Omentum Is Better Site Than Kidney Capsule for Growth, Differentiation, and Vascularization of Immature Porcine β-Cell Implants in Immunodeficient Rats

Kim Bartholomeus,[1] Daniel Jacobs-Tulleneers-Thevissen,[1,2] Sun Shouyue,[1,3] Krista Suenens,[1] Peter A. In't Veld,[1] Miriam Pipeleers-Marichal,[1] Daniel G. Pipeleers,[1] and Karine Hellemans[1,4]

Background. Rapid revascularization of islet cell implants is important for engraftment and subsequent survival and function. Development of an adequate vascular network is expected to allow adaptive growth of the β-cell mass. The present study compares omentum and kidney capsule as sites for growth and differentiation of immature β-cell grafts.

Methods. Perinatal porcine islet cell grafts were implanted in omentum or under kidney capsule of nondiabetic nude rats. Implants were compared over 10 weeks for their respective growth, cellular composition, number and size of β cells, their proliferative activity, and implant blood vessel density.

Results. In both sites, the β-cell volume increased fourfold between weeks 1 and 10 reflecting a rise in β-cell number. In the omental implants, however, the cellular insulin reserves and the percent of proliferating cells were twofold higher than in kidney implants. In parallel, the blood vessel density in omental implants increased twofold, reaching a density comparable with islets in adult pig pancreas. A positive correlation was found between the percent bromodeoxyuridine-positive β cells and the vessel density.

Conclusions. Growth of the β-cell volume proceeds similarly in the omentum and under the kidney capsule. However, the omentum leads to higher insulin reserves and an increased pool of proliferating cells, which might be related to a more extended vascular network. Our observations support the omentum as an alternative site for immature porcine islet cells, with beneficial effects on proliferation and implant revascularization.

Keywords: Cell therapy, Endocrine pancreas, Islet transplantation, Revascularization, Omentum.

Islet intraportal transplantation is since long considered as therapy for type 1 diabetes (*1*). Clinical proof-of-concept has been provided since the early 1980s and initiated clinical trials worldwide (*2–4*). In most trials with human donor islets, the liver is still used as implant site, although there is growing awareness that this site is not optimal (*5*). It has been shown that several of its immunologic, anatomic, and physiologic features contribute to a significant early graft loss and β-cell dysfunction. Moreover, because this implant site is inaccessible, it can also not be considered when it comes to the use of alternative β-cell sources, such as stem cell–derived β cells or the use of xenografts (*6–8*). Several alternative sites have been tested in animal models to improve engraftment and long-term survival and to minimize surgical complications (*6, 8*). Few of these alternative sites hold the potential to be translated into clinical trials, and in general, evidence of posttransplantation functions better than those reached after intraportal infusion are lacking.

[1] Unit Diabetes Pathology and Therapy, Diabetes Research Center, Brussels Free University-VUB, Brussels, Belgium.

[2] Department of General and Abdominal Surgery, Universitair Ziekenhuis Brussel, Brussels, Belgium.

[3] Department of Endocrinology and Metabolism, Rui-Jin Hospital, Shanghai, China.

[4] Address correspondence to: Karine Hellemans, Prof., Dr., Unit Diabetes Pathology and Therapy, Diabetes Research Center, Brussels Free University-VUB, Laarbeeklaan 103, B-1090 Brussels, Belgium.

E-mail: Karine.Hellemans@vub.ac.be

The present study further assesses the omentum as implant site. This site was previously shown successful for rat islet isografts, which is in itself not particular because these preparations have functioned well in many sites (9, 10). When testing human islet cell grafts in diabetic immunodeficient rats, we found a better survival in the omentum than in the liver: omental implants exhibited little infiltration and were capable to correct hyperglycemia, whereas intraportal implants lost function after a heavy inflammatory infiltration (11).

We now assess whether the omental site would also provide an adequate environment for growth of the β-cell mass as is known to occur in implants of immature pancreatic cell preparations (12–14). In a previous study, we have shown the growth potential of perinatal porcine β-cell grafts implanted under the kidney capsule of nude mice (15). The implants became structurally organized as homogenous endocrine clusters with predominantly insulin-positive cells and few other endocrine cells in the periphery; the increase in β-cell mass generated the potency to normalize diabetes (15, 16). Efficient revascularization is considered to be a prerequisite for this process (17). Within 2 days after implantation, we recognized the first endothelial cells and small blood vessels in the proximity of the implants; the revascularization process, however, proceeded beyond the 20-week study period (15, 18). It is conceivable that the rapidity and extent of this process determines initial engraftment as well as subsequent adaptive growth. Because the omentum is well vascularized and has been described as a rich source of angiogenic and neurogenic factors (19–21), we wanted to compare the growth of immature porcine β-cell grafts in omentum and kidney and relate it to the vessel density that has developed in both implants.

Implants in the omentum reached vessel densities comparable with those in endogenous adult porcine islets within 10 weeks after implantation, which was not the case in the kidney subcapsular space. In parallel, implant volumes were increased reflecting a beneficial effect on β-cell proliferation and numbers as well as on cellular insulin content. Our data elaborate our prior findings with human islets implants and add further support on the potential use of the omentum as an alternative site for islet transplantation. Whether the omentum could offer an alternative to intraportal transplantation in humans remains to be evaluated.

RESULTS

Both Sites Exhibit Structural Organization of Immature Porcine Islet Cell Implants with Increase in Total β-Cell Volume but Not α-Cell Volume

The composition of the implants was determined immediately before transplantation (Pre-Tx) and 1 and 10 weeks after transplantation (PT) (Table 1). At the day of transplantation, they were composed of single cells or small cell clusters, which were mainly endocrine (78%±11%), corresponding to mostly insulin- and glucagon-positive cells in slightly different proportions (54%±10% and 42%±6% of the endocrine fraction, respectively). Grafts contained in average 1.2×10^6 β cells with 8.8±2.0 μg insulin per 10^6 β cells.

At PT week 1, the relative proportion of insulin- and glucagon-positive cells was still similar, but after 10 weeks, the proportion of insulin-positive cells was threefold higher than that of glucagon-positive cells as reported previously (15); this was the case for both implant sites. Parallel to this change in cellular composition, implants became vascularized (Fig. 1) and structurally organized with the formation of endocrine cell clusters (Fig. 1). At the same time, the total volume of insulin-positive cells had fourfold increased in both sites (Fig. 2A): from 124±37 to 521±226 μm^3 for omental implants and from 105±53 to 473±450 μm^3 for kidney implants (Fig. 2B). The total volume of glucagon-positive cells in the implants did not change with time and remained similar for both sites (Fig. 2B).

Increase in β-Cell Number and Cellular Insulin Content Leads to Threefold Higher Insulin Reserves in Omental Implants

At PT week 1, only 15% of the initial β-cell number was recovered in the implants (for 1.2×10^6 β cells in graft, only $0.18 \pm 0.05 \times 10^6$ were found engrafted), as described previously (15). This number then increased threefold to fourfold in both sites resulting in $0.72 \pm 0.39 \times 10^6$ β cells in omentum and $0.53 \pm 0.37 \times 10^6$ β cells in kidney (Fig. 2C). A similar four-fold increase was seen in the insulin content of the kidney implants, but that of omental implants increased twelve-fold from 2.5±2.1 μg at PT week 1 to 31±8 μg, bringing their insulin reserve three-fold higher than that in the kidney implants (10±11 μg) (Fig. 2C). When calculated

TABLE 1. Composition of porcine Islet cell grafts and implants

		Pre-Tx	Week 1	Week 10
Cellular composition				
Insulin positive (%)	Kidney	54±9 (11)	52±1 (6)	76±3 (5)[a,b]
	Omentum	54±10 (10)	48±4 (5)	73±5 (5)[a,b]
Glucagon positive (%)	Kidney	39±4 (11)	46±5 (6)[c]	22±4 (5)[a,b]
	Omentum	44±6 (10)	52±6 (5)[c]	23±7 (5)[a,b]
Insulin content (μg/10^6 β cells)	Kidney	9±2 (12)	15±7 (5)[c]	20±21 (7)[c]
	Omentum	9±2 (9)	14±12 (5)	43±11 (4)[a,d]

[a] $P<0.001$ compared with Pre-Tx.
[b] $P<0.001$ compared with PT week 1.
[c] $P<0.05$ compared with Pre-Tx.
[d] $P<0.05$ compared with PT week 1.
Values represent means±SD from (n) animals.
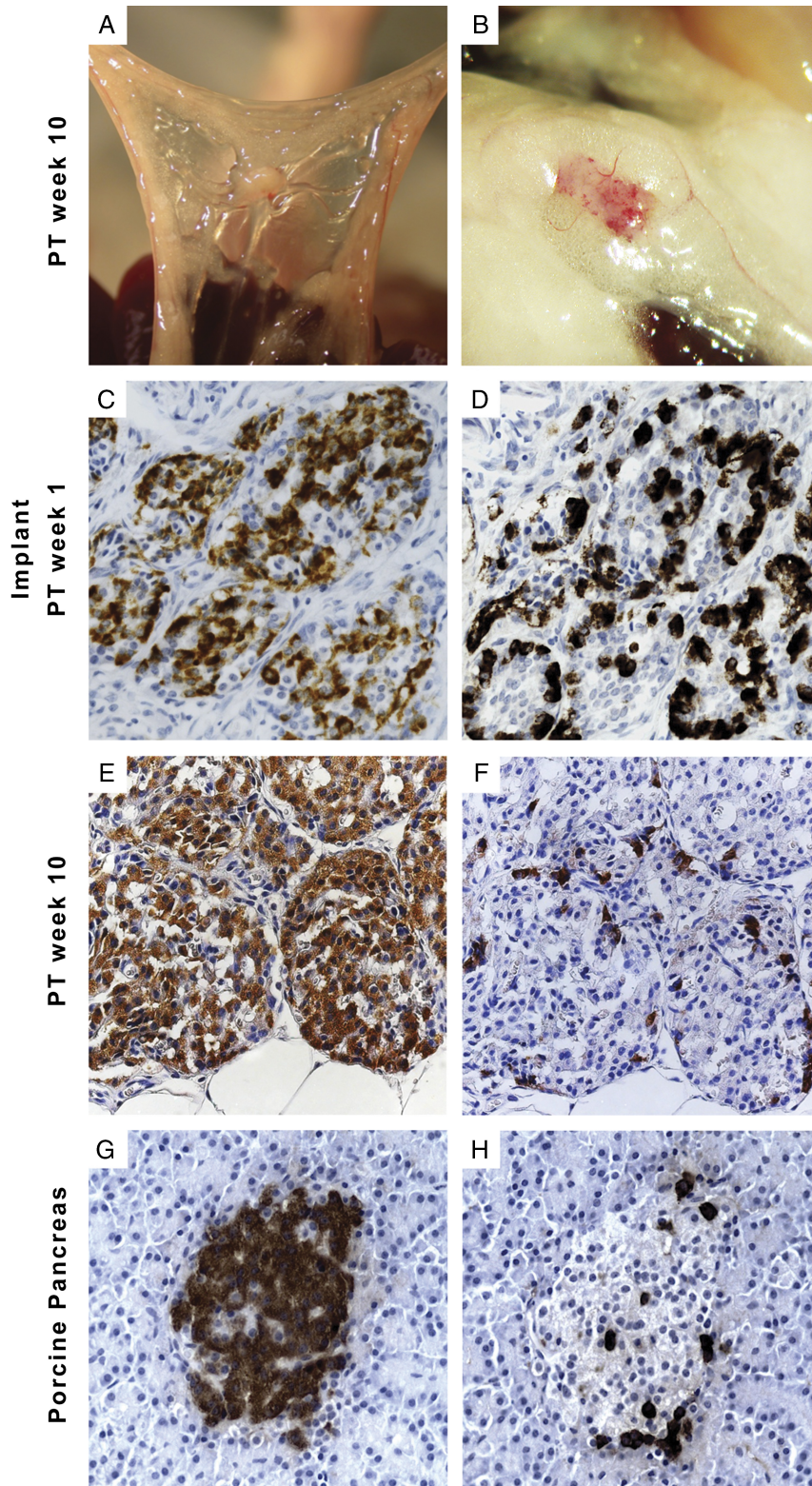
FIGURE 1. Morphology of perinatal porcine islet cell implant in omentum compared with that of islets in adult porcine pancreas. Macroscopic view of omental implant at PT week 10 (A), exhibiting revascularization (B). Structural organization of insulin-positive (C and E) and glucagon-positive (D and F) cells in omental implants and in islets of adult porcine pancreas (G and H).
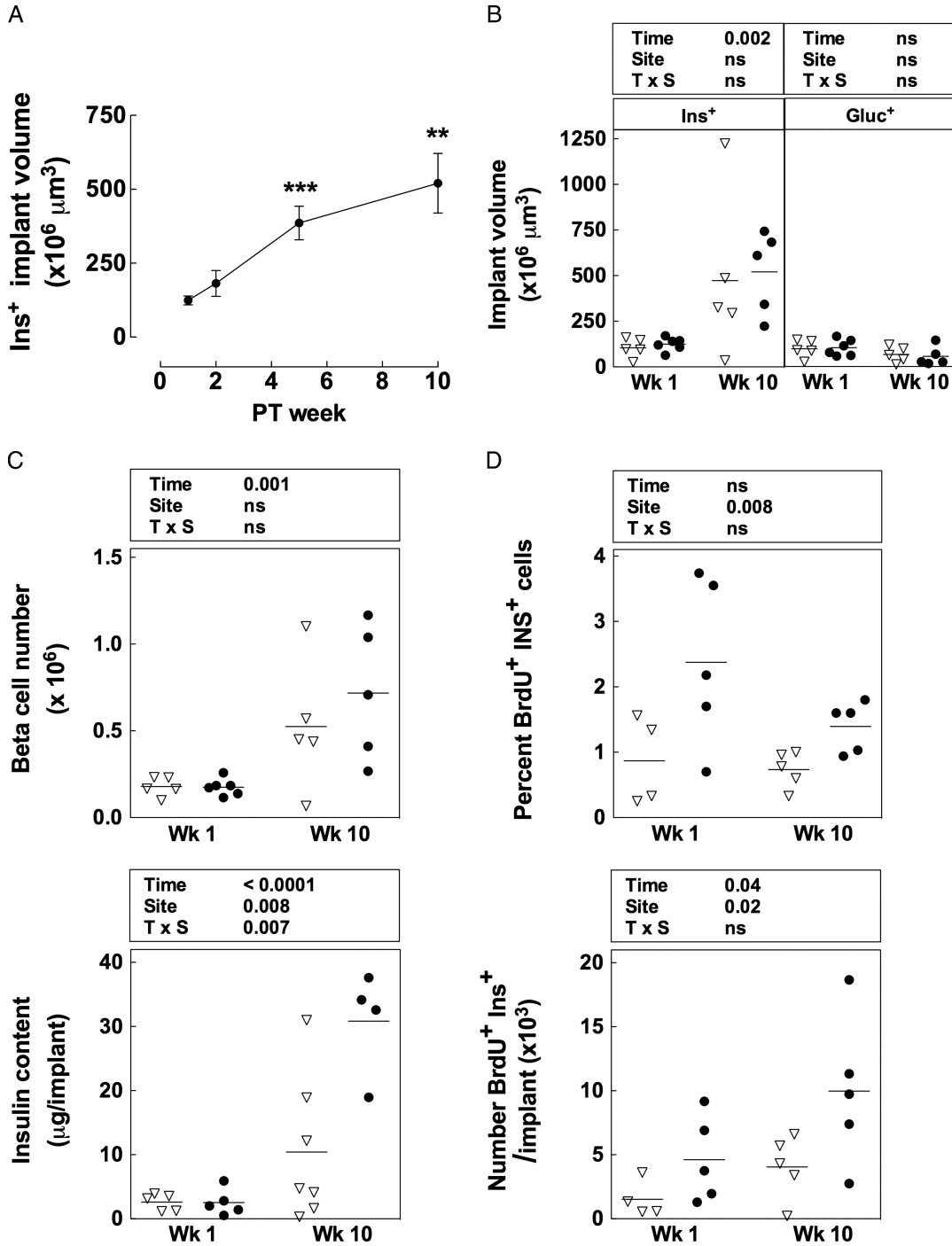
A

B

Time	0.002	Time	ns
Site	ns	Site	ns
T x S	ns	T x S	ns

C

Time	0.001
Site	ns
T x S	ns

Time	< 0.0001
Site	0.008
T x S	0.007

D

Time	ns
Site	0.008
T x S	ns

Time	0.04
Site	0.02
T x S	ns

FIGURE 2. Comparison of growth and differentiation in perinatal porcine islet cell implants in kidney and omentum. Growth of insulin-positive volume in omental (A and B) and kidney (B) implant between PT weeks 1 and 10 without increase in glucagon-positive volume. C, associated increase in total β-cell number and insulin content. D, number of β cells in proliferative activity as percent of β cells and as total number in the implant. Results are presented as mean±SE or as dot plot and mean, for n=4–7 in each group and time point. **$P<0.01$; ***$P<0.001$ versus the PT week 1 time point (t test). Time=Effect of transplantation time, Site=Effect of implant site, T×S=Interaction between transplantation time and implant site (two-way ANOVA).

per million β cells, the average cellular insulin content in omental implants (43±11 μg/10^6 β cells) had threefold increased between PT weeks 1 and 10, although this was not the case in kidney implants (Table 1).

Parallel with the increase of implant insulin contents over 10 weeks, a significant decline of basal 2 hr fasting blood glucose levels was observed in both recipient groups: kidney (4.3±0.7 mmol/L; $P<0.05$) and omentum (4.0±0.9 mmol/L;

$P<0.001$) versus Pre-Tx (5.5±0.8 mmol/L) and normal control levels (5.0±0.7 mmol/L; $P<0.05$). No difference in fasting glucose levels was observed between omentum or kidney transplanted animals.

Larger Pool of Proliferating β Cells in Omental Implants

Bromodeoxyuridine (BrdU) was injected 1 hr before sacrifice of the implanted animals. At PT week 1, 2.4%±1.3% of the insulin-positive cells in the omental implants was labeled with BrdU versus only 0.9%±0.7% in kidney implants. There was still a significant difference at PT week 10 (1.4%±0.4% vs. 0.7%±0.3%) (Fig. 2D). At both time points, the total number of β cells caught in proliferative activity was 2.5-fold higher in omental implants than in kidney implants (Fig. 2D).

Revascularization of Implants Leads to Higher Vascular Density in Omentum

The vascularization significantly increased in both implant sites. At PT week 1, blood vessels were mainly found at the periphery of the small endocrine aggregates, whereas, at PT week 10, many were also present inside the aggregates, as is the case in adult porcine islets in situ (Fig. 3A). Blood vessel density was assessed as the endothelial cell surface area that was associated with the insulin- and glucagon-positive area (Fig. 3A). At PT week 10, the omental implants showed a sevenfold higher density than at PT week 1 compared with only a twofold increase in kidney implants (14%±4% vs. 7%±3%; $P<0.01$). The average value was comparable with the average vessel density measured in islets of the adult porcine pancreas (13%±2%) and the nude rat pancreas (17%±7%) (Fig. 3B). This was not the case for omental implants of adult rat islets; their blood vessel density at PT week 10 was only 7.1%±1.3%, 40% lower than that in islets of the adult rat pancreas ($P<0.001$) and 50% lower than the density in porcine islet cell implants ($P<0.01$) (Fig. 3B). There was a positive correlation between blood vessel density and the total number of proliferating β cells in the porcine implants (r=0.95; $P<0.05$) (Fig. 3C); such correlation was not found for the kidney implants, where the pool of cells in proliferative activity was also significantly smaller.

DISCUSSION

Previous studies have indicated the omentum as a favorable site for islet graft survival in rodent and large animals models, with possible translation to clinical trials (9, 22). However, whereas the omentum of rodents forms a thin gossamer membrane, adult human omentum, in general, shows an extensive fatty degeneration, the extent of which varies with body mass index. It thus remains to be evaluated how these features would affect implantation and islet function under clinical settings and whether an advantage could be obtained using the omentum compared with the intraportal site or other implant sites (23). Several other properties, however, support the choice of the omentum as an islet transplant site (9, 24, 25). In surgery, the omentum is since long used for its wound-healing abilities (26); technically, the double-layered structure of the omentum

offers an advantage, as it allows pouch formation or implantation between the two sheets (9, 20, 27); this can facilitate implantation of larger cell volumes, including transplant devices, or implants consisting of cell mixtures (28–31). Moreover, its blood flow provides hepatic portal delivery, which approaches a physiologic route for released insulin (32). The omentum is also seen as an immunologically privileged site in which local regulatory T cells are generated (33).

Another potentially relevant characteristic of the omentum, on which we focused for the current study, is its high vascular density and angiogenic capacity, which may lead to a better revascularization and engraftment than in other sites (19, 21, 34). In prior work, we showed that human islet cells survived and functioned better in rat omentum than after intraportal injection (11). We now evaluated the omentum as an implant site for immature β cells. The capacity of immature β cells to grow and to mature toward functional β cells makes them an attractive alternative source (14, 35, 36).

Grafts consisted of purified endocrine cells prepared from perinatal porcine organs containing similar percentages of insulin- and glucagon-positive cells. At this age, β cells are considered immature in view of their smaller average cell volumes, low insulin content, and their ability to replicate and differentiate to cells with a larger insulin content and a capacity to correct diabetes as we documented previously after their implantation under the kidney capsule of mice (15, 16). The associated growth in β-cell mass was now also observed in nude rats both in kidney and omental implants. In both sites, total β-cell volume and total number of β cells increased threefold to fourfold between PT weeks 1 and 10. The total α-cell volume did not increase, resulting in a marked reduction in their relative contribution to the endocrine surface area. This is consistent with prior data (14, 15, 37). As a result, in both sites, endocrine cell clusters were formed with a comparable percentage composition than the islets in adult porcine pancreas, with insulin-positive cells in the center and glucagon-positive cells at the periphery (15, 37, 38).

The increase of the implant volume and β-cell number corresponded with a significantly higher pool of BrdU-labeled β cells in the omentum, both at PT weeks 1 and 10. This explains, in part, the higher total insulin content in the omental implants; in addition, however, there was also a marked increase in the average cellular insulin content in the omental implants, reaching twofold higher values than in kidney implants, and approaching the cellular insulin content of mature β cells. We found no evidence for α-cell proliferation. These observations indicate that whereas the structural reorganization of the endocrine implants proceeds similar in both implant sites, the omentum shows a clear positive influence on β-cell replication and leads to higher β-cell numbers and higher insulin reserves per 10^6 cells. This last feature suggests the omentum as a better environment for maintaining the balance between insulin synthesis and release based on a larger insulin storage compartment.

The increase in cell number and differentiation of immature porcine β-cell implants thus occurred to a larger extent in the omentum than under the kidney capsule. This difference appeared not caused by a better engraftment because both implants exhibited similar high losses during the
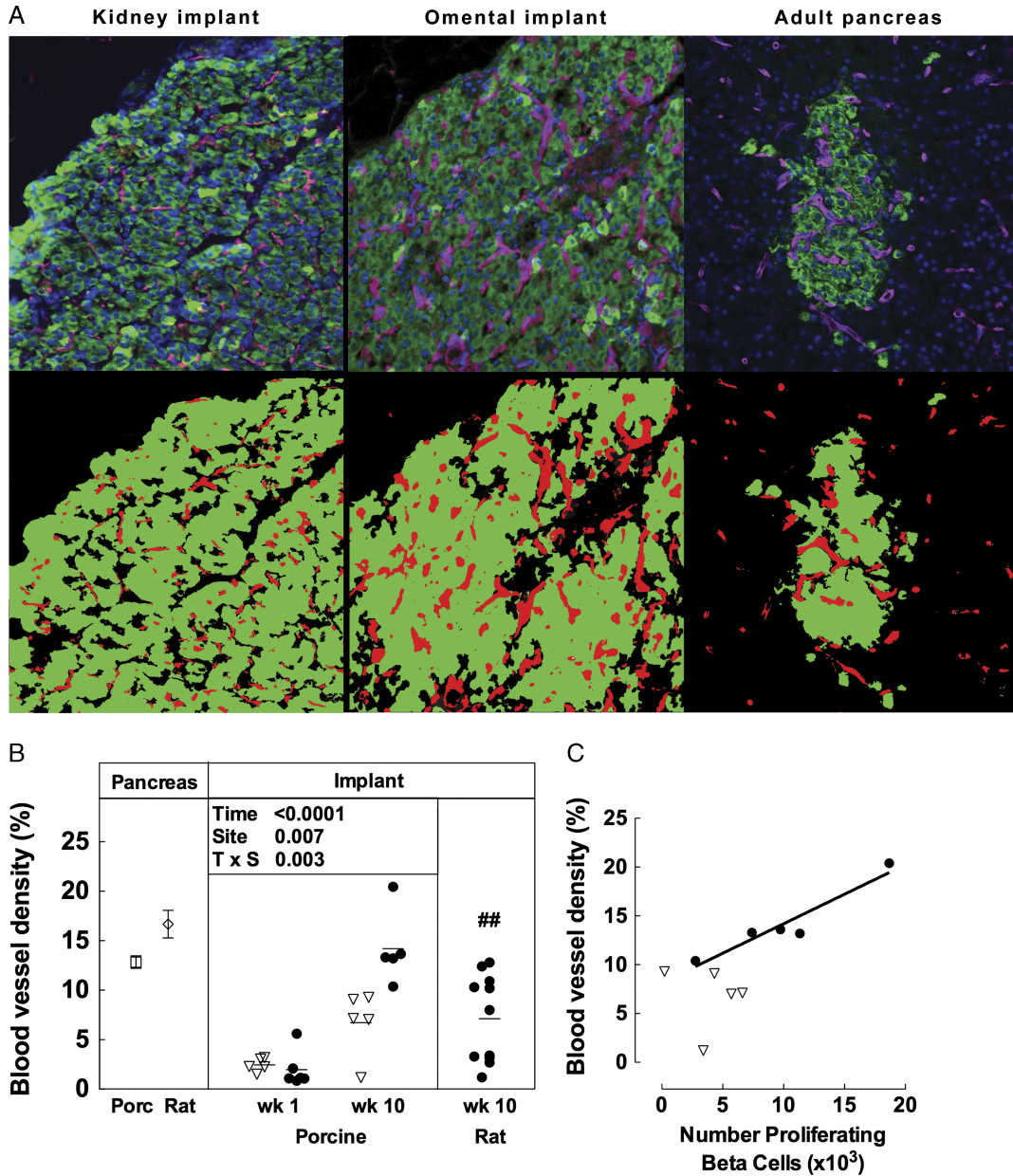
FIGURE 3. Blood vessel density in perinatal porcine islet cell implants. Immunofluorescent (top) and binarized (bottom) images (A) used for measurement of blood vessel density, as percentage of lectin-positive area (pink/red) in the insulin-positive area (green): kidney (left) and omental (middle) implants at PT week 10 and islets in an adult porcine pancreas (right). B, blood vessel density in islets of porcine and rat pancreas (left), implants of perinatal porcine islet cells under kidney capsule (triangle) and omentum (black dot), and implants of adult rat islets in omentum (right). C, correlation for blood vessel density and number of proliferating β cells in perinatal porcine islet cell implants at PT week 10. Results are presented as mean±SE or as dot plot and mean, for n>5 in each group. [##]$P<0.01$ versus the porcine omental implant site at PT week 10 (t test). Time=Effect of transplantation time, Site=Effect of implant site, T×S=Interaction between transplantation time and implant site (two-way ANOVA).

first PT week. At PT week 1, their vessel density was equally low and represented only 15% of that in pancreatic islet tissue of adult rats or pigs, which can account for the massive cell death. During the subsequent 9 weeks, both implants became vascularized. Consistent with our hypothesis, the revascularization process was twofold more pronounced in the omentum, as reflected by the larger lectin-positive

area and larger vessel diameter in the insulin-positive space. The omentum thus provides a more favorable environment for the revascularization of these immature islet cell implants reaching a vessel density that is comparable with that in islet tissue of adult rats and pigs. The latter was, however, not the case in omental implants of adult rat islets, which might be attributed to their larger size or preserved

islet integrity (*39*). Alternatively, our observations also indicate that the immature endocrine islet cells exert a stronger angiogenic action. The possible role of vascular endothelial growth factor needs to be investigated, as this growth factor has been found to regulate the balance between vessel density and islet cell mass during pancreatic development (*40*). We noticed a positive correlation between the vessel density in the omental implants and the number of β cells in proliferative activity; such correlation has also been observed in a recent comparative analysis of rat pancreatic islets with low and high blood perfusion (*41*).

In conclusion, both omentum and kidney subcapsular space provide an environment for growth and differentiation of immature β-cell implants using a rat model. The omental implants exhibit a twofold higher pool of proliferating β cells during this process and result in β cells with a threefold higher insulin content, approaching the values in adult β cells. This difference is attributed to a twofold higher revascularization, reaching the vessel densities measured in endogenous islets of adult rats and pigs.

MATERIALS AND METHODS

Preparation of Islet Cell Grafts

Cultured perinatal porcine islet cell preparations were obtained from Beta Cell NV (Zellik, Belgium) according to isolation and culture conditions that were previously described (*15*). Belgian land race sows at 112 to 115 days of gestation or newborn piglets 1 day after birth were used as source. Samples were taken to determine cell numbers, cellular composition, and hormone content. Implants were prepared by embedding $2.3\pm0.4\times10^6$ endocrine cells in a fibrin matrix (Tissucol; Baxter, Vienna, Austria) Pre-Tx. Rat islets were prepared from adult male Wistar rats (200–250 g; Elevage Janvier, Le Genest-Saint-Isle, France) as described previously (*11*).

Transplant Procedures

All animal experiments and procedures were approved by the local ethical committee for animal experimentation of the Vrije Universiteit Brussel; manipulations were carried out in accordance with the European Community Council Directive (86/609/EEC). Normoglycemic male immunodeficient Rowett nude rats (Hsd:RH-Foxn1$^{rnu/rnu}$; 7–9 weeks old; Harlan, Horst, The Netherlands) were selected as recipient. Animals had free access to water and standard laboratory animal food. Animals received an implant under the kidney capsule (*42*) (n=22) or between the omental sheets (n=29) as described previously (*11*). Surgical procedures were performed under general anesthesia (ketamine and xylazine). After transplantation, body weight and basal and stimulated glucose level were measured weekly until the end of the experiment. Tail vein blood was analyzed after a 2 hr morning fasting period and 30 min after an intragastric administration of a single bolus of 30% glucose solution (2 g/kg body weight) (Glucocard Memory PC; A. Menarini Diagnostics, Florence, Italy). Animals were sacrificed and implants were removed 1, 2, 5, or 10 weeks PT and processed to determine their insulin content (n=4–7 per transplantation site per time point) (porcine insulin radioimmunoassay; Linco Research, St. Charles, MO) or fixed in 4% neutral phosphate-buffered formalin for histology (n=4–6 per transplantation site per time point). In the latter group, BrdU (50 mg/kg) was injected intraperitoneally 1 hr before the implant was removed.

Immunohistochemistry

Paraffin-embedded implants were completely sectioned at 4 μm section thickness. For analysis of cellular composition, sections were stained for islet hormones using anti-synaptophysin antibody (Dako, Glostrup, Denmark) and anti-glucagon and anti-insulin antibodies (both a gift from C. Van Schravendijk, DRC-VUB). β cells in proliferating activity were identified with an anti-BrdU antibody in combination with protease antigen retrieval (MP Biomedicals, Eschwege, Germany). Secondary antibodies were biotin-linked anti-rabbit IgG (Amersham International, Amersham, UK), anti-guinea pig IgG (Vector Laboratories, Peterborough, UK), or anti-mouse IgG (Amersham International). Detection was performed with the Vectastain Elite ABC kit (Vector Laboratories). The peroxidase reaction was developed with a DAB development kit (Dako, Glostrup, Denmark). Digital images were acquired using a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany) fitted with a Axiocam MRc5 camera (Carl Zeiss) and processed using Axio Vision software (Carl Zeiss).

Implant Morphometry

The size of the insulin- and glucagon-positive cell population in the implants was determined according to Cavalieri's principle. The total volume of insulin- and glucagon-positive cells was estimated by point counting (*43*). Every 40th section was immunostained for insulin or glucagon and stereologically analyzed using a light microscope that projects the image onto a grid. The total volume of insulin- and glucagon-positive cells was calculated by multiplying the total number of points overlaying the antibody-positive cells in each section with the area per point (816 μm^2) and the distance between subsequent stained sections (160 μm). Total insulin-positive cell number was estimated by dividing the total insulin-positive implant volume by the calculated mean individual insulin-positive cell volume (*44*).

Implant Blood Vessel Density

Blood vessel density in the implants was measured after incubation of tissue sections with neuraminidase V (Sigma-Aldrich, Poole Dorset, UK) for retrieval of endothelial carbohydrates and using biotin-labeled *Bandeiraea simplicifolia* lectin (1:500; Vector Laboratories) in combination with Alexa Fluor 647–conjugated streptavidin (Invitrogen, Carlsbad, CA). Nuclei were counterstained using 4′,6-diamidino-2-phenylindole (Sigma-Aldrich). Sections were photographed using a Pathway 435 imager (BD Biosciences, Rockville, MD). First, the endocrine areas were defined based on anti-insulin and anti-glucagon staining; the lectin-positive endothelial cell areas were then determined within these boundaries using IP lab 4.0 software (BD Biosciences). The blood vessel density was determined as the ratio of both areas.

Statistical Analysis

Results are expressed as means±SE as mentioned. Statistical analysis was carried out using Prism4 (GraphPad, San Diego, CA). Differences between experimental groups were calculated with an unpaired two-tailed *t* test for one variable and a two-way analysis of variance (ANOVA) test for two independent variables. The degree of correlation between two variables was tested using Pearson's correlation coefficient. Statistical significance was assumed when $P<0.05$.

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