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REGULAR ARTICLE

Nerve/glial antigen 2 is crucially involved in the revascularization of freely transplanted pancreatic islets

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Abstract

Pancreatic islets are highly vascularized endocrine units. Accordingly, their adequate revascularization is of major importance for successful islet transplantation. The proteoglycan, nerve/glial antigen 2 (NG2) expressed in pericytes is a crucial regulator of angiogenesis. Therefore, we herein analyze whether this surface protein contributes to the revascularization of grafted islets. Islets were isolated from $NG2^{+/+}$ (wild-type) and $NG2^{-/-}$ mice and their cellular composition was analyzed by immunohistochemical detection of insulin, glucagon, somatostatin and CD31. Moreover, insulin secretion was assessed by enzyme-linked immunosorbent assay (ELISA). In addition, isolated islets were transplanted into dorsal skinfold chambers of wild-type mice and their revascularization was determined by intravital fluorescence microscopy and immunohistochemistry. $NG2^{+/+}$ and $NG2^{-/-}$ islets did not differ in their cellular composition and insulin secretion. However, transplanted $NG2^{-/-}$ islets exhibited a significantly lower functional capillary density and a reduced number of CD31-positive microvessels. These findings demonstrate that the loss of NG2 impairs the revascularization of transplanted islets, underlining the importance of this pericytic proteoglycan for islet engraftment.

Keywords Pericytes · Type 1 diabetes mellitus · Islet transplantation · NG2 · Angiogenesis

Introduction

In type 1 diabetes, daily insulin applications are still the standard therapy to maintain physiological blood glucose levels. Nonetheless, this approach bears the risk of severe secondary diseases, such as retinopathies and nephropathies (Gargiulo et al. 2004; Vincent et al. 2004). The transplantation of pancreatic islets to cure diabetes may represent an alternative minimal-invasive therapeutic strategy (Bruni et al. 2014). However, for this purpose a great number of islets are required to achieve insulin independency. Accordingly, islets are often pooled from 2 to 4 donor pancreata for successful islet transplantation (Goss et al. 2002; Shapiro et al. 2000). A major reason for this poor donor-to-recipient ratio is the insufficient revascularization of the grafts, resulting in a rapid loss of β-cell mass (Henriksnas et al. 2012; Lau and Carlsson 2009).

The revascularization of pancreatic islets is not only dependent on sprouting angiogenesis of the host tissue but also on the survival and angiogenic activity of intraislet microvessels. This view is supported by the observation of Brissova et al. (Brissova et al. 2004) that individual blood vessels within transplanted islets consist of donor or recipient endothelial cells or are even a chimera of both. Endothelial cells closely interact with pericytes, which are embedded within the basement membrane of microvessels and are involved in regulating vessel permeability, diameter and blood flow (Bergers and Song 2005). During angiogenesis, pericytes communicate with endothelial cells by either direct contact or paracrine signaling (Franco et al. 2011; Ribatti et al. 2011) and, thus, contribute to vessel formation and maturation (Armulik et al. 2011). Pericytes have been shown to maintain the β-cell mass of transplanted islets and to contribute to their neurovascular regeneration (Juang et al. 2015; Sasson et al. 2016). Recently, Almaca et al. (Almaca et al. 2018) reported that pericytes also play an important role in regulating islet blood flow and have

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pathophysiological implications. However, the function of intraislet pericytes in graft revascularization has not been studied so far.

The angiogenic activity of pericytes is mainly induced by the expression of various surface proteins (Ribatti et al. 2011), including nerve/glial antigen 2 (NG2) (Nishiyama 2007; Schlingemann et al. 1990). This single transmembrane proteoglycan consists of a large extracellular N-terminal domain, which is characterized by disulfide bonds and chondroitin sulfate glycosaminoglycan chains (Yadavilli et al. 2016). The C-terminus contains several acceptor sites for various proteins, such as multi-PDZ domain protein (MUPP)-1 and extracellular signal-related kinases (ERK) (Barritt et al. 2000; Makagiansar et al. 2007). Ozerdem et al. (2001) identified high levels of NG2 on nascent microvascular pericytes during developmental angiogenesis. NG2 is further upregulated during microvascular remodeling in postischemic vessels of the rat mesentery (Murfee et al. 2006). In line with these findings, mice lacking NG2 exhibit a significantly reduced angiogenic response during ischemic retinal neovascularization (Ozerdem and Stallcup 2004), indicating a crucial function of this proteoglycan in the process of blood vessel formation.

Based on these findings, we herein hypothesize that pericytic NG2 contributes to the revascularization of transplanted islets. To test this, we used conditional homozygous NG2-knockout ($NG2^{-/-}$) and wild-type ($NG2^{+/+}$) mice as pancreatic donors for islet isolation. The cellular composition and insulin secretion of their islets were analyzed by immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). Subsequently, the islets were transplanted into dorsal skinfold chambers of wild-type mice to analyze their revascularization by means of repetitive intravital fluorescence microscopy.

Material and methods

Chemical and biological reagents

Tamoxifen, fluorescein isothiocyanate (FITC)-labeled dextran 150,000, rhodamine 6G and Hoechst 33342 were purchased from Sigma-Aldrich (Taufkirchen, Germany). Collagenase NB 4G was received from SERVA Elektrophoresis GmbH (Heidelberg, Germany) and corn oil from Merck (Darmstadt, Germany). Ketamine (Ursotamin®) was purchased from Serumwerke Bernburg (Bernburg, Germany) and xylazine (Rompun®) from Bayer (Leverkusen, Germany). HepatoQuick® was received from Roche (Basel, Switzerland). The antibodies anti-α-smooth muscle actin (α-SMA), anti-insulin, anti-glucagon, anti-α-tubulin and anti-somatostatin were purchased from Abcam (Cambridge, UK); anti-NG2 antibody (sc-166251) and anti-PDGFR-β (sc-432) were from Santa Cruz Inc. (Heidelberg, Germany) and anti-CD31 (DIA310) was from Dianova (Hamburg, Germany). Goat-anti-rat IgG Alexa555, goat-anti-rabbit IgG Alexa488 and goat-anti-mouse IgG Alexa555 antibodies were from Thermo Fisher Scientific GmbH (Dreieich, Germany).

Animals

Homozygous NG2-CreERT2 knock-in mice (TgH(NG2-CreERT2)) (Huang et al. 2014), were crossbred with reporter mice (B6;129S6-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze/J}$ (Ai14)) (Madisen et al. 2010). Homozygous NG2-CreERT2xRosa26-tdTomato mice, lacking NG2 ($NG2^{-/-}$) (Huang et al. 2014), as well as C57BL/6N wild-type mice ($NG2^{+/+}$) with a body weight of 30–35 g in the age of 35–45 weeks were used as donors for pancreas tissue and islets. C57BL/6N mice (Institute for Clinical & Experimental Surgery, Saarland University, Homburg/Saar, Germany) aged 8–10 weeks with a body weight of 22–27 g served as recipients for the transplantation of islets into dorsal skinfold chambers. The mice were housed one per cage in a temperature-controlled environment under a 12/12 h day/night cycle and received water and standard pellet food (Altromin, Lage, Germany) ad libitum.

Tamoxifen injection

To induce reporter expression in the inducible CreERT2 line, tamoxifen (10 mg/mL) was dissolved in corn oil and injected intraperitoneally (i.p.) into nine $NG2^{+/+}$ and nine $NG2^{-/-}$ donor mice (100 mg/kg body weight) for two consecutive days.

Isolation of pancreatic islets

Fourteen days after the first tamoxifen injection (Bai et al. 2013), the donor mice were anesthetized by i.p. injection of xylazine (75 mg/kg body weight) and ketamine (75 mg/kg body weight). Following midline laparotomy, the pancreatic duct was flooded with 1 mg/mL collagenase NB 4G in Hank's balanced salt solution (HBSS). The whole pancreas tissue of $NG2^{+/+}$ and $NG2^{-/-}$ mice was then collected to isolate pancreatic islets, as previously described in detail (Beger et al. 1998).

Glucose-stimulated insulin secretion assay

The glucose-dependent secretion of insulin from isolated $NG2^{+/+}$ and $NG2^{-/-}$ islets was determined by a static glucose-stimulated insulin secretion (GSIS) assay, as described by Kelly et al. 2010. For this purpose, islets were isolated and handpicked as groups of five islets ($n = 3$ per group). The islets were incubated for 40 min at 37 °C in Krebs Ringer Buffer (KRB) containing 1.1 mM glucose. Subsequently, glucose concentration was enhanced to 25 mM for an additional 20 min to induce insulin secretion.

Insulin concentrations in the supernatants were quantified by means of an ELISA kit from Merck-Millipore (Darmstadt, Germany), according to the manufacturer's instructions.

Western blot analysis

Tissue extracts were separated through a 7.5% SDS polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% dry milk in phosphate-buffered saline (PBS) (0.1% Tween20) for 1 h and then incubated with anti-NG2 anti-PDGFR-β and anti-α-tubulin antibodies, which were diluted (1:500) in PBS (0.1% Tween20) containing 1% dry milk. The membrane was incubated with the corresponding peroxidase-coupled secondary antibodies for 1 h. Protein expression was visualized by luminol-enhanced chemiluminescence (ECL; GE Healthcare).

Dorsal skinfold chamber preparation and islet transplantation

Dorsal skinfold chambers were prepared in a total of nine $NG2^{+/+}$ mice and served for the repetitive analysis of islet revascularization. The preparation and implantation of the chamber has been described previously in detail (Laschke et al. 2011). Briefly, two symmetrical titanium frames were implanted on the extended dorsal skinfold of anesthetized wild-type mice, resulting in the doubling of the skin in two layers. One skin layer, including skin, subcutis and the retractor muscle, was completely removed in a circular area of 15 mm in diameter. This area was then covered by a removable cover slip and snap ring, providing direct microscopic access to the microcirculation of the chamber. After the procedure, the animals were allowed to recover for 48 h.

For the transplantation of pancreatic islets, the mice were anesthetized and the cover glass of the dorsal skinfold chamber was removed. After the chamber tissue was flushed with NaCl, freshly isolated, neutral red stained $NG2^{-/-}$ ($n = 4$; placed caudal into the chamber) and $NG2^{+/+}$ islets ($n = 4$; placed cranial into the chamber) with a diameter of 150–200 μm were transplanted onto the striated muscle tissue within each chamber. Finally, the chamber was closed again by a new cover slip.

Intravital fluorescence microscopy

For intravital fluorescence microscopy, anesthetized mice were retrobulbary injected with 0.05 mL of 5% FITC-dextran to stain the blood plasma and 0.05 mL of 2% rhodamine 6G for the visualization of endocrine revascularization (Vajkoczy et al. 1995b). Intravital epi-illumination fluorescence microscopy was performed directly after islet transplantation (i.e., day 0) as well as on days 3, 6 and 10 by means of 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 DVD system (DVD-HR775; Samsung, Eschborn, Germany) for offline evaluation. After the last microscopic observation, the chamber tissue was carefully excised and further processed for additional immunohistochemical analyses.

Microscopic images were analyzed off-line by the computer-assisted image analysis system CapImage (Zeintl, Heidelberg, Germany). The islet size (mm^2), the revascularized area (mm^2), the functional capillary density (cm/cm^2) and the endocrine revascularization (mm^2) of the islets were assessed as previously described (Ampofo et al. 2015; Menger et al. 1992). In addition, we measured diameter (μm), centerline red blood cell (RBC) velocity (μm/s) and volumetric blood flow (pL/s) of 4–8 individual microvessels within the grafts (Ampofo et al. 2015; Menger et al. 1992). Finally, we assessed the take rate (%), i.e., the fraction of engrafted islets in relation to the number of transplanted islets per group on day 10.

Immunohistochemistry

For the preparation of histological sections, specimens of pancreata and dorsal skinfold chamber tissue were fixed for 24 h in 4% formalin. In addition, isolated islets were incubated for 45 min at 37 °C in 100 μL HepatoQuick®, 50 μL human citrate plasma (generated from venous blood by four healthy human volunteers) and 10 μL 10% $CaCl_2$ solution. The resulting clot was also fixed for 24 h in 4% formalin. The formalin-fixed specimens were embedded in paraffin and 2-μm-thick sections were cut.

The sections were stained with anti-α-SMA, anti-insulin, anti-glucagon, anti-somatostatin, as well as anti-CD31 antibodies and visualized by their corresponding fluorescence-coupled secondary antibodies. Cell nuclei were stained with Hoechst 33342. The sections were analyzed by means of fluorescence microscopy (BX60F; Olympus, Hamburg, Germany). The numbers of insulin-, glucagon-, somatostatin-, as well as CD31-positive cells were counted using ImageJ software (National Institutes of Health (NIH), Bethesda, MD, USA) and given in % of all islet cells.

Experimental protocol

Pancreatic islets were isolated from four donor mice, two $NG2^{-/-}$ mice and two $NG2^{+/+}$ mice and a total number of nine $NG2^{+/+}$ were equipped with dorsal skinfold chambers. Four neutral red-stained $NG2^{-/-}$ (placed caudal) and four

neutral red-stained NG2$^{+/+}$ islets (placed cranial) were transplanted into the chamber. Repetitive intravital fluorescence microscopy was performed on days 0, 3, 6 and 10 after islet transplantation. And the end of the experiment the chamber tissue was excised for further immunohistochemical analysis.

Statistical analysis

After testing the data for normal distribution and equal variance, an unpaired Student's *t* test was assessed to analyze the differences between the two groups (SigmaPlot 13.0; Jandel Corporation, San Rafael, CA, USA). All values are expressed as mean ± SEM. Statistical significance was accepted for $P < 0.05$.

Results

Effect of NG2 knockout on the cellular composition of pancreatic islets

To investigate the effect of NG2 on the revascularization of transplanted islets, NG2-CreERT2xRosa26-tdTomato mice were tamoxifen-treated to induce reporter expression (Fig. 1a). Tamoxifen-treated wild-type mice (NG2$^{+/+}$) served as controls. The successful knockout of NG2 was verified by genotyping PCR (Fig. 1b) as well as Western blot analysis of pancreatic tissue (Fig. 1c). To exclude that the loss of NG2 affects the cellular composition of the islets, we prepared tissue sections of pancreata from NG2$^{+/+}$ and NG2$^{-/-}$ mice and assessed the fraction of insulin-, glucagon- and somatostatin-expressing cells (Fig. 1d, d', e, e'). Moreover, we examined the number of pericytes by α-SMA and endothelial cells by CD31-stainings (Fig. 1d", d"', e", e"'). The quantification of the immunohistochemical stainings revealed that islets from NG2$^{-/-}$ mice contained similar numbers and distributions of the different endocrine cell types when compared to wild-type animals (Fig. 1f–h). In addition, loss of NG2 did not affect the number of CD31-positive endothelial cells as well as α-SMA-positive pericytes within the islets (Fig. 1i, j).

Effect of NG2 knockout on the cellular composition and insulin secretion of isolated islets

Next, islets were isolated by enzymatic digestion of pancreatic tissue from NG2$^{+/+}$ and NG2$^{-/-}$ mice. The knockout of pericytic NG2 within the isolated islets was visualized by the expression of the fluorescence protein tdTomato after successful recombination (Fig. 2a–a"). To analyze whether the loss of NG2 affects endocrine islet functions, we determined the insulin secretion of NG2$^{+/+}$ and NG2$^{-/-}$ islets following exposure to high glucose. We found that loss of NG2 did not affect the secretory capacity of insulin-producing β-cells (Fig. 2b). We further analyzed immunohistochemically the number and distribution of endocrine cells, endothelial cells and pericytes within the isolated islets (Fig. 2c–c"', d–d"'). As already described for islets within the pancreas, the fractions and distribution of endocrine cells did not differ between islets isolated from NG2$^{+/+}$ and NG2$^{-/-}$ mice (Fig. 2e–g). However, we found a significantly reduced number of CD31-expressing endothelial cells in isolated islets from NG2$^{-/-}$ mice, whereas the number of α-SMA-positive pericytes was not affected by the loss of NG2 (Fig. 2h, i).

Effect of NG2 on the revascularization of isolated islets

To assess the effect of NG2 on graft revascularization, isolated islets of NG2$^{+/+}$ and NG2$^{-/-}$ mice were transplanted into the dorsal skinfold chamber of NG2$^{+/+}$ wild-type mice and analyzed by repetitive intravital fluorescence microscopy (Fig. 3a, b). Our analyses revealed a comparable take rate of ~ 75% for NG2$^{+/+}$ and NG2$^{-/-}$ islets on day 10 after transplantation (Fig. 3c). In both groups, first angiogenic sprouts could be observed on day 3 in the host microvasculature surrounding the grafts. These sprouts originated from capillaries and post-capillary venules of the host tissue. Throughout the following days, the sprouts progressively grew into the transplanted NG2$^{+/+}$ (Fig. 3d–d"') and NG2$^{-/-}$ (Fig. 3e–e"') islets and finally developed into dense, glomerulum-like microvascular networks. Interestingly, the functional capillary density of these networks was significantly reduced in NG2$^{-/-}$ islets on day 10 when compared to that of NG2$^{+/+}$ islets (Fig. 3f). In addition, we found that NG2$^{+/+}$ and NG2$^{-/-}$ islets exhibit a comparable size, excluding that the deteriorated revascularization of transplanted NG2$^{-/-}$ islets was caused by different graft sizes (Fig. 3g). The analysis of microhemodynamic parameters further revealed that the loss of NG2 did not affect the diameter, centerline RBC velocity and volumetric blood flow of individual microvessels within the grafts (Table 1). Finally, we measured the endocrine revascularization of the islets, which can be assessed by the accumulation of extravasated rhodamine 6G (Fig. 3d"", e""). Of note, this parameter correlated with the onset of a functional blood perfusion within the grafts on day 6 (Fig. 3d", d"", 3e", 3e""). Moreover, the loss of NG2 also significantly reduced the endocrine revascularization on day 10 when compared to NG2$^{+/+}$ islets (Fig. 3h).

Finally, the endocrine and endothelial cell population of tissue sections of dorsal skinfold chambers were performed immunohistochemically to examine the effect of NG2 on the grafts. We found that the numbers of endocrine cells did not differ between islets from NG2$^{+/+}$ and NG2$^{-/-}$ mice (Fig. 4a–

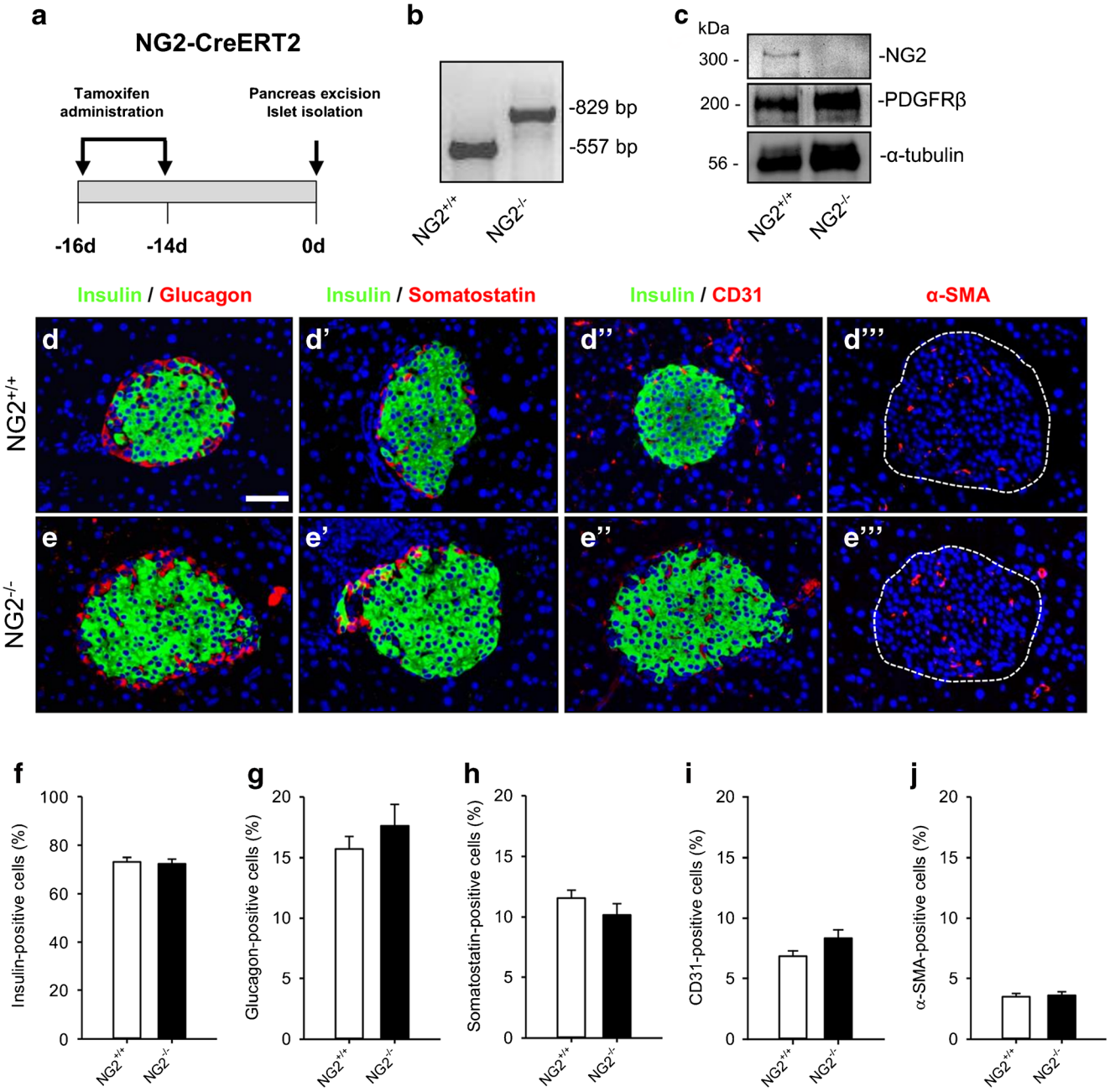

Fig. 1 Effect of NG2 knockout on the cellular composition of pancreatic islets. **a** Schematic illustration of the experimental setup to induce reporter expression in NG2-CreERT2xRosa26-tdTomato mice by tamoxifen administration. **b** Genotyping PCR results of NG2 wild-type ($NG2^{+/+}$, 557 bp) and NG2 knockout ($NG2^{-/-}$, 829 bp) mice. **c** Protein levels of NG2, PDGFR-β and α-tubulin of pancreatic tissue were detected by Western Blot. **d**, **e** Immunofluorescence staining of insulin/glucagon (**d**, **e**), insulin/somatostatin (**d'**, **e'**), insulin/CD31 (**d''**, **e''**) and α-SMA (**d'''**, **e'''**) of islets within the pancreas of $NG2^{+/+}$ and $NG^{-/-}$ mice. Cell nuclei were stained with Hoechst 33342 (blue). Scale bar 50 μm. **f**–**j** Insulin-, glucagon-, somatostatin-, CD31- and α-SMA-positive cells (in % of all islet cells) were assessed in islets of $NG2^{+/+}$ (white bars, $n = 10$ islets) and $NG2^{-/-}$ mice (black bars, $n = 10$ islets) by quantitative analysis of immunohistochemical sections. Mean ± SEM

e). However, in line with our in vivo results, showing a decreased functional capillary density in $NG2^{-/-}$ islets when compared to controls, we also detected a diminished vessel density in islets from NG2 knockout mice as shown by CD31-positive cells (Fig. 4a", b", f).

Discussion

Pancreatic islets are highly vascularized endocrine units and their adequate revascularization after transplantation is essential for their successful engraftment. Besides endothelial cells,

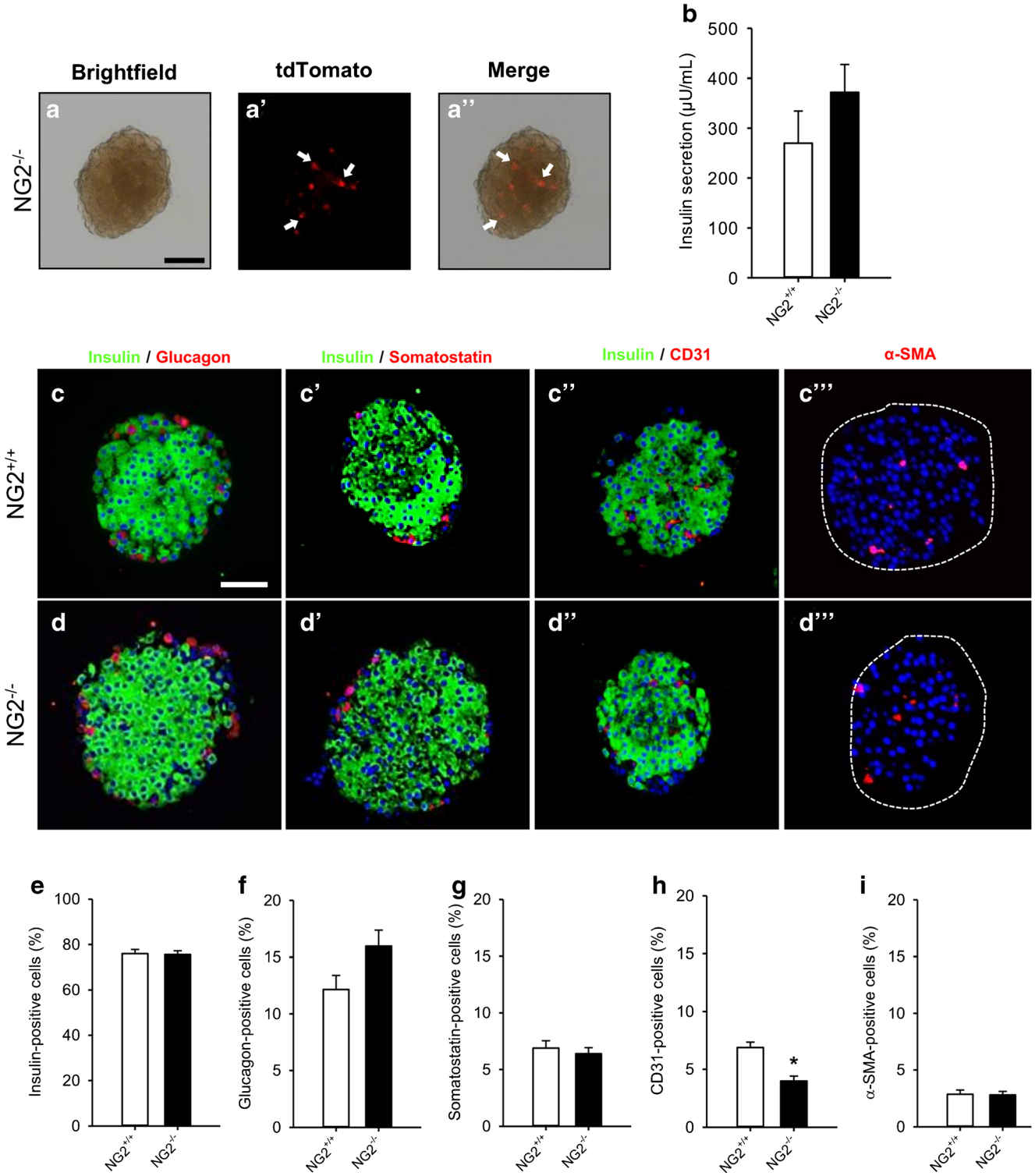

Fig. 2 Effect of NG2 knockout on the cellular composition and insulin secretion of isolated islets. Bright field (**a**), fluorescence (**a'**) and merged microscopic images (**a"**) of an isolated islet from a $NG2^{-/-}$ mouse. Note the tdTomato fluorescence (red) of pericytes (arrows) after tamoxifen-induced recombination. Scale bar 50 µm. **b** Insulin secretion (µU/mL) of $NG2^{+/+}$ (white bar) and $NG2^{-/-}$ islets (black bar) after glucose stimulation (25 mM). Mean ± SEM. **c**, **d** Immunofluorescence staining of insulin/glucagon (**c**, **d**), insulin/somatostatin (**c'**, **d'**), insulin/CD31 (**c"**, **d"**) and α-SMA (**c"'**, **d"'**) of isolated islets from $NG2^{+/+}$ and $NG^{-/-}$ mice. Cell nuclei were stained with Hoechst 33342 (blue). Scale bar 50 µm. **e**–**i** Insulin-, glucagon-, somatostatin-, CD31- and α-SMA-positive cells (in % of all islet cells) were assessed in islets of $NG2^{+/+}$ (white bars, $n = 15$ islets) and $NG2^{-/-}$ mice (black bars, $n = 15$ islets) by quantitative analysis of immunohistochemical sections. Mean ± SEM. *$P < 0.05$ vs. $NG2^{+/+}$

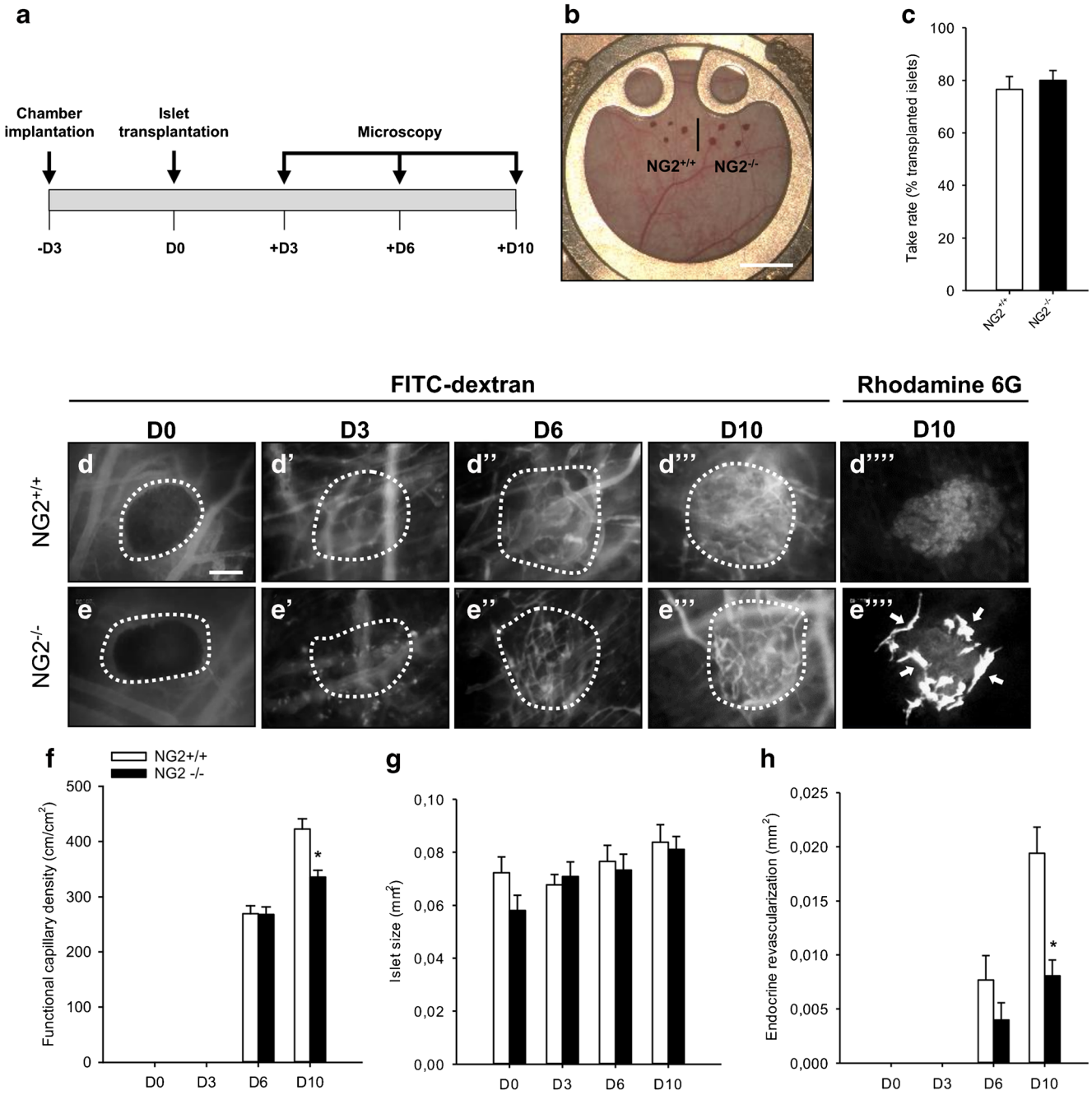

Fig. 3 Effect of NG2 on the revascularization of isolated islets. **a** Schematic illustration of the experimental protocol. **b** Dorsal skinfold chamber containing 4 $NG2^{+/+}$ and 4 $NG2^{-/-}$ neutral red-stained islets. Scale bar 2.5 mm. **c** Take rate (in % of transplanted islets) of $NG2^{+/+}$ (white bar, $n = 9$) and $NG2^{-/-}$ islets (black bar, $n = 9$) on day 10 after transplantation into the dorsal skinfold chamber. Mean ± SEM. **d**, **e** Intravital fluorescence microscopic images of $NG2^{+/+}$ and $NG2^{-/-}$ islets (borders marked by dotted line) on day 0 (**d**, **e**), 3 (**d'**, **e'**), 6 (**d''**, **e''**) and 10 (**d'''**, **d''''** **e'''**, **e''''**) after transplantation into the dorsal skinfold chamber. The plasma marker FITC-dextran was used for the visualization of microvessels; rhodamine 6G was used for the detection of the endocrine vascularization of the islets. Note the bright tdTomato fluorescence of pericytes (arrows) in $NG2^{-/-}$ after tamoxifen-induced recombination. Scale bar 50 μm. **f**–**h** Functional capillary density (cm/cm^2) (**f**), islet size (mm^2) (**g**) and endocrine revascularization (mm^2) (**h**) of $NG2^{+/+}$ (white bars) and $NG2^{-/-}$ islets (black bars) on days 0, 3, 6, and 10 after transplantation into the dorsal skinfold chambers ($n = 9$), as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Mean ± SEM. *$P < 0.05$ vs. $NG2^{+/+}$

pericytes also play a major role in the formation of new blood vessels by expressing angiogenic growth factors and surface proteins (Cai et al. 2008; Franco et al. 2011; Hellstrom et al. 1999; Proebstl et al. 2012). One of these surface proteins is NG2, which has previously been reported to contribute to angiogenesis and vascular remodeling (Murfee et al. 2006).

Table 1 Diameter (μm), centerline RBC velocity (μm/s) and volumetric blood flow (pL/s) of newly formed microvessels within $NG2^{+/+}$ and $NG2^{-/-}$ islets on days 0, 3, 6 and 10 after transplantation into the dorsal skinfold chamber ($n = 9$), as assessed by intravital fluorescence microscopy and computer-assisted image analysis

	D0	D3	D6	D10
Diameter (μm)				
$NG2^{+/+}$	–	–	14.9 ± 0.5	12.5 ± 0.8
$NG2^{-/-}$	–	–	14.6 ± 0.6	14.6 ± 1.5
Centerline RBC velocity (μm/s)				
$NG2^{+/+}$	–	–	395.0 ± 79.4	277.5 ± 32.7
$NG2^{-/-}$	–	–	336.9 ± 39.8	314.3 ± 51.8
Volumetric blood flow (pL/s)				
$NG2^{+/+}$	–	–	51.3 ± 9.8	29.4 ± 5.2
$NG2^{-/-}$	–	–	50.4 ± 8.6	56.7 ± 19.0

Mean ± SEM

In the present study, we analyzed for the first time the effect of NG2 on the revascularization of transplanted islets. Our results show that loss of NG2 significantly deteriorates the revascularization of islets when compared to $NG2^{+/+}$ controls.

We first analyzed the effect of NG2 knockout on the number of insulin-, glucagon-, somatostatin- and CD31-positive cells in islets of pancreatic tissue sections. We did not detect any differences in the quantity of endocrine and endothelial cells between $NG2^{-/-}$ and $NG2^{+/+}$ islets. The isolation of islets by collagenase did also not significantly change the number of glucagon-positive α-cells and somatostatin-positive δ-cells. Moreover, insulin expression and secretion of isolated $NG2^{+/+}$ and $NG2^{-/-}$ islets were comparable in response to high glucose. Furthermore, the number of pericytes was not affected by the loss of NG2. However, the fraction of endothelial cells was significantly reduced in $NG2^{-/-}$ islets when compared to $NG2^{+/+}$ controls. This may be explained by the fact that NG2 interacts with collagen VI and, thus, mediates adhesion and attachment of pericytes to the extracellular matrix (ECM), which in turn stabilizes the microvasculature (Burg et al. 1996; Tillet et al. 2002). In addition, Yotsumoto et al. (2015) demonstrated that the specific loss of NG2 in pericytes decreases the basal lamina assembly by ~31%. Therefore, it is conceivable that the degradation process of the ECM by collagenase digestion of the pancreatic tissue during islet isolation is amplified after loss of NG2, resulting in a reduced number of endothelial cells.

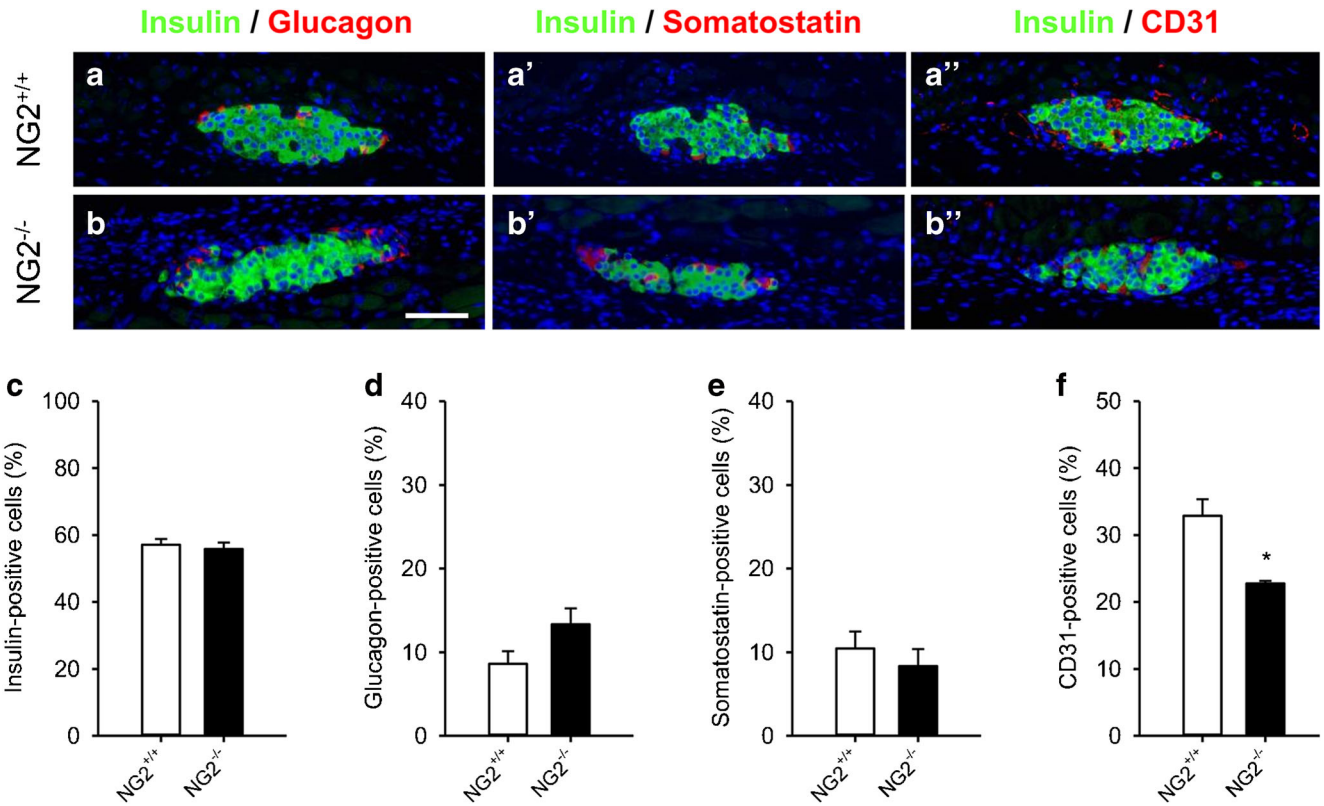

Fig. 4 Cellular composition of transplanted islets. **a**, **b** Immunofluorescence staining of insulin/glucagon (**a**, **b**), insulin/somatostatin (**a'**, **b'**) and insulin/CD31 (**a"**, **b"**) of islets from $NG2^{+/+}$ and $NG^{-/-}$ mice on day 10 after transplantation into the dorsal skinfold chamber. Cell nuclei were stained with Hoechst 33342 (blue). Scale bar 40 μm. **c**–**f** Numbers of insulin-, glucagon-, somatostatin- and CD31-positive cells (in % of all islet cells) in islets of $NG2^{+/+}$ (white bars, $n = 10$) and $NG^{-/-}$ mice (black bars, $n = 10$ islets) analyzed from immunohistochemical sections. Mean ± SEM. *$P < 0.05$ vs. $NG2^{+/+}$

The revascularization of transplanted islets is a limiting factor for islet graft survival (Menger et al. 2001). The role of endothelial proteins during this process is well known (Hogan and Hull 2017); however, the participation of pericytic proteins remains elusive so far. In the present study, we found that NG2 is crucially involved in the revascularization of islets. In fact, we detected a significantly reduced functional capillary density of isolated islets from NG2$^{-/-}$ mice on day 10 when compared to controls. These findings were confirmed by immunohistochemical analyses, demonstrating that loss of NG2 decreases the number of CD31-positive endothelial cells on day 10 without affecting the fractions of endocrine cells within the grafts. In contrast, the functional capillary density of grafted islets from NG2$^{-/-}$ and NG2$^{+/+}$ mice did not differ until day 6. These results indicate that the reduced number of CD31-positive cells in NG2$^{-/-}$ islets directly after their isolation does not affect the early revascularization of the grafts. It may be assumed that NG2 is involved in the maturation of ingrowing vessels in later stages of the angiogenic process, which is triggered by endothelial integrin signaling. Integrins are heterodimeric surface receptors formed by α- and β-subunits (Hynes 2002; Malinin et al. 2012; Ye et al. 2011). It has been shown that germline deletion of integrin β1 leads to embryonic lethality (Tanjore et al. 2008). Recently, Yamamoto et al. (2015) investigated the effects of this protein on blood vessel stability in a model of inducible inactivation of the Itgb1 gene in the postnatal endothelium. They found that integrin β1 is indispensable for the formation of stable and mature vessels. Interestingly, the loss of pericytic NG2 leads to a reduced integrin β1 activation in endothelial cells (You et al. 2014). This raises the possibility that the decreased revascularization of transplanted NG2$^{-/-}$ islets is caused by a reduced NG2/integrin β1 signaling, resulting in higher numbers of immature blood vessels, which have been shown to regress over time (Wietecha et al. 2013).

In the present study, we additionally analyzed the endocrine revascularization of transplanted islets by rhodamine 6G. Of note, transplanted islets develop a dense network of fenestrated blood vessels to facilitate the trans-endothelial transport of secreted hormones (Olsson and Carlsson 2006). We previously demonstrated that rhodamine 6G crosses the fenestrated endothelium and, hence, particularly accumulates in the mitochondria of islet cells. Accordingly, this fluorescence dye is suitable to visualize these endocrine cells after the onset of a sufficient blood supply after transplantation (Vajkoczy et al. 1995a). We herein detected a significantly reduced endocrine revascularization of NG2$^{-/-}$ islets when compared to controls. You et al. (2014) showed a direct involvement of NG2 in improving the barrier function of the endothelium. In fact, they demonstrated that pericyte-specific NG2 ablation leads to structural and functional deficits in tumor blood vessels. This, in turn, results in a disturbed interaction of endothelial cells with pericytes and an increased vessel leakage (You et al. 2014). Based on these findings, a higher rhodamine 6G uptake would be expected in NG2$^{-/-}$ islets. Our opposite observation may be explained by the reduced vascularization of the islets on day 10. Accordingly, the grafts also contained a diminished overall number of fenestrated blood vessels and, hence, less regions allowing rhodamine 6G leakage within the islets.

Taken together, the present study provides the first evidence that NG2 is of major importance for the adequate revascularization of transplanted islets because the loss of NG2 in intraislet pericytes impairs this process. These findings may represent a novel understanding of the role of pericytes in pancreatic islet engraftment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

All experiments were approved by the local governmental animal protection committee (Landesamt für Verbraucherschutz, Abteilung C Lebensmittel- und Veterinärwesen, Saarbrücken, Germany; permit number 45/2018). They were performed according to the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington DC, USA) and the European legislation on protection of animals (Guide line 2010/63/EU).

For the generation of human citrate plasma, venous blood was drawn from four healthy human volunteers after obtaining their written informed consent and with the approval of the local ethics review board.

References

Almaca J, Weitz J, Rodriguez-Diaz R, Pereira E, Caicedo A (2018) The pericyte of the pancreatic islet regulates capillary diameter and local blood flow. Cell Metab 27:630–644 e634

Ampofo E, Rudzitis-Auth J, Dahmke IN, Roessler OG, Thiel G, Montenarh M, Menger MD, Laschke MW (2015) Inhibition of protein kinase CK2 suppresses tumor necrosis factor (TNF)-alpha-induced leukocyte-endothelial cell interaction. Biochim Biophys Acta 1852:2123–2136

Armulik A, Genove G, Betsholtz C (2011) Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell 21:193–215

Bai X, Saab AS, Huang W, Hoberg IK, Kirchhoff F, Scheller A (2013) Genetic background affects human glial fibrillary acidic protein promoter activity. PLoS One 8:e66873

Barritt DS, Pearn MT, Zisch AH, Lee SS, Javier RT, Pasquale EB, Stallcup WB (2000) The multi-PDZ domain protein MUPP1 is a cytoplasmic ligand for the membrane-spanning proteoglycan NG2. J Cell Biochem 79:213–224

Beger C, Cirulli V, Vajkoczy P, Halban PA, Menger MD (1998) Vascularization of purified pancreatic islet-like cell aggregates (pseudoislets) after syngeneic transplantation. Diabetes 47:559–565

Bergers G, Song S (2005) The role of pericytes in blood-vessel formation and maintenance. Neuro Oncology 7:452–464

Brissova M, Fowler M, Wiebe P, Shostak A, Shiota M, Radhika A, Lin PC, Gannon M, Powers AC (2004) Intraislet endothelial cells contribute to revascularization of transplanted pancreatic islets. Diabetes 53:1318–1325

Bruni A, Gala-Lopez B, Pepper AR, Abualhassan NS, Shapiro AJ (2014) Islet cell transplantation for the treatment of type 1 diabetes: recent advances and future challenges. Diabetes Metab Syndr Obes 7:211–223

Burg MA, Tillet E, Timpl R, Stallcup WB (1996) Binding of the NG2 proteoglycan to type VI collagen and other extracellular matrix molecules. J Biol Chem 271:26110–26116

Cai J, Kehoe O, Smith GM, Hykin P, Boulton ME (2008) The angiopoietin/Tie-2 system regulates pericyte survival and recruitment in diabetic retinopathy. Invest Ophthalmol Vis Sci 49:2163–2171

Franco M, Roswall P, Cortez E, Hanahan D, Pietras K (2011) Pericytes promote endothelial cell survival through induction of autocrine VEGF-A signaling and Bcl-w expression. Blood 118:2906–2917

Gargiulo P, Giusti C, Pietrobono D, La Torre D, Diacono D, Tamburrano G (2004) Diabetes mellitus and retinopathy. Dig Liver Dis 36(Suppl 1):S101–S105

Goss JA, Schock AP, Brunicardi FC, Goodpastor SE, Garber AJ, Soltes G, Barth M, Froud T, Alejandro R, Ricordi C (2002) Achievement of insulin independence in three consecutive type-1 diabetic patients via pancreatic islet transplantation using islets isolated at a remote islet isolation center. Transplantation 74:1761–1766

Hellström M, Kalen M, Lindahl P, Abramsson A, Betsholtz C (1999) Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development 126:3047–3055

Henriksnas J, Lau J, Zang G, Berggren PO, Kohler M, Carlsson PO (2012) Markedly decreased blood perfusion of pancreatic islets transplanted intraportally into the liver: disruption of islet integrity necessary for islet revascularization. Diabetes 61:665–673

Hogan MF, Hull RL (2017) The islet endothelial cell: a novel contributor to beta cell secretory dysfunction in diabetes. Diabetologia 60:952–959

Huang W, Zhao N, Bai X, Karram K, Trotter J, Goebbels S, Scheller A, Kirchhoff F (2014) Novel NG2-CreERT2 knock-in mice demonstrate heterogeneous differentiation potential of NG2 glia during development. Glia 62:896–913

Hynes RO (2002) A reevaluation of integrins as regulators of angiogenesis. Nat Med 8:918–921

Juang JH, Kuo CH, Peng SJ, Tang SC (2015) 3-D imaging reveals participation of donor islet Schwann cells and pericytes in islet transplantation and graft neurovascular regeneration. EBioMedicine 2:109–119

Kelly C, Parke HG, McCluskey JT, Flatt PR, McClenaghan NH (2010) The role of glucagon- and somatostatin-secreting cells in the regulation of insulin release and beta-cell function in heterotypic pseudoislets. Diabetes Metab Res Rev 26:525–533

Laschke MW, Vollmar B, Menger MD (2011) The dorsal skinfold chamber: window into the dynamic interaction of biomaterials with their surrounding host tissue. Eur Cell Mater 22:147–164

Lau J, Carlsson PO (2009) Low revascularization of human islets when experimentally transplanted into the liver. Transplantation 87:322–325

Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, Lein ES, Zeng H (2010) A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci 13:133–140

Makagiansar IT, Williams S, Mustelin T, Stallcup WB (2007) Differential phosphorylation of NG2 proteoglycan by ERK and PKCalpha helps balance cell proliferation and migration. J Cell Biol 178:155–165

Malinin NL, Pluskota E, Byzova TV (2012) Integrin signaling in vascular function. Curr Opin Hematol 19:206–211

Menger MD, Vajkoczy P, Leiderer R, Jäger S, Messmer K (1992) Influence of experimental hyperglycemia on microvascular blood perfusion of pancreatic islet isografts. J Clin Invest 90:1361–1369

Menger MD, Yamauchi J, Vollmar B (2001) Revascularization and microcirculation of freely grafted islets of Langerhans. World J Surg 25:509–515

Murfee WL, Rehorn MR, Peirce SM, Skalak TC (2006) Perivascular cells along venules upregulate NG2 expression during microvascular remodeling. Microcirculation 13:261–273

Nishiyama A (2007) Polydendrocytes: NG2 cells with many roles in development and repair of the CNS. Neuroscientist 13:62–76

Olsson R, Carlsson PO (2006) The pancreatic islet endothelial cell: emerging roles in islet function and disease. Int J Biochem Cell Biol 38:710–714

Ozerdem U, Stallcup WB (2004) Pathological angiogenesis is reduced by targeting pericytes via the NG2 proteoglycan. Angiogenesis 7:269–276

Ozerdem U, Grako KA, Dahlin-Huppe K, Monosov E, Stallcup WB (2001) NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. Dev Dyn 222:218–227

Proebstl D, Voisin MB, Woodfin A, Whiteford J, D'Acquisto F, Jones GE, Rowe D, Nourshargh S (2012) Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo. J Exp Med 209:1219–1234

Ribatti D, Nico B, Crivellato E (2011) The role of pericytes in angiogenesis. Int J Dev Biol 55:261–268

Sasson A, Rachi E, Sakhneny L, Baer D, Lisnyansky M, Epshtein A, Landsman L (2016) Islet pericytes are required for beta-cell maturity. Diabetes 65:3008–3014

Schlingemann RO, Rietveld FJ, de Waal RM, Ferrone S, Ruiter DJ (1990) Expression of the high molecular weight melanoma-associated antigen by pericytes during angiogenesis in tumors and in healing wounds. Am J Pathol 136:1393–1405

Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343:230–238

Tanjore H, Zeisberg EM, Gerami-Naini B, Kalluri R (2008) Beta1 integrin expression on endothelial cells is required for angiogenesis but not for vasculogenesis. Dev Dyn 237:75–82

Tillet E, Gential B, Garrone R, Stallcup WB (2002) NG2 proteoglycan mediates beta1 integrin-independent cell adhesion and spreading on collagen VI. J Cell Biochem 86:726–736

Vajkoczy P, Menger MD, Simpson E, Messmer K (1995a) Angiogenesis and vascularization of murine pancreatic islet isografts. Transplantation 60:123–127

Vajkoczy P, Olofsson AM, Lehr HA, Leiderer R, Hammersen F, Arfors KE, Menger MD (1995b) Histogenesis and ultrastructure of pancreatic islet graft microvasculature. Evidence for graft revascularization by endothelial cells of host origin. Am J Pathol 146:1397–1405

Vincent AM, Russell JW, Low P, Feldman EL (2004) Oxidative stress in the pathogenesis of diabetic neuropathy. Endocr Rev 25:612–628

Wietecha MS, Cerny WL, DiPietro LA (2013) Mechanisms of vessel regression: toward an understanding of the resolution of angiogenesis. Curr Top Microbiol Immunol 367:3–32

Yadavilli S, Hwang EI, Packer RJ, Nazarian J (2016) The role of NG2 proteoglycan in glioma. Transl Oncol 9:57–63

Yamamoto H, Ehling M, Kato K, Kanai K, van Lessen M, Frye M, Zeuschner D, Nakayama M, Vestweber D, Adams RH (2015) Integrin beta1 controls VE-cadherin localization and blood vessel stability. Nat Commun 6:6429

Ye F, Kim C, Ginsberg MH (2011) Molecular mechanism of inside-out integrin regulation. J Thromb Haemost 9(Suppl 1):20–25

Yotsumoto F, You WK, Cejudo-Martin P, Kucharova K, Sakimura K, Stallcup WB (2015) NG2 proteoglycan-dependent recruitment of tumor macrophages promotes pericyte-endothelial cell interactions required for brain tumor vascularization. Oncoimmunology 4: e1001204

You WK, Yotsumoto F, Sakimura K, Adams RH, Stallcup WB (2014) NG2 proteoglycan promotes tumor vascularization via integrin-dependent effects on pericyte function. Angiogenesis 17:61–76