissue may require tissue stroma and biomechanical interaction with

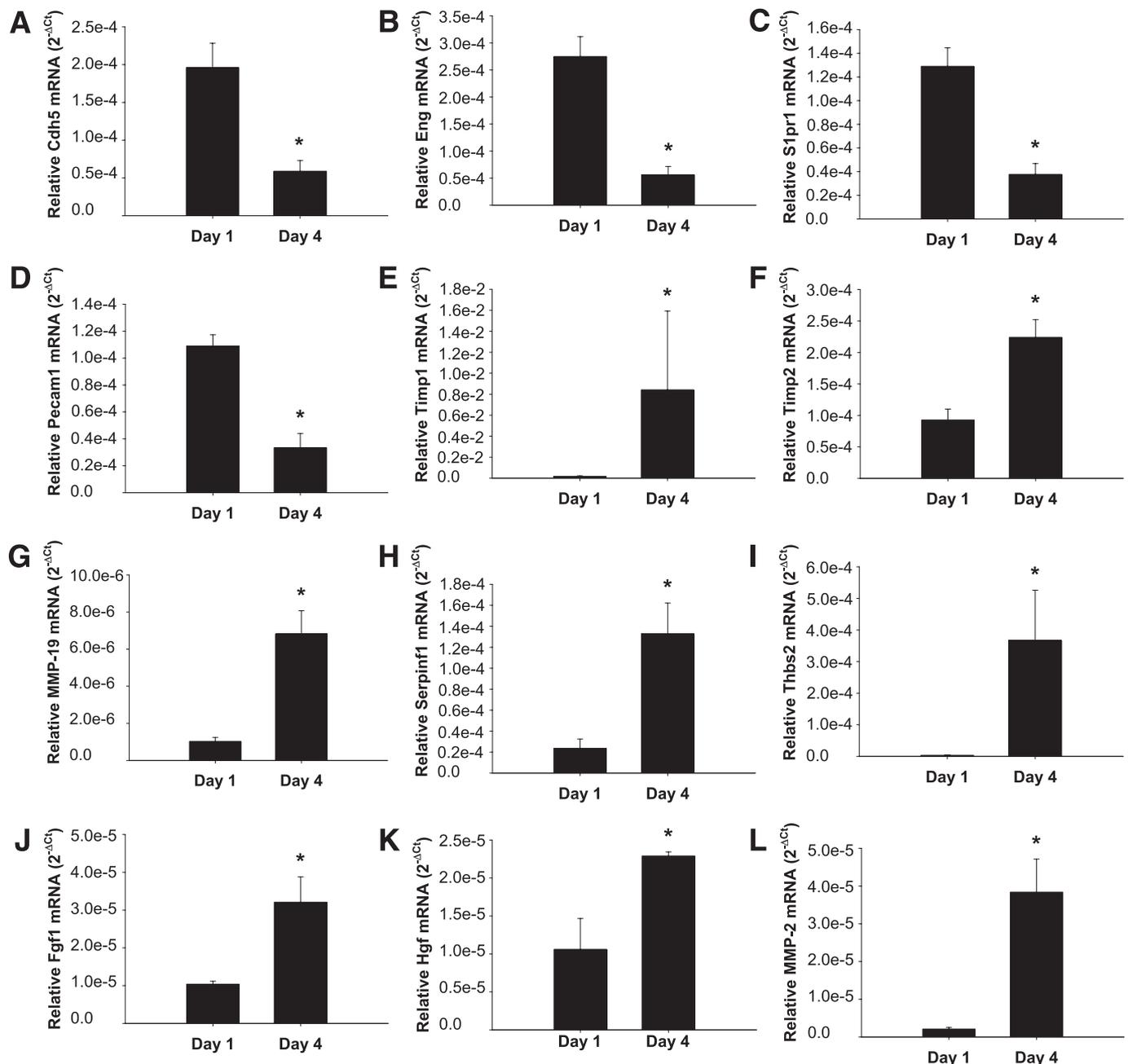


FIG. 6. Changes in the expression of angiogenic factors between overnight-incubated and 4-day-cultured islets. *A*: Cadherin 5 (Cdh5). *B*: Endoglin (Eng). *C*: Sphingosine-1-phosphate receptor 1 (S1pr1). *D*: Platelet/endothelial cell adhesion molecule 1 (Pecam1). *E*: Timp1. *F*: Timp2. *G*: MMP-19. *H*: Ser (or Cys) peptidase inhibitor clade 5 member 1 (Serpinf1). *I*: Thrombospondin-2 (Thbs2). *J*: Fibroblast growth factor 1 (Fgf1). *K*: Hepatocyte growth factor (Hgf). *L*: MMP-2. Data are means \pm SEM for four animals. * $P < 0.05$ compared with overnight-incubated islets. 18S rRNA was used as housekeeping gene.

fibroblasts or myofibroblasts therein (25). Our previous studies also indicate that matrix and tissue degradation by MMP-9 can improve islet graft revascularization (26,27). We therefore measured the release of MMP-9 from intact and fragmented islets in vitro and found that islet fragmentation doubled the release of MMP-9. Moreover, VEGF-A in the medium was similarly increased. A contributing mechanism for the better revascularization of fragmented islets may therefore be activation of MMP-9 with concomitant VEGF-A release from the tissue matrix.

Although transplantation of islets can rapidly restore normoglycemia in rodents, our findings open the possibility that mainly fragmented and distorted islets are functional

posttransplantation, whereas intact islets lack important β -cell/endothelial-cell interactions and suffer from ischemia. In recent work, we showed that $\sim 75\%$ of murine islets stained positive for the hypoxia marker pimonidazole 1 day after intraportal transplantation, and $>60\%$ remained pimonidazole positive at 1-month follow-up (28). In parallel, the apoptosis frequency as measured by caspase-3 staining was increased up to 10% at 1 day after transplantation and remained increased ($\sim 3\%$) 1-month posttransplantation. Decreased oxygenation is well known to impair not only β -cell survival but also β -cell function (29). Indeed, previous studies of intact islets retrieved after transplantation into the liver show marked gene expression

changes and dysfunction (21,22). Human islets have a less developed capsule than rodent islets, possibly predisposing for more fragmentation posttransplantation than their rodent counterpart. However, intact transplanted human islets were previously found poorly revascularized 1 month after experimental intraportal transplantation (19). Poor drainage of islet amyloid polypeptide in such human islets may also predispose for amyloid deposition (7,30,31). In the current study, we used normoglycemic recipients, but at least for islets implanted beneath the renal capsule, there seem to be no differences in revascularization or blood perfusion of transplanted islets given to nondiabetic recipients or to cure diabetic recipients (32,33).

A more substantial decrease in blood perfusion as compared with vascular density was present in the intrahepatic islets when compared with native islets. Dysfunctional blood flow regulation in transplanted islets, as previously observed for islets implanted to the renal subcapsular site (34,35), may explain some of this difference. Most interesting, however, a six-fold higher blood perfusion was observed in the intrahepatic islet grafts composed of islets transplanted after overnight incubation when compared with islets transplanted after 4 days of culture, whereas only a corresponding tendency to increased blood vessel numbers existed. This indicates that some of the vascular structures, especially in the islet grafts composed of cultured islets, lacked perfusion. Freshly isolated murine islets also seem to perform superior to cultured islets after syngeneic transplantation to the renal subcapsular site (36,37). In the clinical allogeneic setting, however, other factors, such as immunosuppressive preconditioning of recipients, necessitate islet culture, and this may be more important for transplantation outcome than the use of freshly isolated islets (38).

During the first 3 days of islet culture, >90% of donor endothelial cells disappear (39). Studies of islets implanted to the renal subcapsular site (39–41) or the anterior chamber of the eye (42) show that donor islet endothelial cells may extensively contribute to islet graft revascularization and become incorporated in the new vascular system. However, in our study, very few Tie-2 GFP positive cells (i.e., donor islet endothelial cells) remained in islets after intraportal transplantation, even when islets were incubated only overnight before transplantation. This indicates a lesser role for donor islet endothelial cells in revascularization of islets at the intrahepatic site. It seems, nevertheless, possible that the remaining vascular channels in freshly isolated islets are beneficial and facilitate resumed blood perfusion after transplantation, thereby explaining differences in blood perfusion of grafts composed of freshly isolated and cultured islets. Moreover, leukocytes residing within the pancreatic islets are known to disappear during culture (43). Such cells are strongly involved in adult blood vessel formation as a result of their local secretion of metalloelastases, which cause the formation of capillary lumens through local tunneling in the parenchyma (44). The higher level of cell death discerned early after islet isolation may also facilitate islet tissue remodeling and tunneling of blood vessels in the parenchyma. When comparing the expression of different genes involved in angiogenesis, most differences (9 of 12) favored blood vessel formation in freshly isolated islets. Among identified factors was lower expression of TIMP1 and TIMP2, as well as thrombospondin-2, in freshly isolated islets. We and others have earlier shown the importance of MMP-9 (see also discussion above), normally inhibited by TIMPs, for

revascularization of pancreatic islets (26,45), as well as demonstrated the importance of the thrombospondin system for the normally low revascularization of transplanted islets (46). Possible predominant presence of duct cells in freshly isolated islet preparations may contribute to angiogenesis by being a source of angiogenic cytokines (47).

Low revascularization and blood perfusion of intrahepatic islets may partially be compensated for by the 10-times increased liver blood perfusion in the vicinity of islets. This suggests that the high number of blood vessels found in this region, when compared with normal liver parenchyma, were mainly of hepatic artery origin. The capacity for liver endothelial cells to migrate and proliferate in response to islet endothelial cell products was recently shown *in vitro* to depend on secretion of VEGF from the transplanted β -cells (48). Some Tie-2 GFP positive cells were also found to have migrated out of islets into surrounding parenchyma and contributed to the vasculature there in a manner similar to what has been previously observed at the renal subcapsular site (40). Noteworthy, the blood perfusion surrounding transplanted freshly isolated islets was higher than in the surroundings of transplanted cultured islets. We have previously observed no differences in either VEGF or fibroblast growth factor 2 content in islet grafts composed of freshly isolated or cultured islets early after transplantation (36), data that were supported by the present angiogenesis gene array results. However, as discussed above, other differences were discerned, including lower levels of TIMPs early after islet isolation, making it possible for MMP-9 to release more tissue-bound VEGF, compared with previous observations (45). We also performed Oil Red O stainings of islet graft-bearing livers to investigate whether the increased blood perfusion in hepatic tissue immediately surrounding the implanted islets reflected insulin-induced increases in hepatic metabolism causing lipid accumulation. However, no or very little lipid accumulation occurred in liver parts close to islets and in the rest of the liver.

We conclude that pancreatic islets transplanted intraportally into the liver have a very low blood perfusion, reflecting few and dysfunctional blood vessels. Donor islet endothelial cells mainly disappear or migrate into surrounding liver parenchyma; therefore, disruption of islet integrity is pivotal to support revascularization by recipient blood vessels. The present identification of the crucial importance to reorganize the islet matrix to support revascularization means that novel strategies to achieve this can be designed. It may not necessarily be obtained by islet fragmentation; less-damaging effects can be obtained by other means, for example, by supporting normal mechanisms for reorganization of the islet matrix by metalloproteinases, as we have previously shown to be effective for islets transplanted to striated muscle (27).

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J.H. and J.L. researched data, contributed to discussion, and reviewed and edited the manuscript. G.Z. researched data and reviewed and edited the manuscript. P.-O.B. and M.K. reviewed and edited the manuscript. P.-O.C. contributed to discussion and wrote the manuscript. P.-O.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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