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**RESEARCH PAPER**

# Erythropoietin accelerates the revascularization of transplanted pancreatic islets

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**Background and Purpose:** Pancreatic islet transplantation is a promising therapeutic approach for Type 1 diabetes. A major prerequisite for the survival of grafted islets is a rapid revascularization after transplantation. Erythropoietin (EPO), the primary regulator of erythropoiesis, has been shown to promote angiogenesis. Therefore, we investigated in this study whether EPO improves the revascularization of transplanted islets.

**Experimental Approach:** Islets from FVB/N mice were transplanted into dorsal skinfold chambers of recipient animals, which were daily treated with an intraperitoneal injection of EPO (500 IU·kg$^{-1}$) or vehicle (control) throughout an observation period of 14 days. In a second set of experiments, animals were only pretreated with EPO over a 6-day period prior to islet transplantation. The revascularization of the grafts was assessed by repetitive intravital fluorescence microscopy and immunohistochemistry. In addition, a streptozotocin-induced diabetic mouse model was used to study the effect of EPO-pretreatment on the endocrine function of the grafts.

**Key Results:** EPO treatment slightly accelerated the revascularization of the islet grafts. This effect was markedly more pronounced in EPO-pretreated animals, resulting in significantly higher numbers of engrafted islets and an improved perfusion of endocrine tissue without affecting systemic haematocrit levels when compared with controls. Moreover, EPO-pretreatment significantly accelerated the recovery of normoglycaemia in diabetic mice after islet transplantation.

**Conclusion and Implications:** These findings demonstrate that, particularly, short-term EPO-pretreatment represents a promising therapeutic approach to improve the outcome of islet transplantation, without an increased risk of thromboembolic events.

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# 1 | INTRODUCTION

Islet transplantation is a promising strategy for the curative treatment of Type 1 diabetes mellitus (McCall & Shapiro, 2012). In comparison to whole organ pancreas transplantation, the minimally invasive transplantation of Langerhans islets reduces the operative trauma and also the risk of complications induced by exocrine pancreatic tissue. However, only 50–70% of patients remain insulin-independent at 5 years after transplantation (Shapiro, Pokrywczynska, & Ricordi, 2017). This can be explained by the fact that the procedure is still hampered by the inefficiency of the islet isolation process and a low rate of successfully engrafted islets (Ahearn, Parekh, & Posselt, 2015). The latter is caused by an insufficient graft revascularization during the initial post-transplantation period, leading to apoptotic and necrotic cell death (Davalli et al., 1996; Schramm, Yamauchi, Vollmar, Vajkoczy, & Menger, 2003). Therefore, it is of great clinical interest to identify novel compounds, which are capable of accelerating the revascularization of transplanted islets.

The glycoprotein **erythropoietin (EPO)** is produced by the kidney and regulates the formation of red blood cells (RBCs) during haematopoiesis (Jelkmann, 1992). EPO binds to its corresponding receptor **EPO receptor**, which is mainly expressed on erythroid cells. Beside the function of EPO on haematopoiesis, several studies also reported a pro-angiogenic activity of this glycoprotein. In fact, **EPO receptor**s are also expressed on endothelial cells and the binding of EPO leads to a signal transduction triggering the expression of pro-angiogenic genes, such as those for **VEGF**, **angiopoietin-1 receptor (TIE)-2**, and **angiopoietin (ang)-2** (Alvarez Arroyo et al., 1998; Li, Lu, Keogh, Yu, & Wei, 2007). This stimulates endothelial cell proliferation and, thus, supports the formation of new blood vessels (Karschnia et al., 2018; Watanabe et al., 2005). EPO has also been shown to protect against hypoxia-induced apoptosis (Grimm et al., 2002; Harder et al., 2009; Heikal et al., 2016). Moreover, EPO increases the expression of **endothelial NOS**, resulting in elevated **NO** levels (Burger et al., 2006). This in turn leads to the generation of **cGMP**, which induces smooth muscle relaxation in blood vessels, resulting in an improved tissue perfusion (Katsuki & Murad, 1977).

Interestingly, Fenjves et al. (2003) reported that pancreatic islets express **EPO receptor**s and the cultivation of islets with EPO protects them from cytokine-induced apoptosis. In addition, EPO reduces pancreatic β-cell damage by promoting anti-oxidative processes (Chen et al., 2015; Choi et al., 2010), and EPO also decreases blood glucose levels in diabetic mouse models (Katz et al., 2010).

Based on these findings, we hypothesized that EPO would accelerate the revascularization and improve the engraftment of freely transplanted pancreatic islets. To test this, islets were isolated from GFP-positive FVB/N donor mice and transplanted into dorsal skinfold chambers of wild-type animals, which were treated or pretreated with EPO or vehicle. The cellular composition, the process of revascularization, and the take rate of the grafts were analysed by means of repetitive intravital fluorescence microscopy and immunohistochemistry. Finally, we assessed the effect of the glycoprotein on the endocrine function of transplanted islets in a diabetic mouse model.

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**What is already known**
- The rapid revascularization of isolated pancreatic islets is crucial for their successful transplantation.
- Erythropoietin exerts pro-angiogenic properties in vitro and in vivo.

**What this study adds**
- Pretreatment with erythropoietin accelerates the revascularization of transplanted pancreatic islets in FVB/N mice.
- Pretreatment with erythropoietin accelerates the restoration of normoglycaemia in mice with streptozotocin-induced diabetes.

**What is the clinical significance**
- Pretreatment with erythropoietin represents a promising pharmacological approach to improve the outcome of islet transplantation.

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# 2 | METHODS

## 2.1 | Animals

All animal care and experimental procedures complied with the German legislation on protection of animals and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington DC, USA), and were approved by the local governmental animal protection committee (permission number: 58/2015). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and with the recommendations made by the *British Journal of Pharmacology*.

For the dorsal skinfold chamber model, 12- to 24-week-old transgenic FVB/N-TgN (Tie2/GFP) 287 Sato mice (Institute for Clinical and Experimental Surgery, Homburg/Saar, Germany) with a body weight of 25–30 g were used as donors for islet isolation. These mice express the reporter gene GFP under the transcriptional control of the endothelial TIE2 promotor (Motoike et al., 2000). Eight- to 10-week-old FVB/N wild-type mice with a body weight of 22–27 g served as recipients for islet transplantation. For the kidney capsule model, we used 12- to 24-week-old C57BL/6J mice (Institute for Clinical and Experimental Surgery, Homburg/Saar, Germany) with a body weight of 25–30 g as donors for islet isolation. Eight-week-old male C57BL/6J mice with a body weight of 25–26 g served as recipients. All mice were housed one per cage under a 12/12-hr day/night cycle and had free access to water and standard pellet chow (Altromin, Lage, Germany). At the end of experiments, the animals were killed by cervical dislocation.

## 2.2 | Isolation of pancreatic islets

Mice were anaesthetized by i.p. injection of ketamine (75 mg·kg$^{-1}$ body weight) and xylazine (25 mg·kg$^{-1}$ body weight). Following midline laparotomy, the pancreatic duct was injected with 1 mg·ml$^{-1}$ of collagenase NB 4G containing 25 μl·ml$^{-1}$ of neutral red solution, and pancreatic islets were isolated as described previously in detail (Beger, Cirulli, Vajkoczy, Halban, & Menger, 1998).

## 2.3 | Dorsal skinfold chamber

To analyse the revascularization of transplanted islets in vivo, we used the dorsal skinfold chamber model. This represents a suitable model to study non-invasively the microcirculatory parameters of striated muscle and s.c. tissue throughout an observation period of 2 weeks (Laschke & Menger, 2016). Moreover, we and others have shown that the dorsal skinfold chamber model can be used to investigate angiogenic processes of transplanted islets (Menger, Vajkoczy, Beger, & Messmer, 1994; Nalbach et al., 2019; Nishimura et al., 2013). The chamber was prepared and implanted as described previously in detail (Laschke, Vollmar, & Menger, 2011). Briefly, mice were anaesthetized as above, and two symmetrical titanium frames were implanted on the extended dorsal skinfold of the animals. Skin, subcutis, and the retractor muscle were completely removed in a circular area of 15 mm. The remaining layers, consisting of striated muscle, subcutis, and cutis, were covered by a removable cover glass, providing direct microscopic access to the microcirculation of the chamber. After the procedure, the animals were allowed to recover for a 72-hr period.

For the transplantation of pancreatic islets, mice were anaesthetized, the cover glass was removed, and the chamber was washed twice with saline. Then, six to eight freshly isolated islets were transplanted onto the striated muscle tissue. After transplantation, the chamber was sealed again with a new cover glass.

## 2.4 | Intravital fluorescence microscopy

The anaesthetized mice were fixed on a plexiglas stage and received a retrobulbary i.v. injection of 0.05-ml 5% fluorescein isothiocyanate (FITC)-dextran for contrast enhancement by staining of blood plasma. Moreover, 0.05-ml 2% rhodamine 6G, which accumulates in endocrine but not in striated muscle tissue by extravasation from fenestrated endothelium, was given i.v. for the visualization of endocrine revascularization (Vajkoczy, Menger, Simpson, & Messmer, 1995). Then, the dorsal skinfold chamber was positioned under a Zeiss microscope (Zeiss, Oberkochen, Germany) with a 100-W mercury lamp attached to a blue (excitation wavelength: 450–490 nm/emission wavelength: >515 nm) and a green (530–560 nm/>585 nm) filter block. The microscopic images were recorded by a charge-coupled device video camera (FK6990; Pieper, Schwerte, Germany) and transferred to a monitor (Trinitron; Sony, Tokyo, Japan) and a DVD system (DVD-HR775; Samsung, Eschborn, Germany) for offline evaluation.

Quantitative analysis of the microscopic images was performed by the computer-assisted image analysis system CapImage (Zeintl, Heidelberg, Germany) and included the determination of the graft initial size (mm$^2$), revascularized area (mm$^2$), functional capillary density (cm·cm$^{-2}$), and endocrine revascularization, as previously described (Ampofo et al., 2015; Menger, Vajkoczy, Leiderer, Jager, & Messmer, 1992). We further measured microhaemodynamic parameters, such as diameter (μm), centreline RBC velocity (μm·s$^{-1}$), and volumetric blood flow (pl·s$^{-1}$), of individual microvessels within the grafts (Ampofo et al., 2015; Menger et al., 1992). Moreover, we assessed the take rate (%), that is, the fraction of engrafted islets in relation to the number of transplanted islets on Day 14. Finally, we investigated the diameter of first-, second-, and third-order arterioles and venules of the chamber tissue to analyse EPO-induced vasodilatation.

## 2.5 | Diabetic mouse model

C57BL/6J mice were anaesthetized, and the kidney was exposed by an incision of the right flank. A small nick was made in the kidney capsule with the bevel of a 10-μl Hamilton syringe over the inferior renal pole. The islets were then deposited under the capsule through the nick towards the superior pole of the kidney. The kidney was returned to the abdomen, and the incisions in the abdominal wall and the skin were closed. After the procedure, the mice received a s.c. injection of carprofen (5 mg·kg$^{-1}$ body weight). Blood glucose levels were monitored at the indicated time points by a blood glucose monitoring system (Beurer Medical, Germany). Hyperglycaemia was defined by blood glucose >12.5 mmol·l$^{-1}$.

## 2.6 | Experimental protocol

Dorsal skinfold chamber model: Pancreatic islets were isolated from 10 GFP-positive FVB/N donor mice. A total number of 32 FVB/N wild-type mice were equipped with dorsal skinfold chambers. The mice were assigned to four randomized experimental groups of eight animals each. In a first set of experiments, EPO (500 IU·kg$^{-1}$ body weight, i.p.) or vehicle (ctrl; 100-μl saline, i.p.) was given daily, starting from the day of islet transplantation (Day 0) until the end of the observation period (Day 14). In a second set of experiments, the animals were pretreated daily with the identical dose of EPO (500 IU·kg$^{-1}$ body weight, i.p.; EPO-pre) or vehicle (100-μl saline, i.p.; ctrl-pre) over 6 days prior to the islet transplantation. Repetitive intravital fluorescence microscopy was performed on Days 0, 3, 6, 10, and 14 after islet transplantation. At the end of the experiments, blood samples were drawn from the vena cava, and the haematocrit was determined by a haematology analyser (Abaxis VetScan HM5; scil animal care company GmbH, Viernheim, Germany). Subsequently, the islet-containing chamber tissue was excised for further immunohistochemical analyses. Moreover, three additional vehicle- and EPO-pretreated mice were already killed on Day 6 after islet transplantation for immunohistochemical analyses of early engrafted islets. The animal

experiments and the histological analysis were performed under blinded conditions.

Diabetic mouse model: Seven days before transplantation, a diabetic phenotype was induced in recipient mice by an injection of 150 mg·kg$^{-1}$ i.p. streptozotocin (STZ) in citrate buffer, pH 6.4. In addition, mice were pretreated with EPO (500 IU·kg$^{-1}$ body weight, i.p.; EPO-pre) or vehicle (100 μl saline, i.p.; ctrl-pre) over 6 days before transplantation. Blood glucose levels were measured before STZ application as well as 4 days after the STZ injection. Mice with a blood glucose level > 12.5 mmol·l$^{-1}$ and a body weight of 24–26 g were used as recipients for islet transplantation. Seven days after STZ application, a suboptimal number of islets (300) that did not restore normoglycaemia within 10 days was transplanted. After 28 days, the kidneys were excised for immunohistochemical analyses.

## 2.7 | Plasma insulin content

The insulin content of plasma samples was quantified by means of an ELISA kit from Thermo Fisher Scientific (Braunschweig, Germany), according to the manufacturer's instructions.

## 2.8 | Immunohistochemistry

For the preparation of histological sections, specimens of islet-containing dorsal skinfold chamber or kidney capsule preparations were fixed for 24 hr in 4% formalin. In addition, freshly isolated islets were incubated for 45 min at 37°C in 100-μl HepatoQuick®, 50-μl human citrate plasma, and 10-μl 10% CaCl$_2$ solution. The resulting clot was also fixed for 24 hr in 4% formalin. The formalin-fixed specimens were then embedded in paraffin, and 3-μm-thick sections were cut.

The antibody-based procedures used in this study comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). The sections were stained with anti-insulin, anti-glucagon, anti-somatostatin, anti-CD31 and anti-GFP antibodies, which were detected by corresponding fluorescence-coupled secondary antibodies. Cell nuclei were stained with Hoechst 33342. The sections were analysed by means of fluorescence microscopy (BX60F; Olympus, Hamburg, Germany). The numbers of insulin-, glucagon-, somatostatin-, and CD31-positive cells were counted using ImageJ software (ImageJ, RRID:SCR_003070) and given in % of all islet cells. In addition, the numbers of GFP/CD31-positive cells were counted and given in % of all CD31-positive cells.

## 2.9 | Data and statistical analysis

We decided sample size for animal studies based on a survey of data from published studies. The number of mice was not predetermined by a statistical method, and no mice were excluded from statistical analysis. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2015). After testing the data for normal distribution and equal variance, differences between two groups were assessed by the unpaired Student's *t* test. To test for time effects within individual groups, one-way ANOVA for repeated measures was applied. This was followed by the Student–Newman–Keuls post hoc test, including the correction of the α-error according to Bonferroni probabilities to compensate for multiple comparisons (SigmaPlot, RRID:SCR_003210). All values are expressed as mean ± SEM. Statistical significance was accepted for $P < .05$. The declared group size is the number of independent values, and the statistical analysis was done using these independent values.

## 2.10 | Materials

Collagenase NB 4G was purchased from SERVA Elektrophoresis GmbH (Heidelberg, Germany). Neutral red solution, FITC-dextran 150,000, rhodamine 6G, Hoechst 33342, STZ and avidin-peroxidase were purchased from Sigma-Aldrich (Taufkirchen, Germany). Mayer's hemalaun solution was received from Merck (Darmstadt, Germany), ketamine (Ursotamin®) from Serumwerke Bernburg (Bernburg, Germany) and xylazine (Rompun®) from Bayer (Leverkusen, Germany). HepatoQuick® and EPO-β (recombinant human EPO, NeoRecormon®) were purchased from Roche (Basel, Switzerland). The antibody anti-CD31 (Dianova Cat# DIA-310, RRID: AB_2631039) was received from Dianova (Germany), the antibody anti-GFP from Rockland Immunochemical Inc. (Limerick, USA) and the antibodies anti-insulin, anti-glucagon and anti-somatostatin as well as 3-amino-9-ethylcarbazole (AEC Substrate System) from Abcam (Cambridge, UK).

## 2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

## 3 | RESULTS

### 3.1 | Characteristics of isolated islets before and after transplantation

Islets were isolated and stained for the detection of insulin-, glucagon-, somatostatin-, and CD31-positive cells (Figure 1a–d). The quantitative analysis of these stains revealed that ~70% of the cells
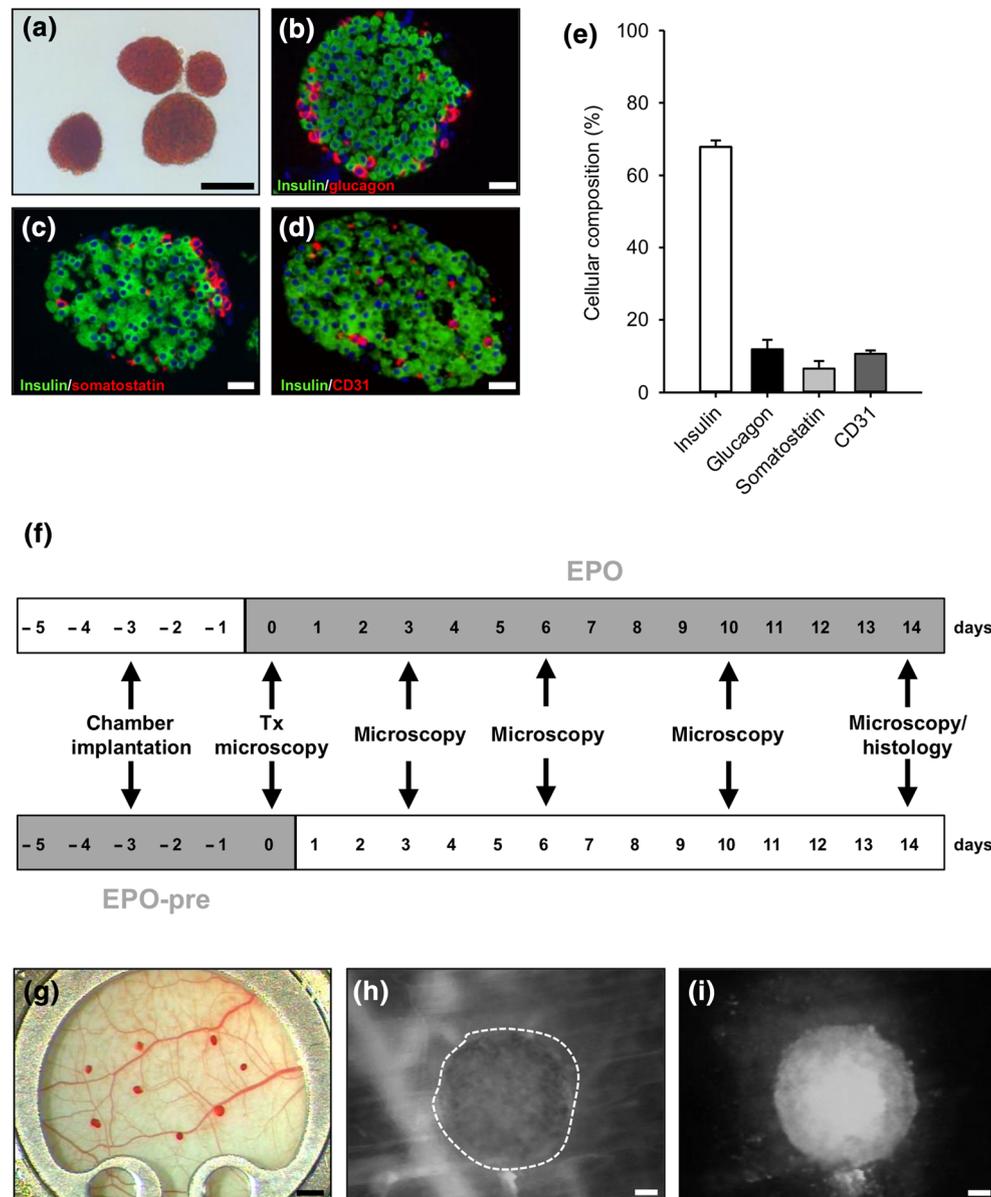
**FIGURE 1** Characteristics of isolated islets before and after transplantation. (a) Bright field microscopy of neutral red-stained isolated islets from GFP$^+$/Tie$^+$ FVB/N donor mice. Scale bar: 200 μm. (b–d) Immunofluorescence staining of insulin/glucagon (b), insulin/somatostatin (c), and insulin/CD31 (d) within isolated islets. Cell nuclei were stained with Hoechst 33342 (blue). Scale bar: 25 μm. (e) Insulin-, glucagon-, somatostatin-, and CD31-positive cells (in % of all islet cells) within isolated islets ($n$ = 10). Mean ± SEM. (f) Schematic illustration of the experimental setting of EPO administration and islet transplantation. In a first set of experiments, EPO or vehicle was administered daily, starting from the day of islet transplantation (Tx, Day 0) until the end of the observation period (Day 14). In a second set of experiments, the animals were only pretreated daily with EPO or vehicle over 6 days prior to islet transplantation (EPO-pre). Intravital fluorescence microscopy was performed on Days 0, 3, 6, 10, and 14 after islet transplantation. (g) Observation window of a dorsal skinfold chamber containing eight transplanted neutral red-stained islets on Day 0. Scale bar: 1 mm. (h,i) Intravital fluorescence microscopic images of a neutral red-stained islet within the dorsal skinfold chamber of a vehicle-treated animal on Day 0. The plasma marker FITC-dextran was used for the visualization of blood-perfused microvessels surrounding the islet (marked by dotted line) in blue light epi-illumination (h). The islet was detected by neutral red-fluorescence in green light epi-illumination (i). Scale bar: 50 μm

within isolated islets were positive for insulin, whereas ~10% expressed glucagon, ~5% somatostatin, and ~10% the endothelial marker CD31 (Figure 1e).

The effect of EPO on the revascularization of transplanted islets was investigated in the mouse dorsal skinfold chamber model. For this

purpose, the recipient animals were either treated with EPO or vehicle, starting from the day of islet transplantation until the end of the observation period, or pretreated over 6 days prior to islet transplantation (Figure 1f). Using intravital fluorescence microscopy, the position of the grafts within the host tissue of the chamber could easily be
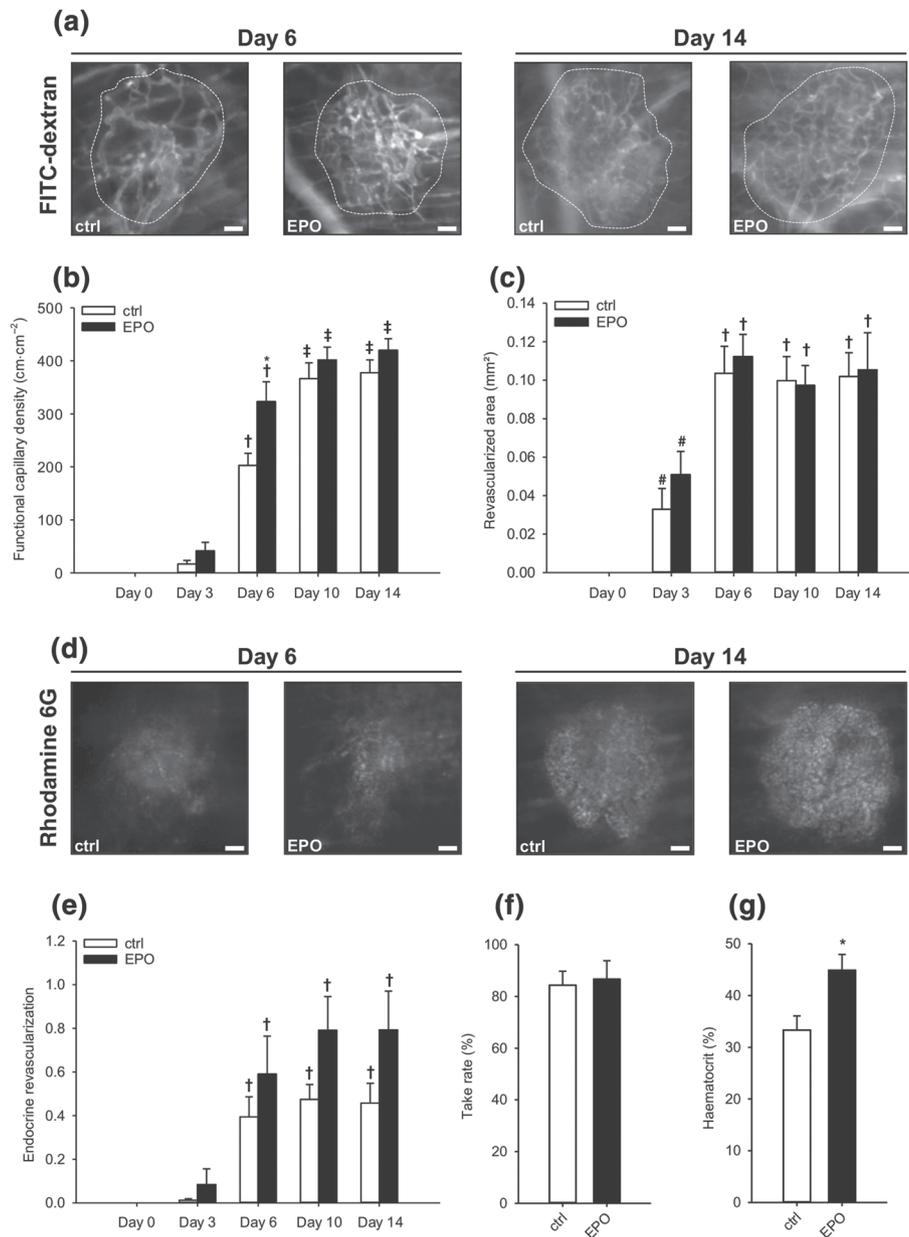
**FIGURE 2** Effect of EPO treatment on the revascularization of transplanted islets. (a) Intravital microscopic images of islets (marked by dotted lines) on Days 6 and 14 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were treated with vehicle (ctrl) or EPO. The plasma marker FITC-dextran was used for the visualization of microvessels in blue light epi-illumination. Scale bar: 50 μm. (b,c) Functional capillary density (cm·cm$^{-2}$) (b) and revascularized area (mm$^2$) (c) of islets on Days 0, 3, 6, 10, and 14 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were treated with vehicle (ctrl, $n = 8$) or EPO ($n = 8$). Mean ± SEM. $^{#}P < .05$, significantly different from Day 0 in each individual group; †$P < .05$, significantly different from Days 0 and 3 in each individual group; ‡$P < .05$, significantly different from Days 0, 3, and 6 in each individual group; $^{*}P < .05$, significantly different from ctrl. (d) Intravital microscopic images of islets as described in (a). Rhodamine 6G was used to visualize endocrine revascularization in green light epi-illumination. Scale bar: 50 μm. (e) Endocrine revascularization of islets on Days 0, 3, 6, 10, and 14 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were treated with vehicle (ctrl, $n = 8$) or EPO ($n = 8$). Mean ± SEM. † $P < .05$, significantly different from Days 0 and 3 in each individual group. (f,g) Take rate (%) (f) and systemic haematocrit (%) (g) on Day 14 after islet transplantation into the dorsal skinfold chamber of FVB/N mice, which were treated with vehicle (ctrl, $n = 8$) or EPO ($n = 8$). Mean ± SEM. $^{*}P < .05$, significantly different from vehicle

determined by their strong neutral red-staining on Day 0 (Figure 1g–i). Of note, the initial size of the transplanted islets did not markedly differ between the experimental groups (EPO: 0.08 ± 0.01 mm$^2$ vs. ctrl: 0.11 ± 0.02 mm$^2$ and EPO-pre: 0.10 ± 0.01 mm$^2$ vs. ctrl-pre: 0.10 ± 0.02 mm$^2$).

## 3.2 | Effect of EPO treatment on the revascularization of transplanted islets

In the first set of experiments, we examined the efficiency of EPO treatment in accelerating the revascularization of transplanted islets.

We found that the functional capillary density and revascularized area of the grafts progressively increased in vehicle- and EPO-treated animals (Figure 2a–c). EPO slightly accelerated the revascularization process, as shown by a significantly higher functional capillary density on Day 6 when compared to controls (Figure 2a,b). The diameters of the microvessels within the islets of both the vehicle- and the EPO-treated animals decreased over time, which is a typical sign for blood vessel stabilization and maturation (Diez et al., 2017). The centreline RBC velocity and volumetric blood flow increased throughout the observation period of 14 days without significant differences between the groups (Table 1).

Next, we assessed the effect of EPO treatment on the islets' endocrine revascularization; that is, the ratio between the size of the microvascularly perfused endocrine tissue and the original islet size after transplantation (Figure 2d,e). EPO treatment increased the endocrine revascularization of the islets, but without a significant difference compared with controls (Figure 2e). Moreover, EPO treatment did not improve the take rate of the grafts, which was ~85% in both groups (Figure 2f). Finally, we found that the daily administration of EPO over 14 days did significant raise the systemic haematocrit levels of the animals (Figure 2g).

## 3.3 | Effect of EPO-pretreatment on the revascularization of transplanted islets

In the second set of experiments, mice were only pretreated with EPO over 6 days prior to islet transplantation. Of interest, this short-term pretreatment was much more effective when compared to the administration of EPO during the first 14 days after transplantation. We detected a significantly higher functional capillary density during the first 10 days after transplantation and a markedly larger revascularized islet area in EPO-pretreated mice when compared with controls (Figure 3a–c). Consistent with these findings, EPO also significantly improved the endocrine revascularization of the grafts during the entire observation period (Figure 3d,e). The additional analysis of

microhaemodynamic parameters revealed an accelerated decrease of the diameter as well as an increase of the centreline RBC velocity and the volumetric blood flow of microvessels within transplanted islets of EPO-pretreated animals (Table 2). Furthermore, the number of successfully engrafted islets was significantly elevated in EPO-pretreated animals when compared with controls, as indicated by a take rate of almost 100% (Figure 3f). In contrast to EPO treatment (Figure 2g), the 6-day administration of the glycoprotein prior to islet transplantation did not elevate the systemic haematocrit levels of the animals at the end of the 14-day observation period (Figure 3g).

## 3.4 | Effect of EPO on vasodilatation

We further determined the diameter of microvessels within the dorsal skinfold chamber of EPO-treated and EPO-pretreated animals. Our analyses revealed that EPO-pretreatment significantly increases the diameter of arterioles and venules during the first 6 days after islet transplantation when compared to vehicle-pretreated controls (Figure 4a–d). Of note, this vasodilator effect of the glycoprotein occurred in EPO-treated animals only 6 days after islet transplantation (Figure 4e,f).

## 3.5 | Immunohistochemical analysis of transplanted islets

Because the revascularization of grafted islets was much improved in EPO-pretreated mice, compared with EPO-treated animals, we only analysed the islet grafts of the pretreated mice in more detail by immunohistochemistry. These analyses confirmed our intravital fluorescent microscopic findings. On Day 6 after transplantation, islets in EPO-pretreated mice exhibited a significantly higher fraction of CD31-positive endothelial cells, compared with controls (Figure 5a,b), indicating an improved revascularization at this early time point. As expected, this difference was not present on Day 14 after islet

**TABLE 1** Diameter (μm), centreline RBC velocity (μm·s$^{-1}$), and volumetric blood flow (pl·s$^{-1}$) of newly formed microvessels within islets on Days 3, 6, 10, and 14 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were treated with vehicle (ctrl, $n = 8$) or EPO ($n = 8$)

| | D3 | D6 | D10 | D14 |
|---|---|---|---|---|
| Diameter (μm) | | | | |
| ctrl | 11.8 ± 1.8 | 12.1 ± 0.8 | 9.4 ± 0.5[#] | 9.1 ± 0.2[#] |
| EPO | 12.4 ± 0.7 | 10.7 ± 0.6* | 9.6 ± 0.4* | 9.5 ± 0.5* |
| Centreline RBC velocity (μm·s$^{-1}$) | | | | |
| ctrl | 51.9 ± 31.7 | 330.6 ± 54.8* | 431.0 ± 67.1* | 425.9 ± 69.4* |
| EPO | 73.0 ± 40.6 | 322.8 ± 57.5[8] | 337.8 ± 75.7* | 236.0 ± 74.7[8] |
| Volumetric blood flow (pl·s$^{-1}$) | | | | |
| ctrl | 3.1 ± 1.8 | 30.3 ± 5.7* | 28.7 ± 6.8* | 23.0 ± 3.8* |
| EPO | 7.6 ± 4.3 | 25.4 ± 4.7* | 21.5 ± 3.7* | 12.5 ± 4.1* |

*Note.* All data are mean ± SEM.
*$P < .05$, significantly different from Day 3.
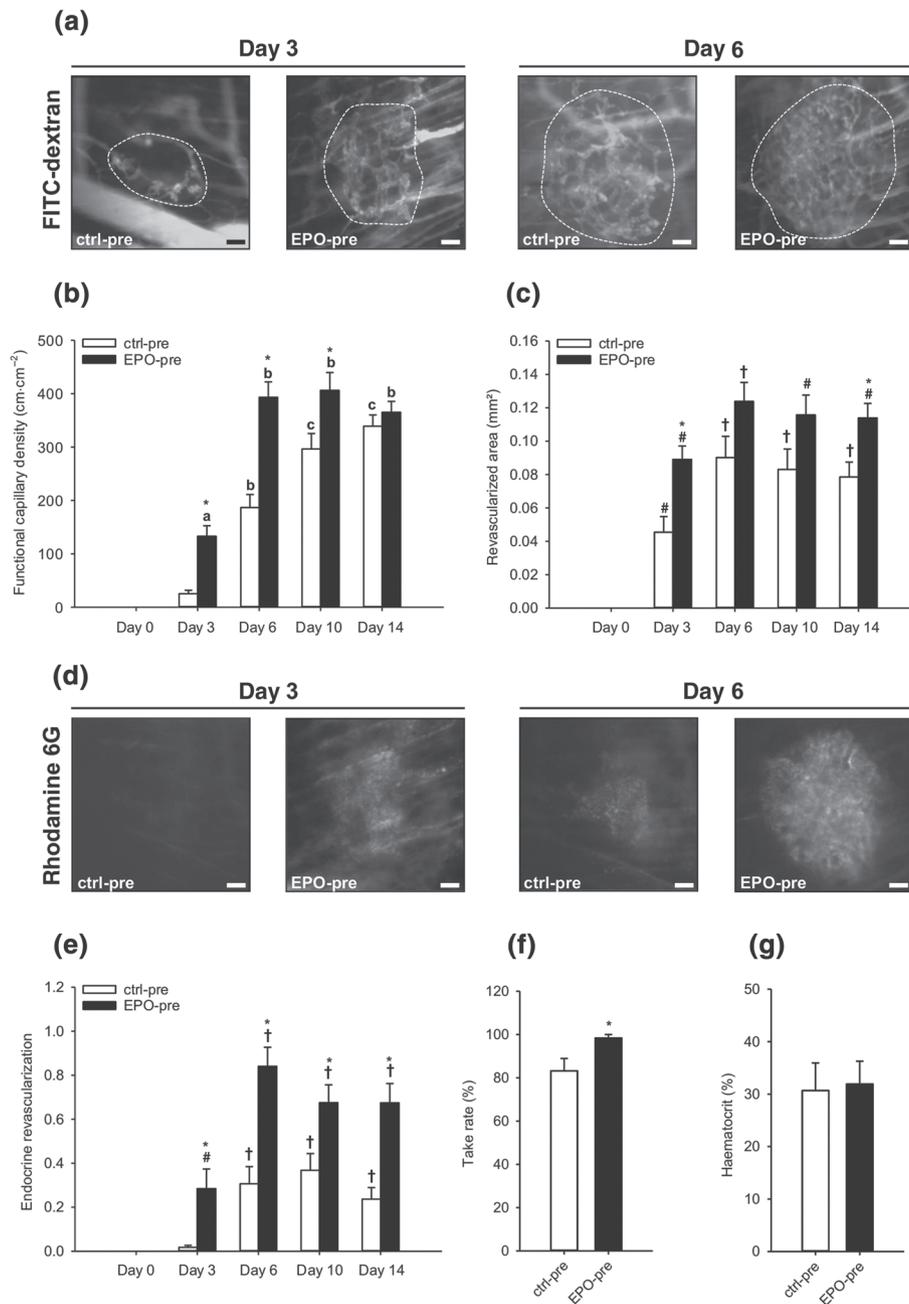[#]$P < .05$. significantly different from Day 6.

**FIGURE 3** Effect of EPO-pretreatment on the revascularization of transplanted islets. (A) Intravital microscopic images of islets (marked by dotted lines) on Days 3 and 6 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre) or EPO (EPO-pre). The plasma marker FITC-dextran was used for the visualization of microvessels in blue light epi-illumination. Scale bar: 50 μm. (b,c) Functional capillary density (cm·cm$^{-2}$) (b) and revascularized area (mm$^2$) (c) of islets on Days 0, 3, 6, 10, and 14 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre, n = 8) or EPO (EPO-pre, n = 8). Mean ± SEM. [#]$P$ < .05, significantly different from Day 0 in each individual group; †$P$ < .05 versus Days 0 and 3 in each individual group; ‡$P$ < .05 versus Days 0, 3, and 6 in each individual group; *$P$ < .05, significantly different from ctrl-pre. (d) Intravital microscopic images of islets as described in (a). Rhodamine 6G was used to visualize endocrine revascularization in green light epi-illumination. Scale bar: 50 μm. (e) Endocrine revascularization of islets on Days 0, 3, 6, 10, and 14 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre n = 8) or EPO (EPO-pre, n = 8). Mean ± SEM. [#]$P$ < .05, significantly different from Day 0 in each individual group; †$P$ < .05, significantly different from Days 0 and 3 in each individual group; *$P$ < .05, significantly different from ctrl-pre. (f,g) Take rate (%) (f) and systemic haematocrit (%) (g) on Day 14 after islet transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre, n = 8) or EPO (EPO-pre, n = 8). Mean ± SEM. *$P$ < .05, significantly different from ctrl-pre

**TABLE 2** Diameter (μm), centreline RBC velocity (μm·s$^{-1}$), and volumetric blood flow (pl·s$^{-1}$) of newly formed microvessels within islets on Days 3, 6, 10, and 14 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre, $n = 8$) or EPO (EPO-pre, $n = 8$)

| | D3 | D6 | D10 | D14 |
|---|---|---|---|---|
| Diameter (μm) | | | | |
| ctrl-pre | 11.2 ± 1.6 | 11.9 ± 0.6 | 9.0 ± 0.5[†] | 8.8 ± 0.4[†] |
| | 12.0 ± 0.6 | 9.0 ± 0.3[#] * | 8.2 ± 0.4[#] | 8.7 ± 0.7[#] |
| Centreline RBC velocity (μm·s$^{-1}$) | | | | |
| ctrl-pre | 73.1 ± 31.9 | 240.5 ± 28.4[#] | 366.4 ± 62.4[#] | 379.2 ± 96.0[#] |
| EPO-pre | 295.6 ± 72.4* | 571.2 ± 75.6[#] * | 671.5 ± 78.2[#] * | 551.8 ± 61.3[#] |
| Volumetric blood flow (pl·s$^{-1}$) | | | | |
| ctrl-pre | 6.6 ± 4.0 | 18.5 ± 1.7[#] | 18.0 ± 2.9 | 17.4 ± 3.6 |
| EPO-pre | 30.9 ± 8.1* | 32.0 ± 5.1* | 32.2 ± 7.1 | 31.7 ± 6.6 |

*Note.* All data are mean ± SEM.

[#]$P < .05$, significantly different from Day 3. [†]$P < .05$, significantly different from Days 3 and 6. [*]$P < .05$, significantly different from vehicle.

transplantation, when the revascularization process was completed in both groups (Figure 5c,d). The analysis of insulin-, glucagon-, and somatostatin-positive cells revealed no significant differences between EPO-pretreated and control grafts on Days 6 and 14 (Figure 5a–d).

Transplantation of islets from the GFP/Tie2-positive donor mice into the GFP-negative animals further allowed the analysis of the origin of the endothelial cells within the grafts (Figure 5e–g). These analyses showed that the islets in EPO-pretreated mice contained a significantly lower fraction of GFP/CD31-positive endothelial cells on Day 6, compared with controls (Figure 5e,g). In contrast, there was no significant difference in the fraction of GFP/CD31-positive endothelial cells between the two groups on Day 14 (Figure 5f,g).

To assess the effect of EPO-pretreatment on the endocrine function of the grafts, we performed a suboptimal transplantation of islets under the kidney capsule of diabetic mice. For this purpose, we first treated mice with STZ to induce a diabetic phenotype. Six days before transplantation of 300 islets under the kidney capsule, the animals were pretreated with EPO or vehicle (Figure 6a). Blood glucose levels and body weight of the recipient mice were repetitively determined (Figure 6a). STZ-induced diabetic mice of both groups showed an initial decrease in body weight due to the STZ treatment (Figure 6b). However, after transplantation, they recovered and gained body weight until the end of the experiment (Figure 6b). Analyses of blood glucose levels revealed a STZ-induced hyperglycaemia, as indicated by blood glucose levels up to 25 mmol·L$^{-1}$ before transplantation (Figure 6c). The transplantation of islets resulted in a reduction of blood glucose levels throughout the observation period of 28 days. Noteworthy, on Days 7 and 10 after EPO-pretreatment, blood glucose levels were found significantly more reduced than those in controls. This led to an accelerated recovery to normoglycaemia (Figure 6c). In addition, the AUC of blood glucose levels was slightly but not significantly reduced in EPO-pretreated mice (Figure 6d). The analyses of plasma samples after 28 days demonstrated that the insulin levels of EPO-pretreated animals did not significantly differ from that of controls (Figure 6e). Moreover, after 28 days, the glycoprotein did not alter the β-cell mass and the number of endothelial cells within the islet grafts (Figure 6f,g).

## 4 | DISCUSSION

The aim of the present study was to analyse the effects of EPO on the revascularization of transplanted pancreatic islets. Our results demonstrated that EPO accelerated the revascularization of transplanted islets, resulting in an increased take rate of the grafts and an improved perfusion of the endocrine tissue. Of interest, this beneficial effect of EPO is especially pronounced if the glycoprotein is administered prior to islet transplantation.

Insufficient revascularization is thought to be the major cause for the poor survival of grafted islets during islet transplantation for the therapy of Type I diabetes (Brissova & Powers, 2008). To overcome this problem, previous studies suggested the co-transplantation of stem cells or endothelial cells with islets to enhance the angiogenic potential of the grafts (Quaranta et al., 2014; Rackham et al., 2011). However, the application and manipulation of specific cell types is difficult to realize under clinical conditions not only due to the regulatory hurdles but also due to time-consuming isolation and cultivation procedures and difficulties in the standardization of cell quality and islet yield. Accordingly, the use of pharmacological drugs, which specifically stimulate angiogenic pathways, may be a more feasible strategy to improve islet engraftment in clinical practice. Hence, it is necessary to identify novel pro-angiogenic compounds, which are effective for this approach without inducing severe side effects.

The haematopoietic cytokine EPO is required for the generation of RBCs in response to low tissue oxygen levels. EPO binds to its corresponding receptor on erythroid precursor cells and stimulates their survival, proliferation, and differentiation via signalling through kinases such as JAK2, MAPK, and PI3K (Elliott & Sinclair, 2012). Interestingly, studies have demonstrated that beside this effect on erythropoiesis, EPO also promotes angiogenesis (Anagnostou, Lee, Kessimian, Levinson, & Steiner, 1990). For instance, Ribatti et al. (1999) found
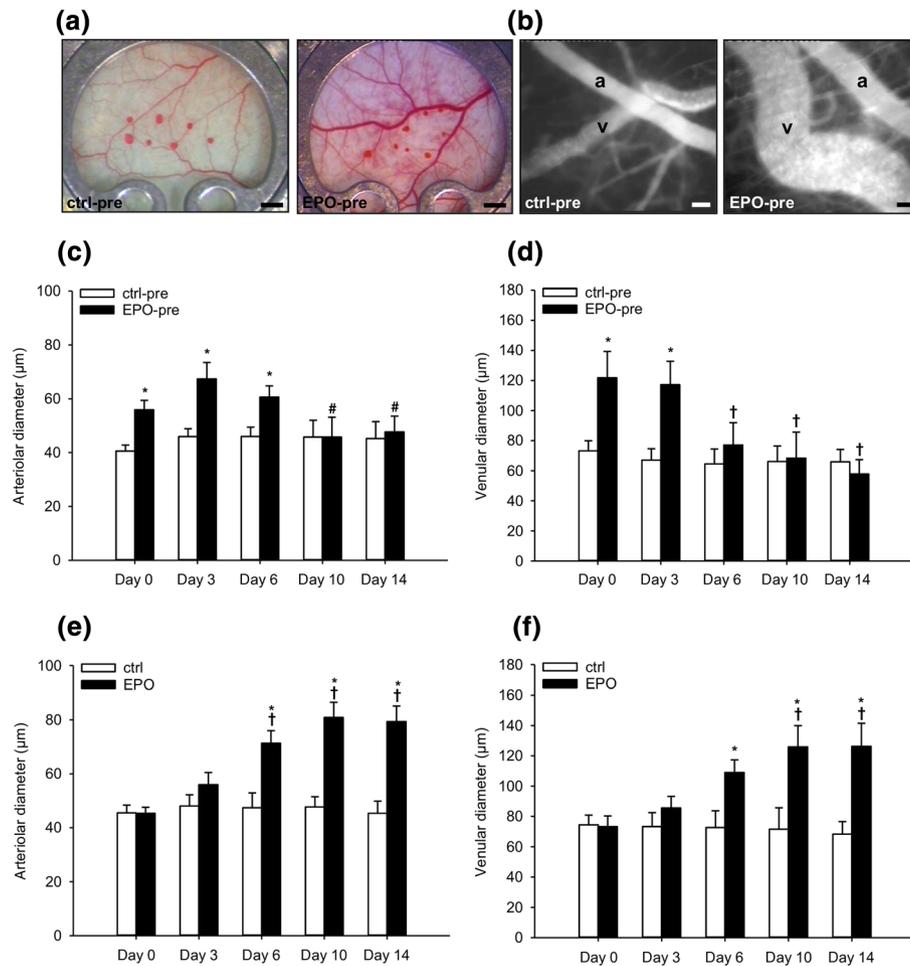
**FIGURE 4** Effect of EPO on vasodilatation. (a) Observation window of islet-containing dorsal skinfold chambers of vehicle- (ctrl-pre) and EPO-pretreated (EPO-pre) FVB/N mice on Day 0. Scale bar: 1 mm. (b) Intravital microscopic images of arterioles (a) and venules (v) within dorsal skinfold chambers of vehicle- (ctrl-pre) and EPO-pretreated (EPO-pre) FVB/N mice on Day 0. The plasma marker FITC-dextran was used for the visualization of microvessels in blue light epi-illumination. Scale bar: 50 μm. (c,d) Diameter of arterioles (c) and venules (d) on Days 0, 3, 6, 10, and 14 after islet transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre $n = 8$) or EPO (EPO-pre, $n = 8$). Mean ± SEM. [#]$P < .05$, significantly different from Day 3 in each individual group; [†]$P < .05$, significantly different from Days 0 and 3 in each individual group; [*]$P < .05$, significantly different from ctrl-pre. (e,f) Diameter of arterioles (e) and venules (f) on Days 0, 3, 6, 10, and 14 after islet transplantation into the dorsal skinfold chamber of FVB/N mice, which were treated with vehicle (ctrl, $n = 8$) or EPO ($n = 8$). Mean ± SEM. [b]$P < .05$, significantly different from Days 0 and 3 in each individual group; [*]$P < .05$, significantly different from ctrl-pre

that EPO stimulates the formation of new blood vessels in the chick embryo chorioallantoic membrane. However, this pro-angiogenic effect seems to depend on the administered dose of EPO. In fact, previous studies suggest that only low doses (500 IU·kg$^{-1}$) but not high doses (5000 IU·kg$^{-1}$) of EPO induce angiogenesis (Harder et al., 2009). Accordingly, we also used a dose of 500 IU·kg$^{-1}$ of EPO, in our experiments, to stimulate the revascularization of transplanted islets.

In a first set of experiments, we administered EPO daily, starting from the day of islet transplantation. This treatment protocol resulted in a higher functional capillary density and slightly increased the endocrine revascularization of the grafts compared with controls. However, the effect of the glycoprotein was only marginal in this experimental setting. This may be explained by the fact that it takes several days until VEGF expression is up-regulated in ischaemic tissue in response to low-dose EPO (Rezaeian et al., 2010). Hence, on Day 3 after

transplantation, the functional capillary density and revascularized area of islets did not differ between EPO- and vehicle-treated animals. In contrast, we detected a significantly higher functional capillary density on Day 6 in EPO-treated mice, while the grafts in both groups exhibited a comparable vascularization on Day 14. Taken together, these findings indicate that EPO is capable of accelerating the revascularization process.

To further improve the pro-angiogenic effect of EPO on islet grafts, we performed a second set of experiments, in which we pretreated recipient mice with the glycoprotein 6 days prior to islet transplantation. According to our previous assumptions, we found that EPO-pretreatment more effectively accelerated the revascularization process of the grafts. This was shown by a significantly higher functional capillary density of islets in EPO-pretreated mice between Days 3 and 10, compared with controls. Moreover, these islets also
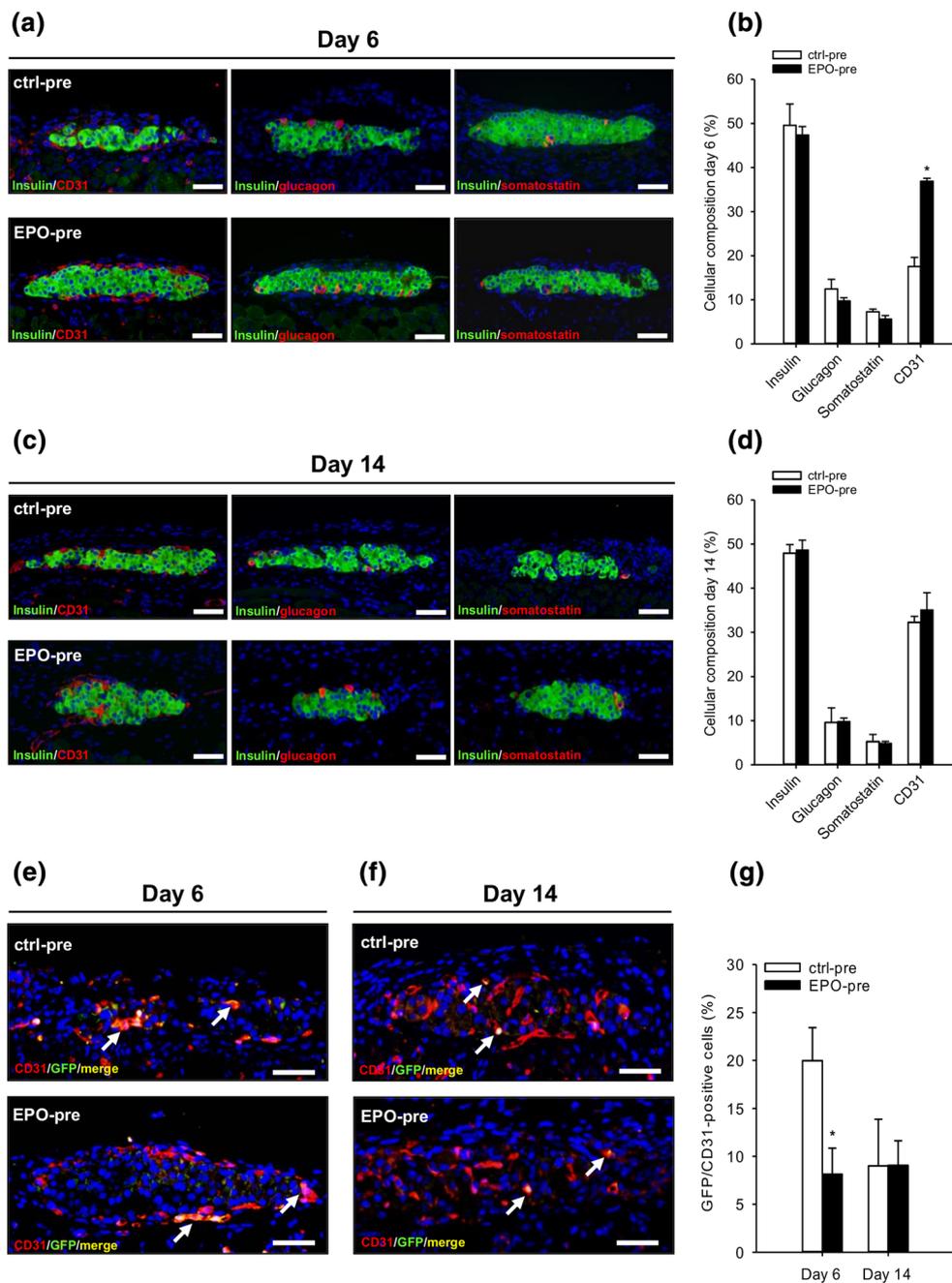
**FIGURE 5** Immunohistochemical analysis of transplanted islets. (a) Immunofluorescence staining of insulin/CD31, insulin/glucagon, and insulin/somatostatin within transplanted islets on Day 6 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre) or EPO (EPO-pre). Scale bar: 50 μm. (b) Insulin-, glucagon-, somatostatin-, and CD31-positive cells (in % of all islet cells) within transplanted islets on Day 6 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre, $n = 3$) or EPO (EPO-pre, $n = 3$). Mean ± SEM. *$P < .05$, significantly different from ctrl-pre. (c) Immunofluorescence staining of insulin/CD31, insulin/glucagon, and insulin/somatostatin within transplanted islets on Day 14 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre) or EPO (EPO-pre). Scale bar: 50 μm. (d) Insulin-, glucagon-, somatostatin-, and CD31-positive cells (in % of all islet cells) within transplanted islets on Day 14 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre, $n = 8$) or EPO (EPO-pre, $n = 8$). (e ,f) Immunofluorescence staining of CD31 and GFP within transplanted islets on Day 6 (e) and Day 14 (f) after transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre) or EPO (EPO-pre). Arrows = GFP/CD31-positive cells. Scale bar: 50 μm. (g) Number of GFP/CD31-positive cells (in % of all CD31-positive cells) within transplanted islets on Days 6 and 14 after transplantation into the dorsal skinfold chamber of FVB/N mice, which were pretreated with vehicle (ctrl-pre, $n = 3$ (Day 6) and $n = 8$ (Day 14)) or EPO (EPO-pre, $n = 3$ (Day 6) and $n = 8$ (Day 14)). Mean ± SEM. *$P < .05$, significantly different from ctrl-pre
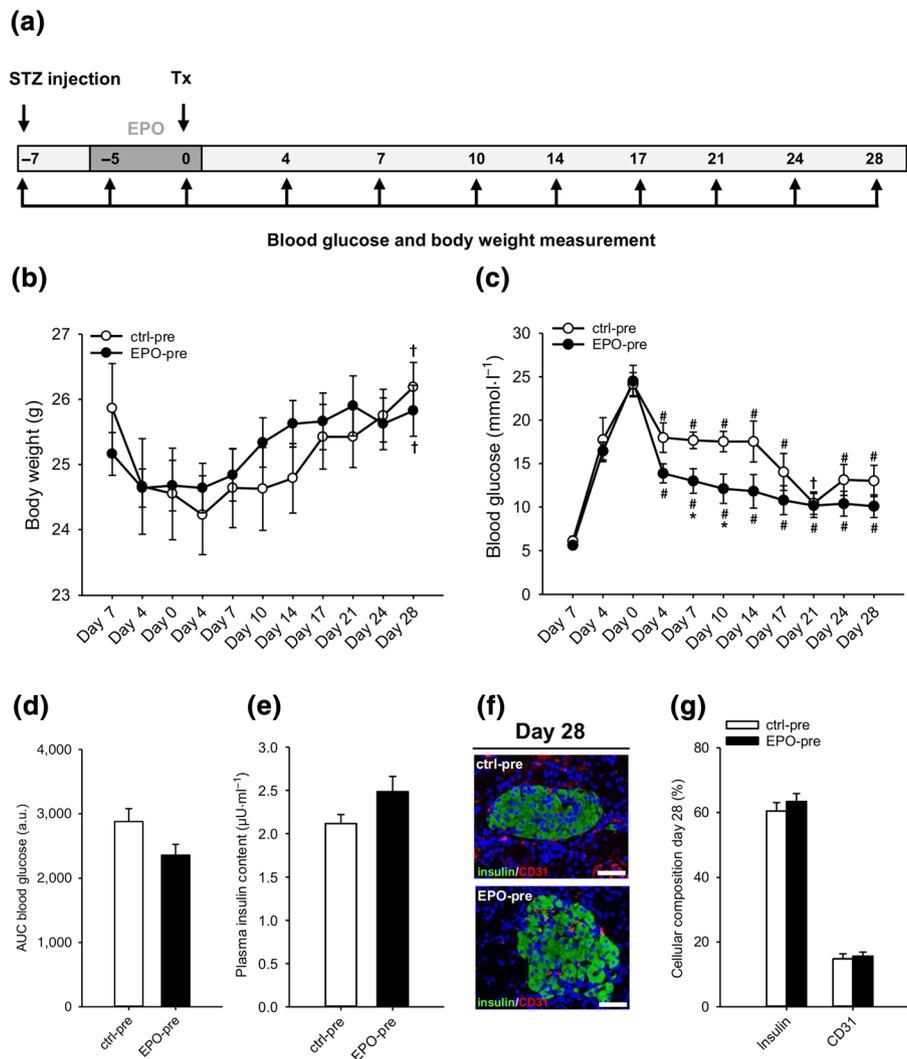
**FIGURE 6** Effect of EPO-pretreatment on the endocrine function of transplanted islets. (a) Schematic illustration of the experimental setting of EPO administration and islet transplantation under diabetic conditions. STZ (150 mg·kg$^{-1}$, i.p.) was administered 7 days before islet transplantation (Tx, Day 0). Mice received daily EPO or vehicle over 6 days. On Day 0, 300 islets were transplanted under the kidney capsule. Blood glucose levels were measured on Days 7 and 4 before islet transplantation as well as on Days 0, 4, 7, 10, 14, 17, 21, 24, and 28 after islet transplantation. (b,c) Body weight (b) and blood glucose levels (c) on Days 7 and 4 before transplantation as well as on Days 0, 4, 7, 10, 14, 17, 21, 24, and 28 after islet transplantation in diabetic C57BL/6J mice, which were pretreated with vehicle (ctrl-pre, $n = 8$) or EPO (EPO-pre, $n = 8$). Mean ± SEM. $^a P < .05$, significantly different from Day 0 in each individual group; †$P < .05$, significantly different from Days 0, 4, 7, 10, and 14 in each individual group; $^*P < .05$, significantly different from ctrl-pre. (d) AUC of blood glucose levels (a.u., arbitrary units). Mean ± SEM. (e) Plasma insulin content (µU·ml$^{-1}$) of vehicle (ctrl-pre)- and EPO (EPO-pre)-pretreated diabetic C57BL/6 J mice on Day 28 after islet transplantation. Mean ± SEM. (f) Immunofluorescence stainings of insulin/CD31 within transplanted islets on Day 28 after transplantation under the kidney capsule of C57BL/6J mice, which were pretreated with vehicle (ctrl-pre) or EPO (EPO-pre). Scale bar: 35 µm. (g) Insulin- and CD31-positive cells (in % of all islet cells) within transplanted islets on Day 28 after transplantation under the kidney capsule of C57BL/6J mice, which were pretreated with vehicle (ctrl-pre, $n = 5$) or EPO (EPO-pre, $n = 5$). Mean ± SEM

contained a significantly lower fraction of GFP/CD31-positive endothelial cells on Day 6, indicating an increased ingrowth of GFP-negative endothelial cells from the surrounding host tissue in this initial phase of islet revascularization. This was associated with a markedly elevated endocrine revascularization throughout the observation period and higher number of engrafted islets on Day 14.

The endocrine revascularization of transplanted islets only represents an indirect marker for their endocrine function. Therefore, we

additionally used the kidney capsule model of diabetic mice (van Suylichem, Strubbe, Houwing, Wolters, & van Schilfgaarde, 1994) to confirm an improved endocrine function due to an accelerated revascularization. For this purpose, we transplanted 300 islets under the kidney capsule of STZ-treated diabetic mice. This represents a suboptimal number of islets because they do not restore normoglycaemia within 10 days (Lai et al., 2005). Of interest, we measured physiological blood glucose levels in EPO-pretreated animals already 7 days

after islet transplantation. In contrast, normoglycaemia was not observed in controls until Day 17. On Day 28 after transplantation, immunohistochemical analyses revealed similar numbers of endothelial cells and beta-cells within the islet grafts of both groups. These data strengthen our results from the dorsal skinfold chamber model, showing that EPO-pretreatment accelerated the revascularization of the grafts without increasing the final overall number of blood vessels. This can be explained by the fact that angiogenesis depends on the balance of stimulatory and inhibitory factors (Kota et al., 2012). It is well known that hypoxia modulates EPO levels by increasing expression of the EPO gene (Stockmann et al., 2006). EPO, in turn, triggers angiogenic pathways (Alvarez Arroyo et al., 1998; Li et al., 2007). In case of the present experimental setting, the exogenous administration of EPO may already induce these angiogenic pathways within the grafts on Day 0, independently of hypoxia, resulting in an acceleration of islet revascularization. However, after the development of a dense microvascular network, which provides a sufficient oxygen and nutrient supply to the islet cells, anti-angiogenic factors, such as thrombospondin-1 (Chen et al., 2000) or IL-1β (Cozzolino et al., 1990), may contribute to the termination of the angiogenic process. Hence, the final revascularization of EPO-pretreated and control islets was comparable.

Interestingly, EPO up-regulates the expression of endothelial NO synthase, resulting in elevated NO levels (Burger et al., 2006). On the other hand, Chen, Wang, Asavaritkrai, and Noguchi (2010) reported that NO stimulates the expression of EPO receptors and, thus, contributes to EPO-mediated protection against hypoxia-induced apoptosis. In addition, it is well known that NO is a potent vasodilator. In line with these findings, we observed a strong vasodilatory effect of EPO on arterioles and venules of the host tissue. EPO-pretreatment caused a vasodilatation during the first 6 days, whereas EPO treatment increased the vessel diameters between Days 6 and 14 after islet transplantation. Therefore, it may be speculated that several days are required until the repetitive application of low-dose EPO results in NO levels sufficient to induce vasodilatation. Of note, it has also been reported that NO promotes angiogenesis (Ziche & Morbidelli, 2000). This may have further contributed to our observation that short-term EPO-pretreatment is more effective in enhancing islet revascularization than EPO treatment in the post-transplantation phase.

Several studies demonstrated that EPO is also capable of reducing pancreatic beta-cell death (Chen et al., 2015; Fenjves et al., 2003; Fenjves et al., 2004). Watanabe et al. (2016) showed that the pyroglutamate helix B surface peptide ARA 290, which is highly specific for EPO receptors and lacks haematopoietic activity, protects transplanted islets against cytokine-induced apoptosis. Hence, besides its pro-angiogenic effect, this anti-apoptotic activity of EPO may be an additional advantage for its application in clinical islet transplantation.

EPO elevates haemoglobin levels and activates platelets, resulting in an increased risk of thrombosis (Jelkmann, 1992; Kato et al., 2010; Stohlawetz et al., 2000). In the present study, the analysis of blood samples on Day 14 revealed that a daily EPO treatment over 14 days significantly elevates systemic haematocrit levels when compared to controls. Importantly, this was not the case when mice were only pretreated with the glycoprotein for a 6-day period. In fact, these observations are in line with those of Galeano et al. (2006), demonstrating that daily EPO administration (400 IU·kg$^{-1}$) over 2 weeks increases haematocrit levels not earlier than Day 14. Accordingly, the short-term EPO-pretreatment in the present study did not trigger the haematopoietic cascade but was capable of inducing angiogenesis. This is of crucial clinical interest, because islet transplantation is often associated with portal thrombosis (Kawahara et al., 2011).

In conclusion, we demonstrate that EPO-pretreatment effectively improved the revascularization of transplanted islets and, thus, accelerated restoration of normoglycaemia without increasing systemic haematocrit levels. These novel findings indicate that EPO-pretreatment may represent a promising therapeutic approach to improve the clinical outcome of islet transplantation.

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## AUTHOR CONTRIBUTIONS

M.M.M., L.N., L.P.R., C.K., and S.W. performed the overall experiments and analysed the data. E.A., M.W.L., and M.M.M. designed the study and prepared the manuscript. E.A., L.P.R., C.K., S.W., M. G, M.W.L., and M.D.M reviewed, revised, and approved the manuscript.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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