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**Abstract**: The liver as transplantation site for pancreatic islets is associated with significant loss of islets, which can be prevented by grafting in a prevascularized, subcutaneous scaffold. Supporting vascularization of a scaffold to limit the period of ischemia is challenging and was developed here by applying liposomes for controlled release of angiogenic factors. The angiogenic capacity of platelet-derived growth factor, vascular endothelial growth factor, acidic fibroblast growth factor (aFGF), and basic FGF were compared in a tube formation assay. Furthermore, the release kinetics of different liposome compositions were tested. aFGF and L-α-phosphatidylcholine/cholesterol liposomes were selected to support vascularization. Two dosages of aFGF-liposomes (0.5 and 1.0 μg aFGF per injection) were administered weekly for a month after which islets were transplanted. We observed enhanced efficacy in the immediate post-transplant period compared to the untreated scaffolds. However, on the long-term, glucose levels of the aFGF treated animals started to increase to diabetic levels. These results suggest that injections with aFGF liposomes do improve vascularization and the immediate restoration of blood glucose levels but does not facilitate the long-term survival of islets. Our data emphasize the need for long-term studies to evaluate potential beneficial and adverse effects of vascularization protocols of scaffolds. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 105A: 2533–2542, 2017.

**Key Words**: type 1 diabetes, islet transplantation, controlled growth factor release, vascularization, scaffold

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## INTRODUCTION

Type 1 diabetic patients require daily injections of insulin to control their blood glucose levels in addition to monitoring their blood glucose several times per day. This intensive therapy is life-saving, but cannot prevent frequent hypoglycemia and diabetic complications.[1] These complications can be prevented by transplanting an endogenous insulin source such as pancreatic islets that regulate glucose levels from a minute-to-minute level.[2] Currently islets are infused into the liver as clinical standard, but widespread application of this therapy is limited due to the lack of long-term success: within 5 years after clinical islet transplantation <50% of the patients remain normoglycemic.[3,4] The liver as transplantation side is considered to be a major contributor to

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these low success rates. Inadequate revascularization,[5] the large numbers of natural killer T cells in the liver,[6] high concentrations of immunosuppressive drugs,[7,8] and the instant blood-mediated inflammatory reaction[9] play a role in islet graft failure after intraportal transplantation. Therefore, a more adequate transplantation site is required for this technology to gain widespread acceptance and practice.

The subcutaneous site might be a suitable alternative for the liver as it is easily accessible, able to bear an adequate tissue volume, and enables monitoring of the islet graft after transplantation. However, up to now the efficacy of subcutaneous islet transplantation is low due to poor revascularization of islets in the subcutaneous site.[10] During islet isolation, the vasculature is disrupted while in the native pancreas the islets are highly vascularized and receive 15–20% of the pancreatic blood supply.[11,12] Inadequate revascularization leads to hypoxia and finally to reduced islet viability and function after transplantation. Before the subcutaneous site could be used it might be necessary to stimulate the vascularization degree in order to accommodate the islets.[10]

Several biomaterials were used to modify the subcutaneous site to facilitate islet-survival, but resulted in variable outcomes.[10,13] Our previous study showed that a PDLLCL (poly($^{68}/_{32}$[$^{15}/_{85}$ $^{D}/_{L}$-lactide]-*co*-ε-caprolactone) scaffold exerted protective effects on the viability and function of islets and improved the engraftment of subcutaneous transplanted islets.[14] However, compared to islet transplantation under the kidney capsule, the PDLLCL scaffold was less efficacious in achieving normoglycemia. A conceivable way to further improve the efficacy of the PDLLCL scaffold is by stimulating vascularization in order to reduce the distance between the engrafted islets and blood vessels facilitating faster ingrowth into the islets. Several strategies have been proposed to enhance vascularization. This includes co-transplantation of vascular-inductive cell types,[15,16] delivery of angiogenic genes,[17,18] or growth factor injections.[19,20] Some of these proposed systems are not applicable for islets. For example harvesting vascular-inductive cell types of the same donors as that of islets might be complex, while there are safety concerns associated with the gene transfer technology.[21] By directly injecting the growth factors produced by the vascular-inductive cell types or angiogenic genes, these issues are circumvented. However, bolus delivery of growth factors showed limited effectiveness.[20,22,23] Often multiple injections of high concentrations are required which result in abnormal vascularization and are associated with undesired side effects such as leaky vessels, growth of tumor-like vessels, or hemangiomas.[20] Therefore, several groups including ours focused on the development of a controlled release system for angiogenic growth factors.[24–27]

With the aim of improving vascularization of a subcutaneous islet transplantation site, in this study, we compared the angiogenic capacity of platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (aFGF), and basic FGF (bFGF). These are all growth factors with reported efficacy in stimulation of vascularization.[28] Subsequently, liposomes were designed for delivery of the selected growth factor in the PDLLCL scaffold. Two dosages of growth factor containing liposomes were administered weekly for a month after which efficacy of the subcutaneous scaffold as transplantation site for an islet graft was compared to untreated scaffolds.

## MATERIALS AND METHODS

### Experimental design

The angiogenic capacity of PDGF, VEGF, aFGF, and bFGF were first tested *in vitro* using a tube formation assay (described below). For *in vivo* purposes, controlled release liposomes were designed and tested *in vitro* for release of the most potent angiogenic growth factor. To support vascularization, subcutaneously placed PDLLCL scaffolds received an injection with liposomes containing the angiogenic factor on a weekly basis during a month before islets were introduced. To test the efficacy of the liposome-treated scaffold as transplantation site for islets, 800 rat islets were implanted in scaffolds treated with liposomes loaded with 0.5 and 1.0 μg growth factor and compared with untreated controls. Nonfasting blood glucose levels of the mice were monitored three times a week and 2 weeks after transplantation an intraperitoneal glucose tolerance test (IPGTT) was performed.[29] Islet function was monitored for 75 days after islet transplantation. After 75 days, the scaffolds were removed for histological analysis of the vascularization.

### Cells

Human umbilical vein endothelial cells (HUVECs) were provided by the Endothelial Cell facility of the UMCG (Groningen, The Netherlands). The HUVECs were cultured in RPMI (Lonza, Basel, Switzerland) supplemented with 2 mM L-Glutamine (Gibco™; Thermo Fisher Scientific, Landsmeer, The Netherlands), 5 U/mL heparin (Gibco™), 100 IE/mL penicillin (Gibco™), 100 μg/mL streptomycin (Gibco™), 50 μg/mL crude ECGF solution (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 20% FCS (Sigma-Aldrich) at 37°C and 5% $CO_2$ on 1% gelatin-precoated tissue culture flasks (Corning® Costar®; Sigma-Aldrich).

### Tube formation assay

A HUVEC tube formation assay was performed to compare the *in vitro* effects of human PDGF (Life Technologies, Bleiswijk, The Netherlands), VEGF (Life Technologies), aFGF (Life Technologies), and bFGF (Life Technologies) on the angiogenic activity of vascular endothelial cells. HUVECs (200,000 cells/mL) were plated on to growth factor reduced Matrigel™ (BD Biosciences, Breda, The Netherlands) with the different growth factors in a concentration of 5 ng/mL. Matrigel™ plated HUVECs cultured in basal RPMI without supplements served as control. After overnight incubation at 37°C and 5% $CO_2$, the cells were stained with 1 mM calcein AM (Molecular Probes; Life Technologies) and tube formation was determined with a Leica DM IL fluorescent microscope with a Leica DFC 420 camera (Leica Microsystems B.V., Rijswijk, The Netherlands). The images obtained were analyzed using ImageJ (Version 1.47f; Rasband, W.S., ImageJ, USA National Institutes of Health, Bethesda,

**TABLE I. Liposome Characteristics**

| Lipids | Molar Ratio | Size (nm) | Concentration (μmol TL/ml) |
|---|---|---|---|
| PC:CHOL | 60:40 | 67.9 ± 31.7 | 12.1 ± 1.9 |

The size and concentration (mean ± standard deviation) of liposomes composed of L-α-phosphatidylcholine (PC) and cholesterol (CHOL). The concentration is expressed is the total amount of both PC and CHOL lipids (TL) per ml.

Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012). The angiogenic activity was defined as the percentage of pixels in an image that form the tubes (%Area), the %Area was normalized to the controls (controls set on 1).

### Liposome preparation

Liposomes composed of L-α-phosphatidylcholine (PC; Instruchemie B.V., Delfzijl, The Netherlands) and cholesterol (Sigma-Aldrich) in a molar ratio of 60:40 were prepared by lipid film hydration. Briefly, lipids were dissolved in a 9:1 v/v chloroform/methanol solution (Merck Millipore, Amsterdam, The Netherlands) and dried under a stream of nitrogen for 10 and 30 min of vacuum. The dried lipids were hydrated overnight with HN buffer [5 mM HEPES (Gibco$^{TM}$), 150 mM NaCl (Merck Millipore), pH 7.4]. Liposome size was reduced by repeated extrusion through a polycarbonate membrane (Whatman; Maidstone, Kent, UK) with a pore size of 50 nm using a high-pressure extruder (Lipex, Vancouver, Canada). The concentration of the liposomes was determined by a phosphate assay. The size of the liposomes was determined with a Nicomp 380 ZLS Particle Sizing analyzer (Nicomp particle sizing systems; Port Richey, FL) using dynamic light scattering in the volume-weighing mode (Table I). The liposomes were stored at 4°C under argon gas.

### Growth factor release from liposomes

To determine the release pattern of growth factor from the liposomes, liposomes were incubated in 10 μg/mL growth factor for 60 min at 37°C. To be able to take samples for measuring the release without removing liposomes, the liposomes were immobilized in an alginate microbead.[30] To this end, growth factor loaded liposomes were mixed with 1.9% high-gluconic acid alginate (ISP Alginates, Girvan, UK) and divided over a 96-wells plate. The alginate was crosslinked with calcium chloride (100 mM; Merck Millipore) during 10 min incubation at room temperature. The calcium chloride was replaced with Krebs–Ringer–Hepes (KRH; pH 7.4, 133 mM NaCl, 4.69 mM KCl, 1.18 mM $KH_2PO_4$, 1.18 mM $MgSO_4.7H_2O$, 25 mM HEPES, 2.52 mM $CaCl_2.2H_2O$ (all obtained from Merck Millipore)) solution and the release experiment was carried out at 37°C and 5% $CO_2$ for 14 days. The KRH was replaced first after one hour, and then every 24 h. These samples were used for quantification of the growth factor and were stored at −20°C. Quantikine® Human ELISA (R&D Systems, Oxon, UK) was performed according to manufacturers' protocol to determine the growth factor concentrations in the samples.

### Scaffold preparation

Porous PDLLCL scaffolds were obtained from Polyganics B.V. (Groningen, The Netherlands). Briefly, PDLLCL was dissolved in chloroform (Merck Millipore; 4% (w/v)) and thoroughly mixed with 250–425 μm sodium chloride particles (Sigma-Aldrich; 10:1 w/w). The solvent was allowed to evaporate overnight. To remove the sodium chloride particles from the 5 mm thick polymer sheet, it was washed thoroughly with sterile water. The porous polymer sheet was resized into 10 by 15 mm rectangle scaffolds. During the casting process two channels were created by introducing 400 μm iron rods. After casting, these iron rods were substituted by hydrophobic polyethylene tubing (PE50, 0.5 × 1 mm; Brinkman, 's-Gravenzande, The Netherlands) that keep the channels patent during the 4 weeks of engraftment of the scaffold in the mice recipients. The scaffolds were washed and stored in 70% ethanol (Fresenius Kabi, Zeist, The Netherlands) before implantation.

On the day of implantation the pores of the scaffolds were filled with fibrin. This was done by adding 1 μL thrombin IIa (1.0 U/mL; Stago, Leiden, The Netherlands) to 100 μl fibrinogen (2 mg/mL; Stago) solution. During a 1 h incubation at room temperature and a 1 h incubation at 37°C, the fibrin gel was solidified in the pores of the scaffold.

### Islet isolation

The study was conducted according to the NIH guidelines for the care and use of laboratory animals (NIH Publication #85–23 Rev. 1985). The University of California Institutional Animal Care and Use Committee at the University of Irvine approved the beneath described animal procedures for islet isolation and transplantation (IACUC # 2008–2850). Male Sprague-Dawley rats (Harlan, Placentia, USA) with a body weight of 250–279 g served as pancreas donors for islet isolation. Briefly, under anesthesia pancreata were distended with Collagenase V (Sigma-Aldrich) in Hank's balanced salt solution (HBSS; Corning Cellgro, Virginia) and carefully dissected. The distended pancreata were incubated for 18 min at 37°C. After several washes, islets were separated from the exocrine tissue by making use of a Ficoll (Gradient stock solution; Corning Cellgro) density-gradient. Islet yield and purity were quantified by a dithizone staining (MP Biomedicals, Santa Ana).

### Islet transplantation

Male athymic nude mice (Foxn1$^{nu}$; Charles River, Wilmington, USA) of 8 weeks old were used as transplant recipients. The nude mice were rendered diabetic by an intraperitoneal streptozotocin (Sigma-Aldrich) injection of 180 mg/kg. Diabetes was confirmed by a minimum of three consecutive measurements of blood glucose above 350 mg/dL. A Bayer Health Care (Whippany, USA) blood glucose monitor was used to measure nonfasting blood glucose levels. After diabetes confirmation, the mice received an insulin implant (LinBit; LinShin, Scaraborough, Canada) to reduce the discomfort caused by the diabetic state and PDLLCL scaffolds were subcutaneously implanted on the upper back of the

mice 4 weeks before transplantation. The skin was closed using a standard two-layer technique with 3–0 silk sutures (Ethicon, San Angelo, USA) and skin staples (Cellpoint Scientific, Gaithersburg, USA). To stimulate growth of vasculature in and around our scaffold we made use of growth factor-loaded liposomes. Liposomes were loaded with growth factor by an incubation of 60 min at 37°C. Liposomes were loaded with a 10 μg/mL growth factor solution and subcutaneously injected near the implanted scaffold. Injections were started at the day of scaffold implantation and were repeated three more times after every 7 days.

At the day of islet transplantation, the mice were anesthetized by 2–3% of isoflurane (Piramel Healthcare, Morpeth, UK) and a small incision was made at one side of the preimplanted scaffold. The polyethylene tubing was removed and 800 islets were carefully placed in two channels through a PE50 tube connected to a 23G Hamilton syringe (SGE Analytical Science, Austin, USA). The insulin implant was removed directly after transplantation in all groups and all mice received ibuprofen (Banner Pharmacaps, High Point, USA; 0.2 mg/mL) as analgesic postsurgery for 2 days.

The nonfasting blood glucose levels were measured on a weekly basis to monitor the function of the islet graft. Animals were considered to be normoglycemic when blood glucose levels were below 150 mg/dL. When the blood glucose levels remained above 350 mg/dL or higher, the animal was euthanized by a cut through the heart and the scaffold was removed for histological analysis.

### Intraperitoneal glucose tolerance test

To determine the metabolic graft function in terms of glucose clearance, an IPGTT was performed in the second week after islet transplantation.[29] Before the IPGTT was started, mice were fasted overnight. At time point 0 an intraperitoneal glucose (3 g/kg; Sigma-Aldrich) injection was given to these mice. To determine the glucose concentration in the blood at time points 0, 10, 30, 60, 90, and 120 min after this injection, blood samples were collected from the tail and glucose concentration was measured with a Bayer Health Care blood glucose monitor. For analysis the glucose clearance was expressed as the area under the curve (AUC).[31]

### Histological examination

The scaffolds were removed 75 days after islet transplantation. After removal, the nonfasting blood glucose levels were measured for another week to determine graft dependency of the normoglycemia. After this week, the animals were sacrificed. Pancreas biopsies were taken to exclude pancreas regeneration. The islet scaffolds were fixed in fresh 2% paraformaldehyde (Merck Millipore) and processed for glycol methacrylate (GMA; Technovit® 8100; Heraeus Kulzer GmbH, Wehrheim, Germany) embedding. Sections of 2 μm were used for insulin staining. Briefly, sections were dried at 37°C and incubated in 0.01% trypsin [Sigma-Aldrich; in 6.8 mM 0.1% $CaCl_2$ (Merck Millipore) and 0.1*M* Tris–HCl (Merck Millipore), pH 7.8] for 10 min at 37°C. The sections were incubated with a mouse antirat insulin antibody [Sigma-Aldrich; 1:300 in PBS (Lonza) + 1% BSA (Sigma-Aldrich)] for 2 h at 37°C. After this incubation, the staining procedure was performed at 20°C. Nonspecific binding was blocked by 5 min incubation with 10% normal rabbit serum (Sigma-Aldrich). The secondary rabbit antimouse alkaline phosphatase conjugated antibody (Dako, Heverlee, Belgium; 1:100 in PBS + 1% BSA) was applied for 45 min. Alkaline phosphatase activity was demonstrated by incubating the sections for 10 min with SIGMAFAST™ Fast Red (Sigma-Aldrich). A short incubation with hematoxylin (Sigma-Aldrich) was used as counterstain. Furthermore, to observe inflammation and vascularization GMA sections were stained with 1% (w/v) aqueous toluidine blue (Fluka Chemika, Buchs, Switzerland) for 10 s.

The pancreas biopsies were processed for paraffin imbedding. Sections of 5 μm were used for an aldehyde fuchsin staining to determine the presence of viable of beta cells.[32] Briefly, sections were oxidized by 2.5% $KMnO_4$ (Sigma-Aldrich) and 5% $H_2SO_4$ (Merck) in demineralized water for 90 s and cleaned with 2% oxalic acid (Sigma-Aldrich) for 2 min. After an incubation of 2 min with 70% ethanol, sections were incubated with aldehyde fuchsin solution[32,33] for 10 min. An incubation of 6 min with Halmi [0.2% Light-green SF (Sigma-Aldrich) + 1% Orange G (Sigma-Aldrich) + 0.5% Chromotrope 2 R (Sigma-Aldrich) + 0.5% $H_2WO_4$ (Sigma-Aldrich) + 1% $CH_3COOH$ 100% (Merck Millipore) in demi water] was used as counterstain. The absence of beta-cell regeneration in the native pancreas was defined as $<5\%$ of normal controls. All above-mentioned stained sections were analyzed using a Leica DM 2000 LED microscope with a Leica DFC 450 camera (Leica Microsystems BV).

### Statistical analysis

Statistical analysis was carried out in GraphPad Prism (version 5.0b; GraphPad Software, La Jolla, USA). A Shapiro–Wilk normality test was performed to test the data for normality. For statistical analysis of the tube formation assay and IPGTT data a Kruskal–Wallis test with a Dunn's *post hoc* test was applied, $p < 0.05$ were considered significant. The data are respectively presented in mean ± standard deviation in case of parametric distribution and median ± interquartile range in case of nonparametric data distribution.

## RESULTS

### Superior angiogenic capacity by aFGF

Angiogenic growth factors with reported efficacy in stimulating vascularization[28] were compared. To this end, PDGF, VEGF, aFGF, and bFGF were compared in a HUVEC tube formation assay (Fig. 1). The formation of tubes [median %Area (quartile range)] was not stimulated by PDGF [1.1%Area (0.7–1.3)] and VEGF [1.0%Area (0.9–1.3)]; the tube forming capacity of these growth factors was similar to the control condition (set on 1). Addition of aFGF resulted in a two-fold increase of tube formation, 2.0%Area (1.5–2.5). This increase was significantly higher than the %Area of PDGF and VEGF ($p < 0.05$). Furthermore, bFGF slightly enhanced the tube formation to 1.3%Area (1.2–1.6). Based

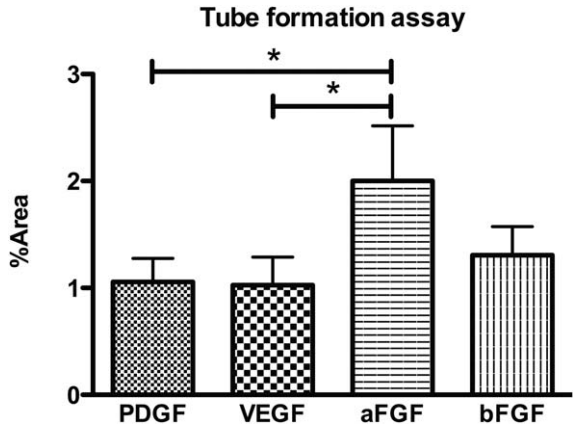


**FIGURE 1**. The effect of PDGF, VEGF, aFGF, and bFGF on the angiogenic capacity of HUVECs in a tube formation assay. PDGF, VEGF, aFGF, and bFGF were added to HUVECs for 24 h in a concentration of 5 ng/ml. The angiogenic activity was defined as the percentage of pixels that form tubes (%Area), the %Area was normalized to the control (control set on 1). HUVECs cultured in basal media without supplements were used as control. Median and interquartile range are plotted ($n = 5$), a statistical analysis was carried out using a Kruskal–Wallis test with a Dunn's *post hoc* test, $p < .05$ (*).

on these results, aFGF was selected for the follow up experiments.

### Design of liposomes for controlled release of aFGF

For delivery of the growth factor over a longer time period, liposomes were applied. As the release kinetics and loading capacity of liposomes can vary, several compositions of liposomes were tested.[34] Based on previous studies with growth factor containing liposomes,[26,35] we tested *in vitro* liposomes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/PC/cholesterol in a molar ratio of 50:50:5, PC/phosphatidylglycerol (PG)/cholesterol in a molar ratio of 50:50:5, and PC/cholesterol in a molar ratio of 60:40. The growth factor release kinetics of these three types of liposomes were determined and found to be similar. PC/cholesterol (60:40) liposomes were able to load slightly higher amounts of growth factor. The PC/cholesterol liposomes were therefore selected for the follow up experiments with aFGF.

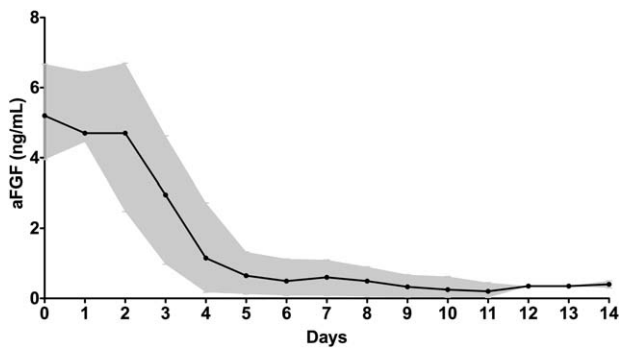


**FIGURE 2**. aFGF release from liposomes. The absolute release of aFGF from our liposomes during 14 days of incubation. Each well contained 0.6 μmol (±0.1) PC:CHOL liposomes and every day the 200 μl KRH was replaced. Data are plotted as median and interquartile range ($n = 4$).

The duration of release of aFGF was monitored *in vitro* to test if we obtained a sustained release of the growth factor (Fig. 2). At the start (day 0), liposomes released 5.2 ng/mL (4.0–6.7) aFGF and during the first 2 days the level was stable at 4.7 ng/mL (2.5–6.7). At day 3, the aFGF concentration decreased to 3.0 ng/mL (1.0–4.6) and this decrease continued till day 5, then the release stabilized at 0.7 ng/mL (0.1–1.3). From day 8 the concentration dropped below 0.5 ng/mL (0.1–0.9), slowly further decreasing till 0.4 ng/mL (0.3–0.5) at day 14. The total amount of aFGF released during the 14 days was 23.8 ng/mL (16.1–29.3).

### Restoration of blood glucose levels immediate following transplantation

Complete normoglycemia after rat islet transplantation was defined as blood glucose levels below 150 mg/dL. An islet graft of 800 islets was transplanted in PDLLCL scaffolds after a prevascularization period of 4 weeks. During this prevascularization period liposomes were injected once a week near the scaffold. The control group received a weekly injection of 100 μL empty liposomes. The 0.5 μg aFGF group received a 100 μL aFGF-loaded liposome injection and the 1.0 μg aFGF group received 200 μL aFGF loaded liposomes. The concentrations were chosen because 0.5 μg aFGF was most effective in enhancing the tube formation and in previous studies concentrations of 0.5–1.0 μg aFGF showed to improve islet engraftment.[36,37] Efficacy of the 800 islet-dose in sham-injected scaffolds was compared with that of scaffold treated with liposomes containing either 0.5 or 1.0 μg aFGF.

From the animals that received empty liposome injections 40% became normoglycemic within 15.5 ± 10.6 days (mean ± standard deviation) (Table II). This percentage increased up to 50% with a dose of 0.5 μg aFGF. The animals of this group became normoglycemic within 7.0 ± 8.5 days. Normoglycemia was obtained within 14.0 ± 11.0 days in 80% of the animals when the dose of aFGF was increased to 1.0 μg aFGF liposomes in the prevascularization period.

### aFGF administration is not compatible with long-term maintenance of normoglycemia

The animals with a successful graft after empty liposome injections remained normoglycemic for the duration of the study, which was a period of 75 days (Fig. 3). Although treatment with aFGF induced a faster return to normoglycemia, we observed an impediment in long-term efficacy of aFGF on graft function. The two animals treated with 0.5 μg aFGF in liposomes became normoglycemic for a period of 37.5 ± 1.5 days. After this period, blood glucose levels started to increase for this group. This happened after 36 days for just one of the four animals of the 1.0 μg aFGF group, the other three of this group remained normoglycemic for the duration of the experiment. This illustrates the aFGF concentration dependent effect. After 75 days, the islet grafts were removed and all animals returned to their

**TABLE II. Transplantation Success Rate**

| 0 μg aFGF | | 0.5 μg aFGF | | 1.0 μg aFGF | |
|---|---|---|---|---|---|
| Days: | Cure Rate: | Days: | Cure Rate: | Days: | Cure Rate: |
| 15.5 ± 10.6 | 2/5 (40%) | 7.0 ± 8.5 | 2/4 (50%) | 14.0 ± 11.0 | 4/5 (80%) |

The mean of days ± standard deviation post transplantation until normoglycemia occurs. Blood glucose values under 150 mg/dL were considered normoglycemic and therefore, also as a successful cure.

pretransplant hyperglycemic state (Fig. 3). As an additional control pancreas biopsies were studied but no regeneration was observed.

### Glucose tolerance changed over time

To test the metabolic function of the islet grafts an IPGTT was performed in the second week after transplantation (Fig. 4). After 2 weeks, both aFGF groups (0.5 and 1.0 μg) had a worse glucose clearance of respectively 20,445 (mg/dL) × min (19,505–21,385) and 29,220 (mg/dL) × min (22,130–31,424) compared to the control group (0 μg aFGF). The glucose clearance of the control group after 2 weeks was 15,180 (mg/dL) × min (13,746–16,614). The twofold increase after injections of 1.0 μg aFGF compared to the empty liposomes was statistically significant ($p < 0.05$).

### Histology

At the end of the experiment, differences were found between the histopathology of the different aFGF treatment groups. The control group, treated with empty liposomes, contained large numbers of insulin positive islets with a normal spherical shape, positioned in the clearly visible channels [Fig. 5(A,B)]. The islet channels were kept patent during the preimplantation period by polyethylene tubing and after 75 days the place of the tubing was easily found by the cell ingrowth into these channels. In the 0.5 μg aFGF group, a small number of insulin positive islets were found after 75 days [Fig. 5C,D]. However, large numbers of insulin positive islets were found in the 1.0 μg aFGF scaffold group [Fig. 5(E,F)].

Toluidine blue staining showed increased vascularization in the aFGF groups compared with the control group (Fig. 6). The number of blood vessels within the scaffold was enhanced in a dose-dependent matter [Fig. 6(A–C)]. In the scaffolds we found no differences in inflammation. Similar amounts and types of inflammatory cells were found in the scaffold. However, at the site where the liposomes were injected, a lot of collagen deposition was observed [Fig. 6(D)].

## DISCUSSION

By applying a stepwise approach in which we compared the angiogenic capacity of VEGF, PDGF, aFGF, and bFGF, and different liposome formulations, we designed a controlled release system for supporting vascularization. With this delivery system we were able to enhance the efficacy of our subcutaneous PDLLCL scaffold as transplantation site for islets in the immediate post-transplant period. The positive effects in the immediate post-transplant period were aFGF concentration dependent. However, long-term function was not improved by the vascularization protocol and was even worse in the aFGF treated scaffolds. After approximately 36 weeks the nonfasting blood glucose levels of the aFGF treated animals started to increase to diabetic levels, which did not happen in the untreated scaffolds. It was due to aFGF treatment and not by the liposomes injections as the controls received sham injections with empty liposomes and

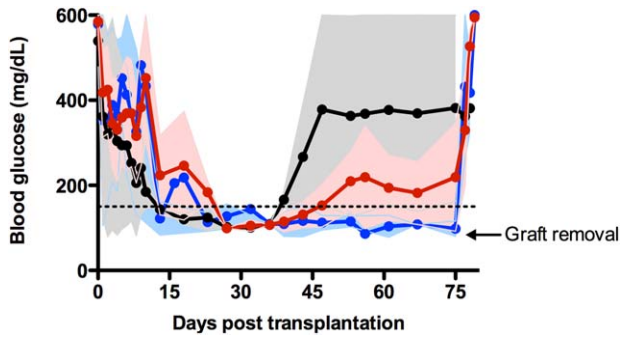


**FIGURE 3**. Nonfasting blood glucose post transplantation and aFGF injections. Blood glucose levels measured after islet transplantation into the subcutaneous scaffold. Normoglycemia was defined as blood glucose levels below 150 mg/dL (dotted line). The control group received empty liposomes (0 μg aFGF, blue line) on a weekly basis during 4 weeks before islet transplantation, the 0.5 μg group received liposomes loaded with 0.5 μg aFGF (black line), and the 1.0 μg group received the double amount of liposomes loaded with 0.5 μg aFGF (red line). After 75 days the scaffolds including the islet graft were removed (arrow). Blood glucose mean and standard deviation are plotted ($n = 2$ for 0 and 0.5 μg aFGF groups, $n = 4$ for 1.0 μg aFGF).

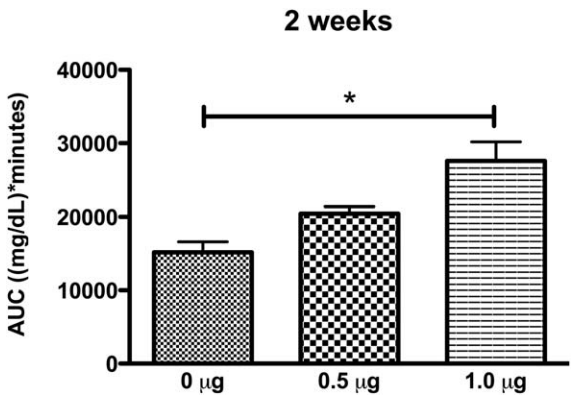


**FIGURE 4**. Glucose clearance after intraperitoneal glucose tolerance test. At 2 weeks after islet transplantation into the scaffolds, the glucose clearance was measured, this is expressed as area under the curve (mg/dL × min) for the 0 μg aFGF (control), 0.5 μg aFGF, and 1.0 μg aFGF groups. Data are plotted as median and interquartile range ($n = 5$), statistical differences between the groups were measured with a Kurskal–Wallis test with a Dunn's *post hoc* test, $p < 0.05$ (*).

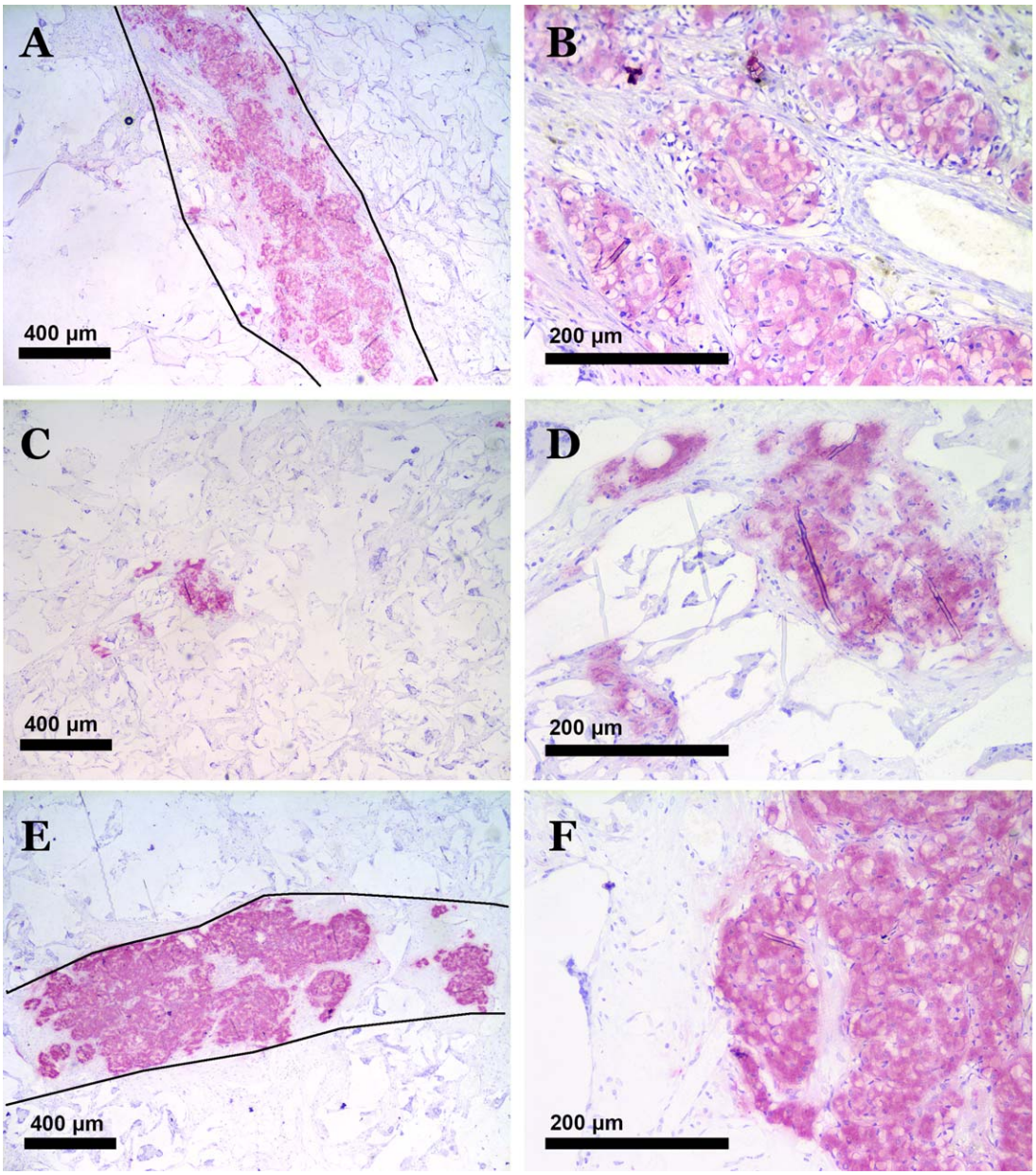


**FIGURE 5**. Insulin positive islets after 75 days in the scaffolds. Insulin staining (SIGMAFAST$^{TM}$ Fast Red) of 800 islets in the nontreated subcutaneous PDLLCL scaffold (A, 5×; B, 20×) 75 days after transplantation. The place of the islet channel (black lines) was clearly visible (A, E). A small number of insulin positive cells was found after 75 days in the 0.5 μg aFGF groups (C, 5×; D, 20×). Insulin positive cells (E, 5×; F, 20×) 75 days after transplantation into a scaffold treated with 1.0 μg aFGF.

remained normoglycemic. Furthermore, the IPGTT data showed that addition of aFGF did not improve the glucose clearance compared with the control.

We hypothesize that the vessels and endothelium attracted by aFGF have a different phenotype. More permeable may be an option but it is difficult to proof. We tried many things to identify the cause for long-term failure. One of the first things was a detailed study on the cell infiltrates and possible immune cells. We found no differences between the controls and aFGF treated scaffolds. This is also not a logical explanation as immediate graft function was improved by aFGF. A proinflammatory microenvironment results in slower return and not faster return to normoglycemia. We think is has to do with differences in the vessel phenotype.

An explanation for the difference in effects of aFGF administration in the immediate and long-term period might be that growth factors such as aFGF induce immediate vascularization, but that for sustained maintenance of the beneficial environment more or other angiogenic factors are required. This suggestion is corroborated by the observations of McQuilling et al.[25] and Khanna et al.[26] who found enhanced vascularization for aFGF-containing capsules implanted into the omental pouch. However, transplantation of islets in these aFGF microcapsules in the omental pouch

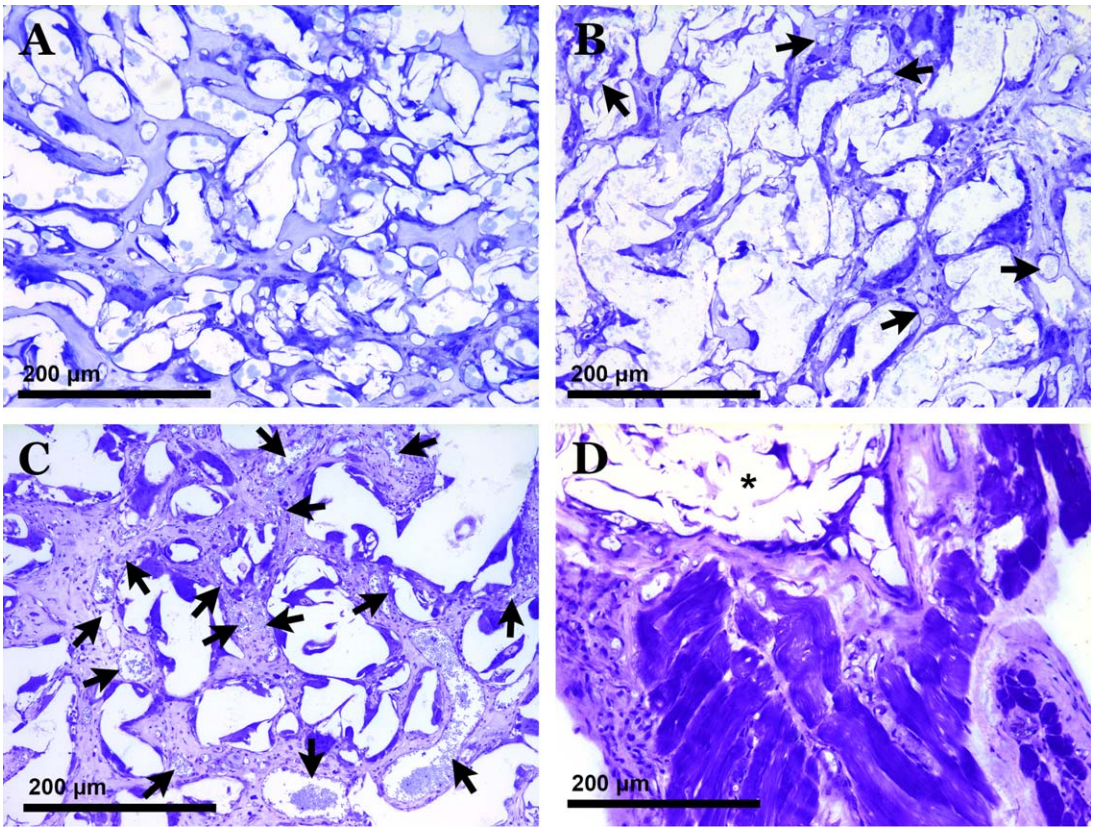


**FIGURE 6**. Histology 75 days after transplantation. Toluidine blue staining (20×) of scaffolds from the control group (A), 0.5 μg aFGF (B), and the 1.0 μg aFGF (C) group. Arrows indicate the blood vessels. (D) (20×) depicts the collagen deposition around the scaffolds (*).

did not result in better transplantation outcomes, c-peptide levels, and glycemic control was less beneficial than in controls not containing aFGF.[36] Their data and ours suggest that establishment of a stable vascular network is complex and might require several angiogenic factors to promote vessel sprouting and remodeling of the vascular network.[38] This is supported by clinical studies demonstrating even detrimental effects after injections of a single growth factor.[39–41]

Liposome injections may also have a negative effect on graft outcome in our study, since in our previous study,[42] transplantation of 800 islets into the preimplanted PDLLCL scaffold (in the absence of liposomes) induced normoglycemia in 80% of the recipients. This was only 40% in the current study where controls received empty liposomes. The large amount of collagen deposition observed in this study was not found in the previous study. This suggests that the four injection procedures or the characteristics of liposomes, that is, size, composition, surface properties, and charge, disturb the scaffold environment before transplantation. Therefore, our liposomes may not have the optimal characteristics for growth factor delivery before islet transplantation. In further research, these characteristics should be optimized. The liposomes itself should not negatively influence the vascularization process and ideally they should release growth factor for a month to exclude the multiple injections. After optimization of the liposomes, it could even be possible to immobilize the liposomes in the scaffold to discard the injections.

Acidic fibroblast growth factor is used in several clinical trials to promote angiogenesis for the treatment of diseases, such as spinal cord injury,[43] coronary artery disease,[44] and critical limb ischemia.[45] In this study, we demonstrated the superior angiogenic capacity of aFGF in the HUVEC tube formation assay. This was described before for endothelial cell assays.[46] To our opinion it is remarkable that in many studies VEGF is used as angiogenic therapeutic. VEGF is also known as vascular permeability factor and was initially described to induce vascular leakage and permeability besides its ability to promote proliferation of endothelial cells.[38,47] We propose to use aFGF as VEGF seems to be involved in the initial permeabilization of vessels and less in tube formation and further maturation of vessels. We are aware that any *in vitro* assay is not more than an indication of what happens *in vivo*. In general, the tube formation assay is considered to be a quick and quantifiable way of measuring angiogenesis *in vitro*.[48,49] It is a useful method to determine growth factors that potentially play a role *in vivo* vascularization. This assay was applied to select the most efficacious growth factor, as it is impossible to test all the factors *in vivo*. Also the *in vitro* growth factor release from liposomes does not reflect the release kinetics *in vivo*. However, this method is suitable for determining if we obtained a sustained release of growth factor.

Vascularization can occur by several mechanisms, including vasculogenesis and angiogenesis. Vasculogenesis occurs when endothelial progenitor cells migrate and form new blood vessels, whereas angiogenesis is the formation of new blood vessels from pre-existing blood vessels.[38,50] Vasculogenesis forms a primitive tubular network, but needs angiogenesis for forming a stable vascular system suggesting that both mechanisms are involved in vascularization of the scaffold. Vasculogenesis starts with the mobilization of angioblasts from the bone marrow by factors such as VEGF. The circulating angioblasts are recruited to the site of vascularization, where they differentiate and proliferate to form a vascular network. Angiogenesis is also initiated by growth factors, such as FGF and VEGF, resulting in the secretion of proteases and plasminogen activators by endothelial cells. These secreted factors increase the permeability of the vessels to allow migration of proliferating endothelial cells into the surrounding tissue. Angiopoietin-1 and ephrin-B2 are important for further maturation and remodeling of migrating endothelial cells into vessels. Angiopoietin-1 also maintains quiescence and stability after maturation of the vessels.[38,50] We believe that both pathways are important for a stable vascular network within our scaffold.

Our findings demonstrate that injections with aFGF liposomes do improve the immediate restoration of blood glucose levels but do not facilitate the long-term engraftment of transplanted islets in our PDLLCL scaffold or improve transplant efficacy in the subcutaneous site. For further research, combinations vasculogenesis and angiogenesis should be tested on long-term survival of islets in scaffolds. Weidling et al.[51] developed a technology by which vascularization can be observed in real time and in a noninvasive manner. This technology will give us more insight in the development of stable vasculature and will be helpful in testing different strategies to deliver growth factors.

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