# Donor Islet Endothelial Cells in Pancreatic Islet Revascularization

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**OBJECTIVE**—Freshly isolated pancreatic islets contain, in contrast to cultured islets, intraislet endothelial cells (ECs), which can contribute to the formation of functional blood vessels after transplantation. We have characterized how donor islet endothelial cells (DIECs) may contribute to the revascularization rate, vascular density, and endocrine graft function after transplantation of freshly isolated and cultured islets.

**RESEARCH DESIGN AND METHODS**—Freshly isolated and cultured islets were transplanted under the kidney capsule and into the anterior chamber of the eye. Intravital laser scanning microscopy was used to monitor the revascularization process and DIECs in intact grafts. The grafts' metabolic function was examined by reversal of diabetes, and the ultrastructural morphology by transmission electron microscopy.

**RESULTS**—DIECs significantly contributed to the vasculature of fresh islet grafts, assessed up to 5 months after transplantation, but were hardly detected in cultured islet grafts. Early participation of DIECs in the revascularization process correlated with a higher revascularization rate of freshly isolated islets compared with cultured islets. However, after complete revascularization, the vascular density was similar in the two groups, and host ECs gained morphological features resembling the endogenous islet vasculature. Surprisingly, grafts originating from cultured islets reversed diabetes more rapidly than those originating from fresh islets.

**CONCLUSIONS**—In summary, DIECs contributed to the revascularization of fresh, but not cultured, islets by participating in early processes of vessel formation and persisting in the vasculature over long periods of time. However, the DIECs did not increase the vascular density or improve the endocrine function of the grafts. ***Diabetes*** **60:2571–2577, 2011**

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Clinical islet transplantation can restore endogenous insulin production and glycemic control in patients with type 1 diabetes, yet increased knowledge, and hence refinement, would allow for a wider application of this therapy (1). Pancreatic islets are interspersed by a dense and tortuous capillary network that facilitates an efficient exchange of oxygen, nutrients, and hormones between the endocrine cells and the bloodstream. Transplanted islets are revascularized by blood vessels that grow into the islets from the host organ via angiogenesis (2), although the acquired vasculature has a significantly lower vessel density compared with the endogenous islets (3). Furthermore, during the initial avascular engraftment period, a dramatic reduction in insulin content and high rate of cell death occur within the islets (4). Therapies that enhance the angiogenic capacity of islets by overexpression of vascular endothelial growth factor-A (VEGF-A) can increase the vascular density of islet grafts and improve metabolic function (5,6).

Recently, we and others showed that donor islet endothelial cells (DIECs) can form functional vessels within transplanted islets (7,8). Immediately after isolation (i.e., in freshly isolated islets), a large number of intraislet endothelial cells (ECs) are present (7–9). However, if the islets are cultured, the intraislet ECs rapidly disappear, and by 4 days, only ~5% of the initial content is detected (7). Therefore, freshly isolated islets, in contrast to cultured islets, contain an extra pool of ECs that potentially could promote islet revascularization and function after transplantation. Here, we have performed a detailed characterization of the role of DIECs in the revascularization of transplanted islets.

## RESEARCH DESIGN AND METHODS

**Mouse models.** C57BL/6J and Tie2–green fluorescent protein (GFP) mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and male nude mice from Taconic M&B (Ry, Denmark). Animal studies were performed under protocols reviewed and approved by the animal ethics committee at Karolinska Institutet and the University of Miami Institutional Animal and Use Committee.

**Pancreatic islet isolation and culture.** Tie2-GFP islets were isolated and cultured as previously described prior to transplantation to nude mice (7). C57BL/6J islets were isolated and cultured as previously described prior to syngeneic transplantation (10).

**Pancreatic islet transplantation and reversal of diabetes.** Freshly isolated or cultured islets from the same batch were transplanted under the kidney capsule or to the anterior chamber of the eye (AC) as previously described (7,11,12). The number of islet equivalents was estimated prior to transplantation to mice rendered diabetic by streptozotocin treatment (11).

**Imaging and image analysis of transplanted islets.** Ex vivo and intravital imaging of islet grafts were performed as previously described in detail (7,11,12). Immunostaining of islet grafts has been described (7). The pixel area of GFP

and CD31 fluorescence was quantified in immunostained kidney islet graft sections to determine the contribution of DIECs to the graft vasculature and the total vascular area. The endocrine area was defined by insulin staining, and the stromal area was defined as the entire graft exclusive of the endocrine area. For each graft, four to nine sections were quantified. All quantifications and image processing were performed with Leica Confocal Software (version 2.61).

**Transmission electron microscopy.** Isolated islets were cultured overnight before transplantation into the AC. Iris tissue, including engrafted islets, and pancreatic tissue of the same mice were extracted 16 weeks after transplantation (for detailed protocol see Supplementary Appendix).

**Statistical analysis.** Data are expressed as median and range or mean and SEM. Two groups were compared using Student *t* test. Survival curves were compared using log-rank test. Data analysis was performed with Excel, SigmaPlot, and Prism (GraphPad Software, Inc., La Jolla, CA).

## RESULTS

**DIECs contribute to the islet graft vasculature but do not increase vascular density.** Pancreatic islets were isolated from Tie2-GFP mice, characterized by EC-specific expression of GFP (13), to allow identification of DIECs after transplantation. Freshly isolated and 4-day-old cultured Tie2-GFP islets were transplanted under the kidney capsule and analyzed 1 and 5 months after transplantation. In accordance with our previous data (7), ex vivo confocal and two-photon laser-scanning microscopy imaging 1 month after transplantation showed that DIECs contributed to functional vessels within fresh islet grafts ($n$ = 5; Fig. 1*A*), whereas only scattered DIECs were found in cultured islet grafts ($n$ = 5; not shown). Ex vivo imaging of fresh islet grafts 5 months after transplantation showed that DIECs remained functionally integrated within the graft vasculature (Fig. 1*B–D*). Immunostaining of graft sections supported these observations (Supplementary Fig. 1) and facilitated quantification of the contribution of DIECs to the graft vasculature (Fig. 1*E*), as well as the total vessel area of the graft (Fig. 1*F*). Together, the quantification shows that, although the DIECs contributed to 7–8% of the vasculature in fresh islet grafts and only to 0.8% in cultured islet grafts, the vascular density was the same in the two groups. It is noteworthy that 50% of the DIECs migrated out from the endocrine areas of the grafts into the surrounding stromal tissue. Notably, the vascular area was 1.5-fold higher in the endocrine versus the stromal areas in all grafts (Supplementary Table 1).

**DIEC participation in early vessel formation correlates with an increased revascularization rate.** Using a newly developed method for intravital laser-scanning microscopy imaging of islets transplanted to the AC (11), we could monitor how DIECs participate in the revascularization process by repetitive imaging of the same islets after transplantation. Freshly isolated and 4-day-old cultured Tie2-GFP islets were imaged after 3, 7, 14, and 28 days after transplantation. At 3 days after transplantation, vessels had started to form between the islets and the iris but were only occasionally found to penetrate into the islet periphery (Figs. 2*C* and 3*A*). Elongated DIECs contributed to the wall of established vessels, whereas round GFP cells were frequently localized close to vessels or in areas with diffuse Texas Red fluorescence (Fig. 2*A–C*). These round cells most likely represent migrating DIECs participating in processes of new vessel formation. At 7 days after transplantation, large vessels were formed, connecting the iris vasculature to the islets, and capillaries partially penetrated into the islets (Figs. 2*F* and 3*A*). Elongated DIECs contributed to vessels both outside and inside the islets

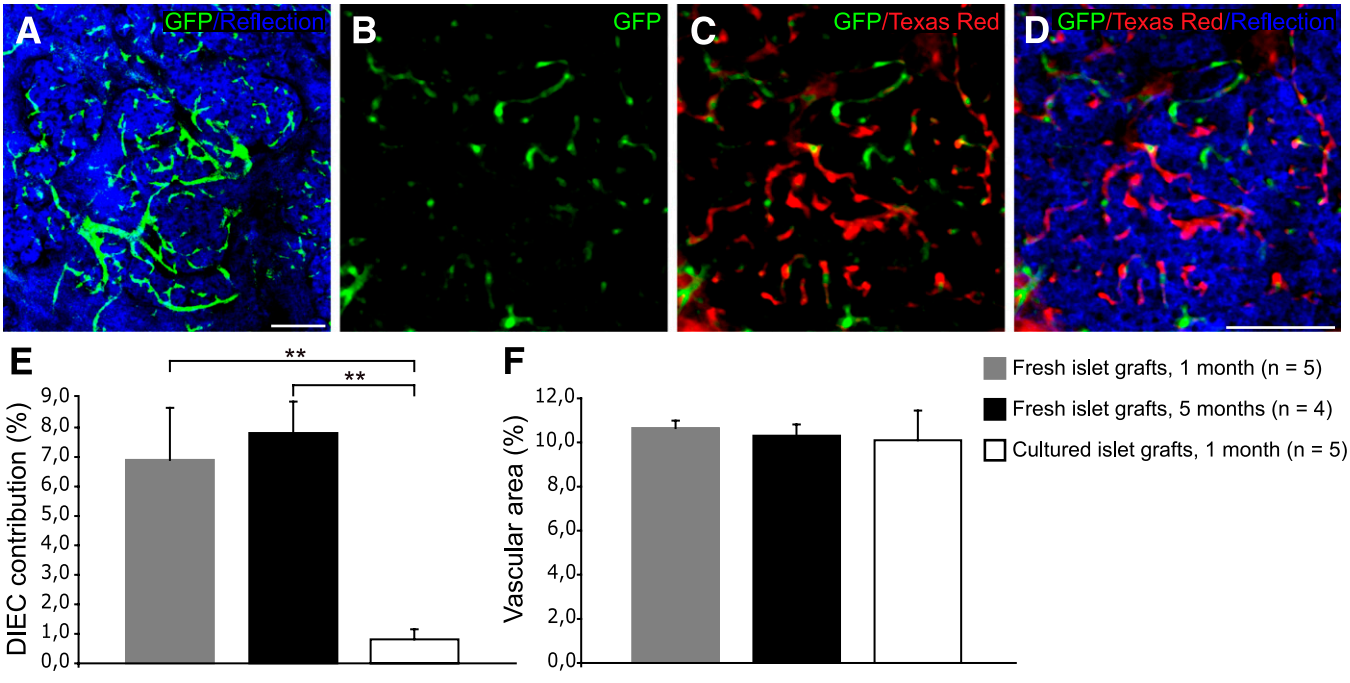


**FIG. 1. DIECs contribute to the vasculature of fresh but not cultured islet grafts. *A–D*: Ex vivo imaging of fresh islet grafts located under the kidney capsule, 1 (*A*, $n$ = 5) and 5 months (*B–D*, $n$ = 4) after transplantation. *A*: Extensive contribution of GFP-fluorescent (green) DIECs is found among the engrafted endocrine cells (blue); note also the contribution to long vessel segments. The image is a projection of an image z-stack corresponding to 30 μm. *B*: GFP-fluorescent DIECs contribute to islet graft vessels. *C*: Perfusion of the graft-bearing kidney with Texas Red (red) shows that vessels with contributing DIECs are connected to the circulation. *D*: DIECs and the perfused vessels are shown together with the reflection of endocrine cells. *B–D*: The images are projections of image z-stacks corresponding to 11 μm. Scale bars correspond to 100 μm. *E*: Quantification of the percentage contribution of DIECs (GFP area) to the graft vasculature (CD31 area). *F*: Quantification of the total vascular area of the graft (CD31 area). Two hundred islets were transplanted per mouse. All values are presented as mean ± SEM; **$P$ < 0.01. (A high-quality digital representation of this figure is available in the online issue.)**

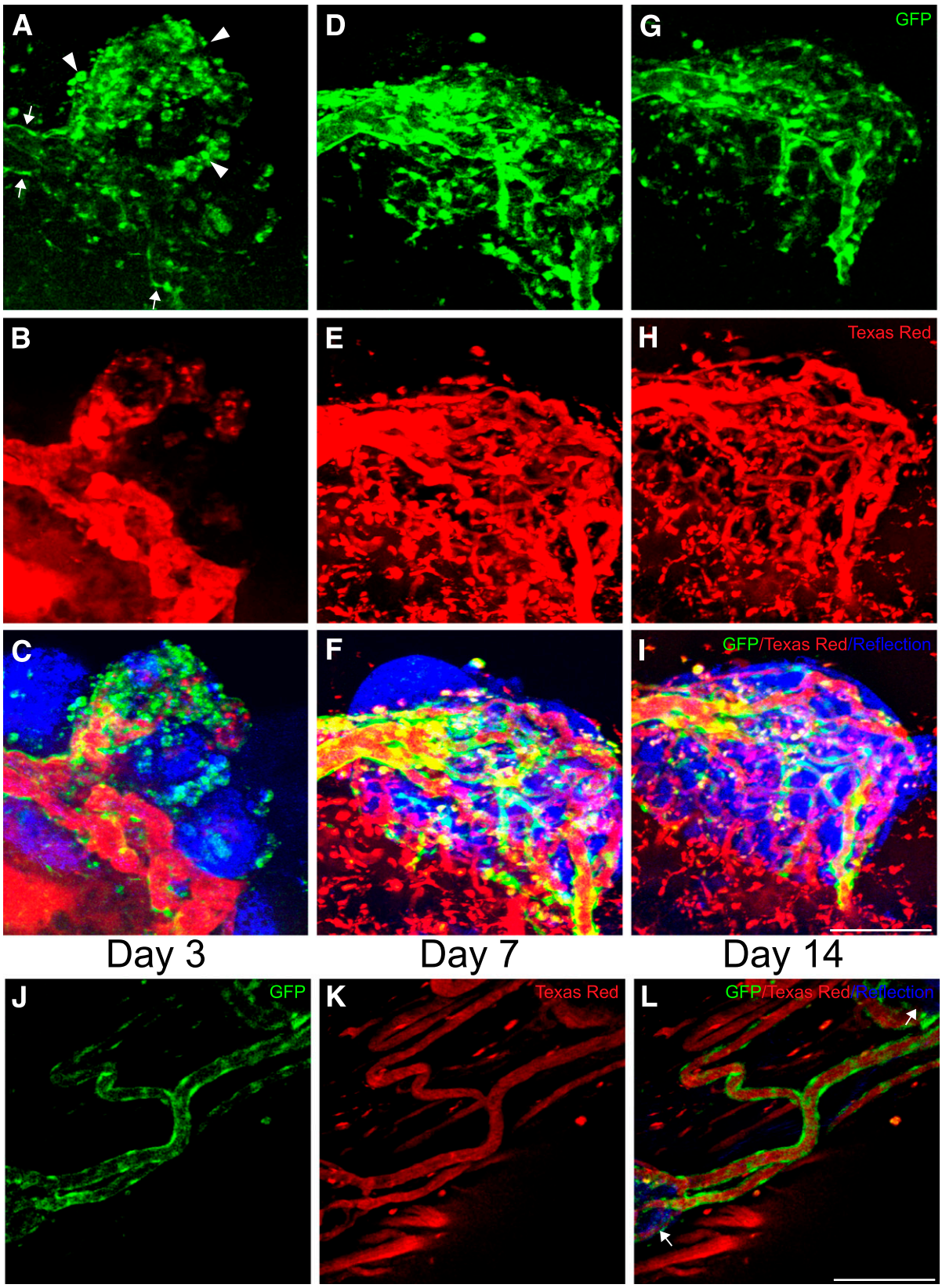


**FIG. 2. Characterization of DIEC participation in islet revascularization. *A–I*: Projections of image z-stacks captured at 3, 7, and 14 days during revascularization of a fresh islet graft in the AC. *A–C*: At day 3, elongated GFP-fluorescent (green) DIECs contribute to the wall of vessels established outside of the islets (*A*, arrows). Round GFP-fluorescent cells are found in the vicinity of areas of diffused Texas Red dye, as well as distant from perfused regions (*A*, arrowheads). *D–F*: At day 7, mostly elongated DIECs contributing to established vessels are found (*D*). *G–I*: At day 14, structural refinements of the vessels are observed with only small changes for the DIECs. The images are representatives of five to seven islets imaged per time point. *J–L*: Projections of an image z-stack captured 14 days after transplantation of freshly isolated islets. GFP (*J*), Texas Red (*K*), and an overlay image of GFP, Texas Red, and reflection (*L*). One islet graft is localized in the upper right image corner (arrow) and another one is localized in the lower left corner (arrow). Large vessels in the iris connecting to the islets show extensive contribution of DIECs. Thirty islets were transplanted per mouse. Scale bars correspond to 100 μm. (A high-quality digital representation of this figure is available in the online issue.)**

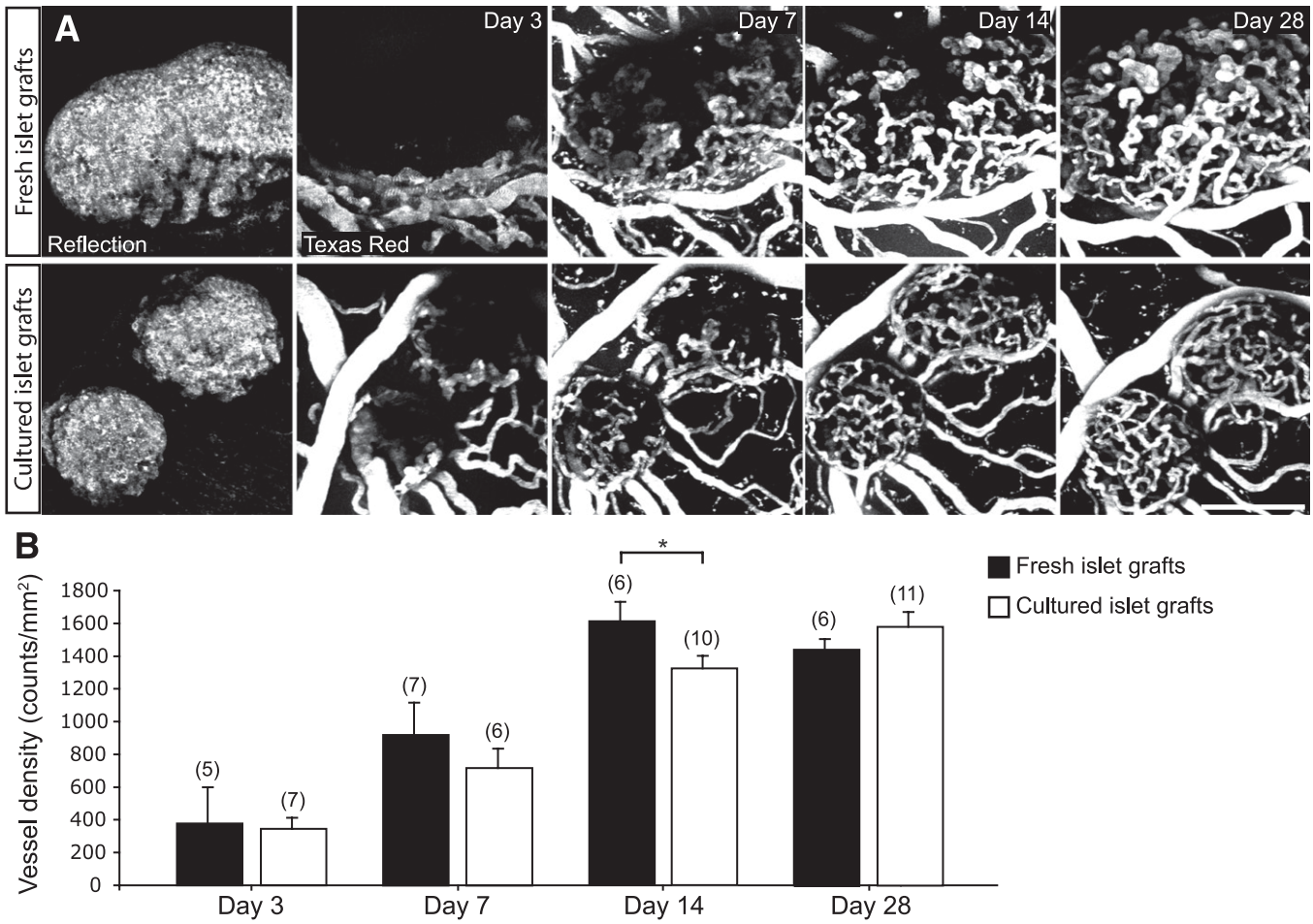


**FIG. 3. Vessel density in fresh and cultured islets during engraftment in the AC. *A*: Image projections of one freshly isolated islet (*top*) and two cultured islets (*bottom*) during the process of revascularization are shown. The left column displays the reflection image captured at day 14. The vasculature was visualized by intravenously administered Texas Red, 70 kDa, at the indicated time points. Scale bar corresponds to 150 μm. *B*: Quantification of vessel density in fresh and cultured islet grafts at indicated time points after transplantation. The number of analyzed islet grafts is indicated within parentheses at the respective time points. The number of recipients was six with fresh islets and five with cultured islets. Thirty islets were transplanted per mouse. All values are presented as mean ± SEM; *$P$ = 0.05.**

(Fig. 2*D–F*). Round GFP cells were only sporadically found at this time point and were absent at later stages. Notably, as also observed in the kidney grafts, large vessels extending far from the islets were occasionally found to be composed, to a great extent, of DIECs (Fig. 2*J–L*). At 14 days after transplantation, the vasculature had extensively increased in density and penetrated most parts of the islets (Figs. 2*I* and 3*A*), whereas only minor rearrangements of the DIECs were observed compared with day 7 (Fig. 2*G–I*). Between days 14 and 28, the vessel density increased further (Fig. 3*A*), whereas no change was observed in the arrangement of DIECs (data not shown). As expected, only a low number of DIECs could be identified after transplantation of cultured islets (data not shown).

During the revascularization process, there was a trend for a higher vessel density within the freshly isolated islets, which was significant at 14 days, indicating a higher revascularization rate of freshly isolated islets compared with cultured islets (Fig. 3*B*). However, after completed revascularization at 28 days, vessel density was similar in both groups (Fig. 3*B*).

**Ultrastructure of islet engrafted in the AC.** Transmission electron microscopy (TEM) was used to characterize the ultrastructure of the graft vasculature after islet engraftment in the AC. Fresh islets cultured overnight were transplanted to the AC, to ensure that the engrafted islets contained a mixed population of host and donor ECs. TEM images of islets from the pancreas (Fig. 4*A* and *C*) and the AC (Fig. 4*B* and *D*), 4 months after transplantation, show endocrine cells with normal fine ultrastructure with intact organelles and characteristic secretory granules. In both compartments, endocrine and ECs within the islets are in close contact, separated only by a thin basement membrane (Fig. 4*A–D*, arrows). In addition, the islet capillaries in both compartments were similar and formed by thin EC bodies with fenestrations covered by a diaphragm (Fig. 4*C* and *D*, arrowheads). The ultrastructural morphology of the ECs in the engrafted islets was homogenous, and no differences were found that suggested different morphologies of ECs of host or donor origin.

**Metabolic evaluation of fresh and cultured islets engrafted in the AC.** The metabolic capacity of islets, cultured overnight or for 4 days before implant (from the same batch), was evaluated after syngeneic transplantation to the AC of diabetic recipients. Freshly isolated islets cultured overnight still contain a 10-fold higher number of DIECs compared with islets cultured for 4 days (7). It is noteworthy that fresh islets reverted diabetes with a longer

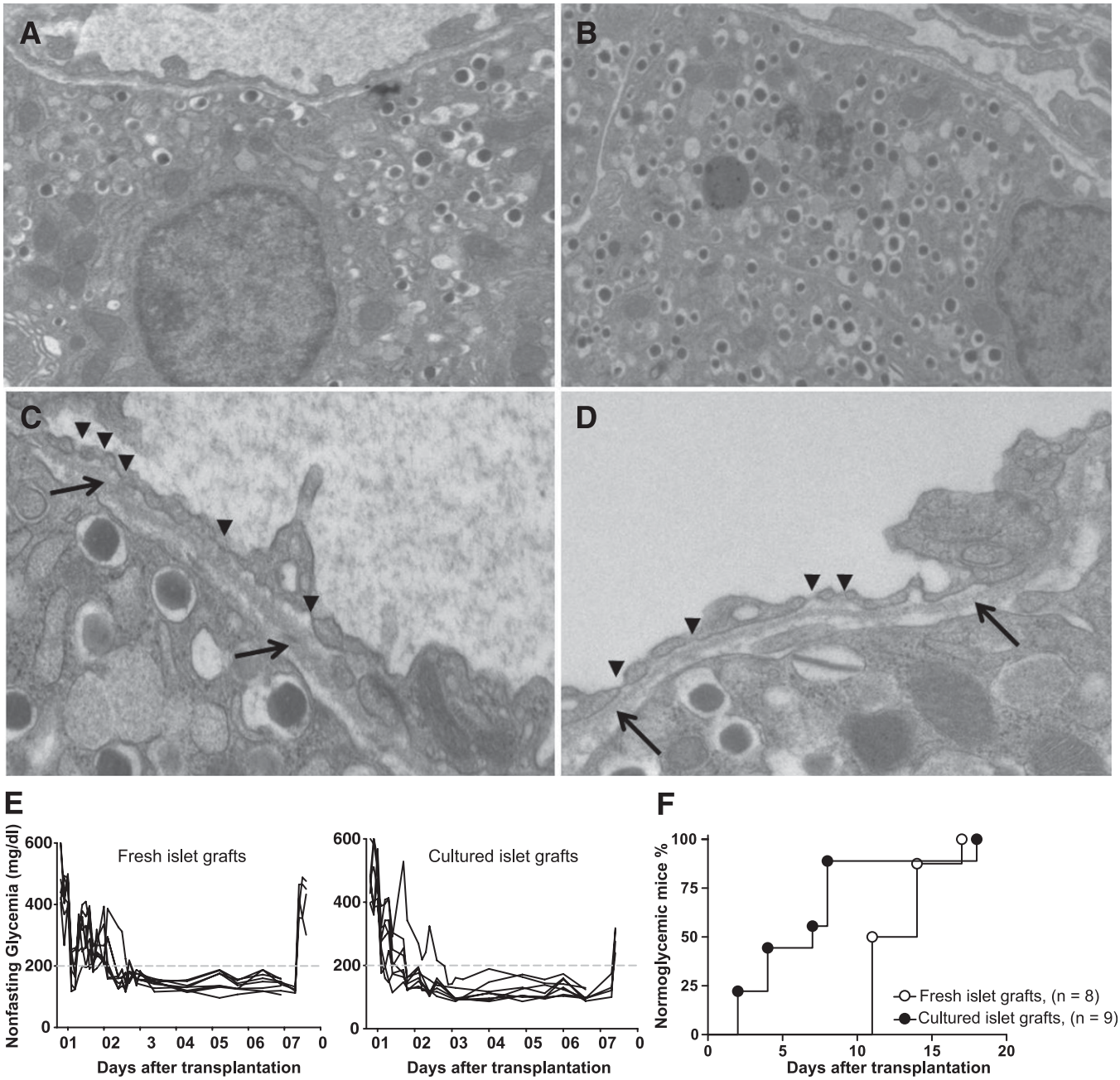


**FIG. 4. Ultrastructural and metabolic evaluation of islets engrafted in the AC. *A–D*: TEM images of a native islet in the pancreas (*A* and *C*) and an islet engrafted in the AC (*B* and *D*). *A* and *B*: The same ultrastructure of the ECs of the endogenous and engrafted islets and the close location of endocrine cells and capillaries. *C* and *D*: Thin ECs of capillaries, which are only separated by a basement membrane (arrows) from the islet cells. In addition, the characteristic abundant diaphragm-covered fenestrations (arrowheads) of the ECs are found in both endogenous and engrafted islets. Thirty islets were transplanted per mouse. *E* and *F*: Metabolic evaluation of AC-engrafted syngeneic islets. *E*: Nonfasting glycemic values in recipients of a syngeneic marginal islet mass transplantation (150 islet equivalents/mouse). At the end of the study, hyperglycemia promptly resumed after enucleation of the graft-bearing eye. *F*: Days needed for return to normoglycemia of diabetic recipients receiving fresh (○) and cultured (●) syngeneic islets.**

median time, 12.5 days (range, 11–17 days; $n = 8$), compared with cultured islets, 7 days (range, 2–18 days; $n = 9$; $P < 0.05$) (Fig. 4*E*). Further evaluation by a glucose tolerance test 1 month after transplantation, when all mice had returned to euglycemia, showed no significant difference (Supplementary Fig. 2).

## DISCUSSION

ECs have been detected inside pancreatic islets after isolation from mouse, rat, pig, and human (5,7–9), indicating that intraislet ECs persist independent of species and isolation procedure. Clinical transplantation protocols using both freshly isolated (1) and cultured islets (14) have been used, underscoring the relevance of characterizing the role of DIECs after islet transplantation. Here we show that DIECs significantly contribute to 7–8% of the islet graft vasculature after transplantation of freshly isolated islets, whereas almost no contribution could be found after transplantation of 4-day-old cultured islets. A similar contribution at both 1 and 5 months after transplantation indicates that

DIECs remain in the vasculature over long time periods. It is noteworthy that after the revascularization process was completed, islet grafts originating from fresh or cultured islets had comparable vascular density, showing that the contributing DIECs did not increase the vascularization of the fresh islet grafts. The DIECs primarily contributed to new vessel formation during the first period after transplantation, which also correlated with a higher revascularization rate of freshly isolated islets compared with cultured islets. Together these results suggest that the addition of ECs per se does not increase the number of formed vessels within the graft but may enhance the rate of vessel formation.

Brissova et al. (8) previously reported, using a different reporter construct, that DIECs of freshly isolated islets might contribute up to 40% of the graft vasculature. However, if our methodology underestimates the contribution of DIECs to the fresh islet grafts, this would further underscore the fact that addition of DIECs does not increase vascular density. Our finding that the vascular density of fresh and cultured islet grafts is similar 1 month after transplantation contradicts the results from Olsson and Carlsson (15), showing a higher vessel density within the endocrine areas of fresh islet grafts compared with cultured islet grafts. However, it is difficult to speculate about the origin of this discrepancy because the description of the general morphology of the islet graft vasculature also strongly differs, as we found a 1.5-fold higher vascularization of the endocrine versus the stromal areas of the grafts (Supplementary Table 1) compared with the opposite finding of a two- to fourfold higher vascularization of the stromal versus the endocrine areas in the aforementioned study (15).

As a higher revascularization rate should reduce the period of hypoxia during engraftment and thereby might increase the survival of β-cells (16), we were surprised to find that the fresh islets were a little slower in reversing diabetes compared with cultured islets. A limited advantage of a higher revascularization rate and a more rapid oxygen supply could be related to site-specific properties of the AC. The AC has a high oxygen tension (63 mmHg; 17), compared with other applied transplantation sites, such as the parenchyma of the kidney (14 mmHg) and the liver (3–4 mmHg; 18), which might reduce or mask a beneficial effect of a higher revascularization rate for the fresh islets. This could contribute to the discrepancy between our result and two previous studies, which found that fresh islets performed better than cultured islets when transplanted under the kidney capsule (15,19). However, differences in other parameters, such as the culture media and islet selection through handpicking, might also be contributing factors. Islet culture could also provide advantages through the removal of injured islets from the isolation process, by lowering the proinflammatory profile of the islets (20), or by affecting other factors that compensate for a slower revascularization rate.

Examination of islets engrafted in the AC using TEM showed a similar ultrastructural morphology compared with endogenous islets in the pancreas, underlining a normal physiological status of the islet cells, as verified earlier in functional studies (11). Most importantly, the ECs in the islets engrafted in the AC acquired a typical intraislet phenotype with thin cell bodies and abundant fenestrations, as also reported after transplantation to other sites (2). In line with this, we could not find any morphological evidence that would suggest differences between host and donor ECs. Long-term incorporation of DIECs into vessels might be of relevance from an immunological aspect, as DIECs have been suggested to play a role in islet allograft rejection (21).

Intra-islet macrophages and other antigen-presenting cells are, in addition to ECs, depleted during islet culture (22). Several subpopulations of macrophages are proangiogenic and may participate in angiogenic processes such as islet revascularization. However, as the number of resident macrophages found per islet in the healthy pancreas is low (two in the mouse [23] and three in nonhuman primates [24]), we find it unlikely that these cells affect the process of islet revascularization.

Our results show that although the contribution of DIECs per se may improve the rate of islet graft vascularization, it does not increase the vascular density or improve the metabolic function. It is possible that a successful future strategy should be to package the islets with both an angiogenic factor and additional ECs (25).

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D.N. performed islet transplantations, ex vivo imaging, in vivo imaging, and immunohistochemical experiments. S.S., S.L., M.R., A.D., and E.I. performed islet transplantations and TEM experiments. R.R.-D., R.D.M., E.Z.-A., J.M., M.L.-C., S.V., M.H.A., C.R., A.C., and A.P. performed islet transplantations and metabolic evaluation of islet grafts. D.N., S.S., A.P., and P.-O.B. analyzed the data. D.N. and P.-O.B. designed the study and wrote the manuscript.

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