

ARTICLE



Effect of recombinant human vascular endothelial growth factor on testis tissue xenotransplants from prepubertal boys: a three-case study



BIOGRAPHY

Elissavet Ntemou graduated from the Aristotle University of Thessaloniki, Greece with a BSc in Biology and obtained her MMedSci in Assisted Reproduction Technology from the University of Nottingham, UK. In 2014, she joined, as a PhD candidate, the Biology of the Testis research group at the Vrije Universiteit Brussel, Belgium.

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KEY MESSAGE

Treatment of testis tissue from prepubertal boys with vascular endothelial growth factor (VEGF) before xenotransplantation resulted in higher vascularized transplants containing more intact seminiferous tubules and increased number of spermatogonia compared with controls. Therefore, VEGF may be considered as a potential candidate for improving the long-term efficiency of immature testis tissue transplantation.

ABSTRACT

Research question: Does recombinant human vascular endothelial growth factor (VEGF-165) improve the efficiency of human immature testis tissue (ITT) xenotransplantation?

Design: ITT fragments from three prepubertal boys were cultured for 5 days with VEGF-165 or without (control) before xenotransplantation into the testes of immunodeficient mice. Xenotransplants were recovered at 4 and 9 months post-transplantation and vascularization, seminiferous tubule integrity, number of spermatogonia and germ cell differentiation were evaluated by histology and immunohistochemistry.

Results: Transplants from donor 1 and donor 2 treated with VEGF demonstrated higher vascular surface ($P = 0.004$) and vessel density ($P = 0.011$) overall and contained more intact seminiferous tubules ($P = 0.039$) with time, compared with controls. The number of spermatogonia was increased over time ($P < 0.001$) irrespective of treatment and donor, whereas, for the VEGF-treated transplants, the increase was even higher over time ($P = 0.020$). At 9 months, spermatocytes were present in the xenotransplants, irrespective of treatment. No transplants could be recovered from donor 3, who had already received treatment with cyclosporine for aplastic anaemia before biopsy.

Conclusions: In-vitro pre-treatment of human prepubertal testis tissue with VEGF improved transplant vascularization in two out of three cases, resulting in improved seminiferous tubule integrity and spermatogonial survival during xenotransplantation. Although further studies are warranted, we suggest VEGF to be considered as a factor for improving the efficiency of immature testis tissue transplantation in the future.

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KEY WORDS

VEGF

Fertility preservation

Immature human testis tissue

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Spermatogonia

Testis tissue transplantation

INTRODUCTION

In mature testis tissue (ITT) cryopreservation is the only fertility preservation option currently offered to prepubertal boys at risk of germ cell loss caused by gonadotoxic treatments (Picton *et al.*, 2015; Onofre *et al.*, 2016). Promising technologies using testis tissue biopsies, containing spermatogonial stem cells (SSC), are being developed: in-vitro spermatogenesis, spermatogonial stem cell transplantation and testis tissue transplantation (TTT) (Keros *et al.*, 2007; Ginsberg *et al.*, 2010; Wynn *et al.*, 2010; Clark *et al.*, 2011; Sadri-Ardekani *et al.*, 2011; Sato *et al.*, 2011; Goossens *et al.*, 2013; de Michele *et al.*, 2017).

The advantage of TTT is that it enables the transfer of the SSCs within their natural environment, without disturbing the interactions between germ cells and supporting cells (Goossens *et al.*, 2013). In several non-primate species (Honaramooz *et al.*, 2002; Schlatt *et al.*, 2002; Shinohara *et al.*, 2002; Oatley *et al.*, 2005; Abrishami *et al.*, 2010; Reddy *et al.*, 2012) and non-human primates (Honaramooz *et al.*, 2004; Ehmcke *et al.*, 2011; Liu *et al.*, 2016) xenotransplantation of ITT into immunodeficient mice led to generation of spermatozoa. Xenotransplantation of human ITT has not yet resulted in complete spermatogenesis (Wynn *et al.*, 2008; Sato *et al.*, 2010; Van Saen *et al.*, 2011; 2013). Long-term survival of human ITT xenotransplants has been demonstrated but the low spermatogonial recovery, the presence of degenerated tubules and the lack of complete spermatogenesis are challenges that need to be overcome (Wynn *et al.*, 2008; Van Saen *et al.*, 2011).

The survival of the transplants depends on the neovascularization and the establishment of a vascular system for sufficient blood supply between the transplant and the host. The availability of oxygen, nutrients and factors supporting cell survival, proliferation and maturation is critical and thus the period of ischaemic hypoxia during transplantation is crucial (Arregui *et al.*, 2008).

Therefore, stimulating neovascularization in the transplants may improve transplant survival and germ cell proliferation and differentiation. Vascular endothelial growth factor (VEGF) is an angiogenesis-

inducing factor. The VEGF family, with five members (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta-derived growth factor) belongs to the vascular endothelium-specific growth factors, also including the angiopoietin family (Ang1, Ang2 and Ang3) and ephrin family (Ephrin-B2) (Gale and Yancopoulos, 1999). As a highly potent stimulator of the vascular endothelium, VEGF promotes endothelial cell migration and proliferation, leading to the formation of new blood vessels either by vasculogenesis or angiogenic sprouting (Ferrara and Davis-Smyth, 1997; Risau, 1997; Yancopoulos *et al.*, 2000; Rafii and Lyden, 2003). Upregulated transcription of VEGF is caused by several factors, including hypoxia, cytokines and growth factors (Shweiki *et al.*, 1992), and biological effects are mediated through binding to two receptor tyrosine kinases, VEGFR-1 and VEGFR-2 (Barleon *et al.*, 1996).

Administration of VEGF, either by culture of the tissue before transplantation or by hydrogel delivery, promoted angiogenesis, follicular survival and enhanced the function of ovarian tissue xenotransplants, including human (Shikanov *et al.*, 2011; Labied *et al.*, 2013; Wang *et al.*, 2013; Henry *et al.*, 2015; Langbein *et al.*, 2016; Tavana *et al.*, 2016). A previous study on autotransplantation of mouse ITT revealed a positive short-term effect of VEGF on transplant neovascularization 5 and 21 days post-transplantation (Poels *et al.*, 2016), whereas the enhancement of germ cell survival has been demonstrated in several species, including mouse, pig and bovine (Schmidt *et al.*, 2006; Caires *et al.*, 2009, 2012; Dores and Dobrinsky, 2014; Poels *et al.*, 2016; Tian *et al.*, 2016).

In the present study, the potential effect of in-vitro pre-treatment with VEGF on human prepubertal testis tissue xenotransplants in terms of vascularization, seminiferous tubule integrity, spermatogonial survival and differentiation over a medium (4 months) and long (9 months) period was investigated.

MATERIALS AND METHODS

Human tissue donors

The present study was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The experiments were

approved by the internal review board of the Universitair Ziekenhuis Brussel (UZ Brussel) (reference number: 2004/17D on 29 April 2004, reference number: 2015/V3 on 28 January 2015 and reference number: 2017/061 on 22 March 2017).

Testis tissue biopsies were obtained from three prepubertal patients aged 3, 8 and 9 years (further referred to as donor 1, 2 and 3, respectively), after written informed consent was obtained from their parents. All patients underwent testicular tissue banking as part of their fertility preservation programme at the Centre for Reproductive Medicine of the UZ Brussel. Ninety per cent of the biopsy was stored for later fertility restoration, and 10% was donated for research purposes. As donor 1 did not survive his disease, all cryopreserved material was donated for research. Donor 1 was diagnosed with a malignant disease (neuroblastoma) and had received treatment with carboplatin before the biopsy was taken. Donor 2 was also diagnosed with a malignant disease (Hodgkin's lymphoma) but the ITT biopsy was taken before the start of the treatment. Donor 3 had a non-malignant disorder (aplastic anaemia) and had received cyclosporine before the biopsy (TABLE 1).

Tissue preparation and in-vitro

treatment with VEGF

Cryopreserved ITT was thawed, as previously described (Van Saen *et al.*, 2011), and cut into smaller fragments (1.0–1.5 mm³). Testis tissue fragments were placed in air-liquid interface culture (34°C and 5% CO₂) on 0.4-μm pore membrane inserts (Millicell Cell culture plate inserts; Sigma Aldrich, Bornem, Belgium). Fragments were cultured in Dulbecco's Modified Eagle Medium (DMEM) F-12 (Life Technologies, Thermo Fischer Scientific, Gent, Belgium) containing 10% human serum albumin (HSA) (Vitrolife, Göteborg, Sweden), 1% Penicillin-Streptomycin (Thermo Fischer Scientific, Gent, Belgium) and 100 ng/ml of recombinant human VEGF-165 (293-VE-010; R&D systems, Abingdon, UK) or without VEGF-165 (control) (FIGURE 1). Tissue culture was maintained for 5 days, with media change every 2 days (Caires *et al.*, 2009). Testis fragments were washed in DMEM before transplantation.

Intratesticular xenotransplantation

All experimental procedures were carried out according to the European Union Directive 2010/63/EU for animal experiments and approved by the

TABLE 1 TESTIS TISSUE DONORS

Patient information					Histology
Donor	Age (years)	Disease	Treatment received before biopsy	Time between treatment and biopsy	Most advanced germ cell type
1	3	Neuroblastoma	Carboplatin (1500 mg/ m ²) Cisplatin (320 mg/ m ²) Cyclophosphamide (4200 g/ m ²), 2 consecutive days / daily dose: 1050 mg/m ² x 2 courses VP16 (1400 mg/ m ²) Vincristine (12 mg/ m ²)	63 days	Spermatogonia
2	8	Hodgkin's lymphoma	None	-	Spermatogonia
3	9	Aplastic anemia	Cyclosporine (85 mg twice a day)	150 days	Spermatogonia

Animal Care and Use Committee of the Vrije Universiteit Brussel (VUB) on 8 December 2014 (reference number: 14-216-4). Twelve fragments of donor 1 were immediately fixed after culture for immunohistochemical analysis (in-vitro study). In total, 64 ITT fragments were transplanted into the testes of 4–5-week-old Swiss Nu/Nu mice ($n = 48$) (Charles River Laboratories, Saint-Germain-Nuelles, France). More specifically, 32 mice received a single ITT fragment from donor 1 in one testis and a frozen-thawed testis tissue (Van Saen *et al.*, 2011) from 5–7-day old SV129 X C57BL green fluorescent protein (GFP⁺) mice (bred at the animal facility of the VUB) in the contralateral testis. The GFP⁺ mouse ITT served as a TTT control in this study to show that the transplantation process worked. All ITT fragments from donor 2 ($n = 16$) and donor 3 ($n = 16$) were used for xenotransplantation. One human ITT fragment was transplanted into both testes of eight mice (TABLE 2).

Briefly, mice were anaesthetized by a single intraperitoneal injection (75 μ l/10 g of body weight) of a mixture of 0.1 mg/ml medetomidine hydrochloride and 0.75 mg/ml ketamine hydrochloride. After exteriorization of the mouse testis, a fine incision was made in the tunica albuginea and the transplant was inserted under the tunica, and the incision was closed using non-absorbable suture (U7003, 10-0) (Ethicon, Instruvet, Beringen-Paal, Belgium).

Mice were housed individually in ventilated cages under a controlled environment of 14-h to 10-h light-dark cycle with access to sterilized diet and water. Cages were enriched with shelter, shredding materials and chewing toys.

Transplant recovery

Collection of mouse testes was carried out at 4 and 9 months post-transplantation. After fixation, the testes were embedded in paraffin and

cut into 5- μ m-thick serial sections. Immunostaining for human-specific vimentin (M072501; Dako, Heverlee, Belgium) was carried out for every tenth section to localize the human testis tissue transplant. Similarly, staining for GFP (SC-9996) (Bioconnect, Huissen, Netherlands) was used to identify mouse GFP⁺ transplants.

Histology and immunohistochemistry

For haematoxylin-periodic acid Schiff (H/PAS) staining, sections were deparaffinized in xylene (2 \times 5 min) and rehydrated in a graded alcohol series (2 \times 100%, 90% and 70% isopropanol). After washing in distilled water (2 \times 5 min), sections were oxidized in periodic acid solution (VWR International) for 5 min. After two more washing steps, slides were placed in Schiff's reagent (VWR International) for 15 min. Next, sections were washed in distilled water (2 \times 5 min) and counterstained with haematoxylin.

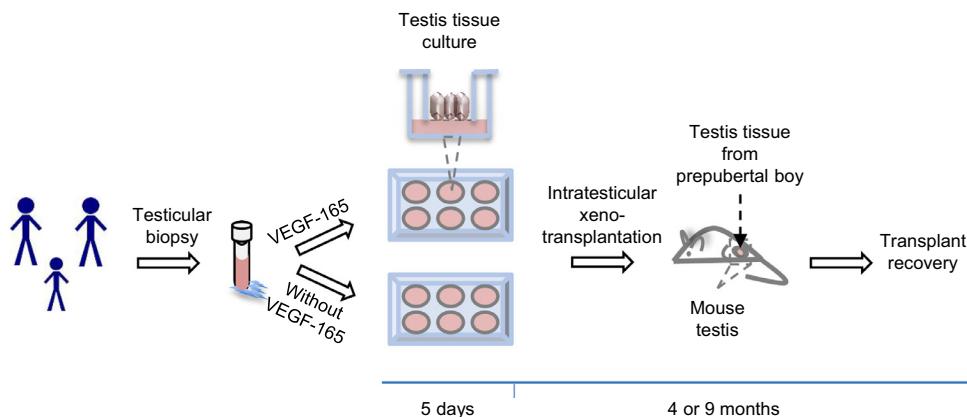


FIGURE 1 Experimental design. Frozen-thawed testis tissue fragments from three prepubertal boys were cultured as tissue explants with vascular endothelial growth factor (VEGF)-165 (100 ng/ml) or without VEGF-165 (control). After 5 days, testis fragments were transplanted into the testes of immunodeficient mice. Xenotransplants were recovered at 4 or 9 months post-transplantation.

TABLE 2 TESTIS TISSUE FRAGMENTS USED PER DONOR AND RECOVERY RATES OF XENOTRANSPLANTS

Donor	Testis tissue (n)	Treatment				Treatment				Treatment			
		Cultured testis tissue (n)	Evaluated cultured tissue (n)	Control VEGF (n)	Transplants* (n)	Mice transplants (n)	Recovered transplants (%)	Control VEGF recovery (n)	VEGF recovery (n)	Transplants ^b (n)	Mice transplants (n)	Recovered transplants (%)	Control VEGF recovery (n)
1	44	12	6	2	4	16	16 ^c	15/15 (100)	7/7	8/8	16	16 ^d	12/15 (80)
2	16	—	—	—	8	4 ^c	5/6 (83)	2/2	3/4	8	4	6/8 (75)	3/4
3	16	—	—	—	8	4	0	0	0	8	4	0	0
Mouse GFP+					16 ^e	16 ^c	15/15 (100)	—	—	16 ^e	16 ^d	14/15 (93)	—

^a Six cultured testis tissue pieces were lost owing to their small size during the preparations of the sections at the microtome.^b Half of transplants were cultured with VEGF and the other half without VEGF (control group).^c One mouse died from the control group.^d One mouse died from the VEGF group.^e GFP⁺ mouse transplants implanted at the contralateral testis of the mice dedicated to donor 1.

GFP, green fluorescent protein; VEGF, vascular endothelial growth factor.

Immunohistochemistry was carried out for several markers (Supplementary TABLE). Sections were deparaffinized in xylene (3×10 min) and rehydrated in a graded alcohol series ($2 \times 100\%$, 90% and 70% isopropanol). After washing with either phosphate-buffered saline (PBS; Life-technologies, Gent, Belgium) or tris-buffered saline (TBS) with 0.05% Tween 20 (Sigma Aldrich) for 5 min, endogenous peroxidase activity was blocked by incubating the tissue sections with hydrogen peroxide (H_2O_2 ; diluted in methanol) for 30 min. After a washing step, antigen retrieval was carried out by incubating the slides in retrieval solution in a water bath ($95^\circ C$). Next, slides were washed, and tissue sections were incubated with normal goat serum (Tebu-bio, Boechout, Belgium) diluted in PBS or TBS with 5% bovine serum albumin (Sigma Aldrich). Primary antibodies were applied to the sections and incubated in a humidified chamber overnight. In general, human adult tissue sections from cadaver testes obtained from the autopsy service of the UZ Brussel, were used as positive controls. Only for the anti-Müllerian hormone (AMH) immunostaining, human prepubertal testis tissue sections from testis fragments donated for research were used, as AMH is not expressed in adult Sertoli cells. As negative controls, the first antibody was replaced by a mouse (sc2025) (Santa Cruz, Biotechnology, Heidelberg, Germany) or a rabbit (sc2027) (Santa Cruz) immunoglobulin G (IgG) isotype control. After three washing steps, sections were incubated with a peroxidase-labelled secondary antibody (Dako Real Envision Detection System; Dako). After 1 h of incubation and three washing steps, visualization with 3,3'-diaminobenzidine (Dako RealTM EnvisionTM Envision system; Dako) and counterstaining with haematoxylin followed.

Transplant vascularization

Von Willebrand factor (VWF), a multimeric plasma glycoprotein produced exclusively by endothelial cells (Jaffe *et al.*, 1973) was used to detect blood vessels. The antibody used is human specific and therefore only blood vessels of human origin were detected. Sections from three different depths of each recovered transplant were used for assessment. Vascular surface, expressed as the percentage of total blood vessel surface towards the total surface of the transplant, and vessel density, as the ratio

between total number of blood vessels and total surface area of the transplant, were calculated. Blood vessel surface, as the area enclosed by endothelial cells, and transplant surface, were calculated by morphometry with CELL F software (Olympus, Berchem, Belgium).

Seminiferous tubule integrity

Sections stained with H/PAS were used to evaluate tubular integrity. The percentage of intact tubules was calculated on sections for every $50\ \mu m$ of each transplant. As intact tubules, we considered tubules with uninterrupted basement membrane, good adhesion of the seminiferous epithelial cells to each other and to the basement membrane and without any sign of degeneration, as previously described (Van Saen *et al.*, 2011). Only round tubular cross sections were included in the analysis. As round tubule, we accepted tubules with diameter maximum 1.5 times of the perpendicular diameter.

Spermatogonial survival

The germ cell marker MAGE-A4 was used to evaluate the survival of spermatogonia after the 5-day culture and at 4 and 9 months post-transplantation. MAGE-A4 is expressed mainly by spermatogonia, but also by primary spermatocytes (Takahashi *et al.*, 1995); therefore, only the positive cells situated at the basement membrane were counted. The number of MAGE-A4 positive cells per mm^2 (MAGE-A4⁺ cells/ mm^2) and the percentage of positive tubules were calculated on sections for every $50\ \mu m$ of each transplant.

Germ cell differentiation

At 9 months post-transplantation, the level of differentiation was determined based on morphological assessment of different germ cell types in all seminiferous tubules on H/PAS stained sections. The percentage of tubules containing the most advanced germ cell type was calculated. Tubules were characterized as tubules with no germ cells, when Sertoli cells were only present; tubules with spermatogonia, when spermatogonia were identified as the most advanced germ cell type present; and tubules with spermatocytes, when spermatocytes were present. Meiotic activity in the transplants was confirmed by immunostaining for the expression of the meiotic marker BOLL. The presence of pachytene spermatocytes was further confirmed by

the typical pattern of γ H2XA expression and the staining of the sex body in the nucleus of the cells.

Niche maturation

Differentiation of SSCs is highly influenced by the niche microenvironment, with Sertoli cells playing a key role in the establishment and function of the niche. Sertoli cell maturation and function was qualitatively assessed by immunostaining for the expression of Sertoli cell markers, including AMH, androgen receptor (AR) (Sharpe *et al.*, 2003) and inhibin A (INHA) (Andersson *et al.*, 1998).

Mouse transplants

Sections from GFP⁺ mouse transplants were used for GFP immunostaining to determine the status of spermatogenesis.

Microscopy

All microscopic evaluations were carried out on an inverted light microscope (Olympus IX81). For the evaluations, whole cross sections of the human testis tissue transplants were analysed with the programme CELL F (Olympus, Berchem, Belgium).

Statistical analysis

RStudio 1.1.463 running on R version 3.5.1 (RStudio, Boston, Massachusetts) was used for all statistical analysis. Fisher's exact test was used to compare recovery rates between VEGF-treated and control transplants at 4 and 9 months, respectively. When describing the transplant analysis, frequencies and percentages were used. For expressing relationships for vascularization, seminiferous tubule integrity, spermatogonial survival and differentiation of human spermatogonia between VEGF-treated transplants and controls, the linear mixed-effects model was used provided by the package lme4 (Bates *et al.*, 2015) in R. The lme4 package in the CRAN repository is a package designed for fitting linear and generalized linear mixed-effects models. It provides functions for fitting and analysing mixed models for which we used the linear variant by using the lmer function. Each time six covariates (treatment [VEGF and control], time [4 months versus 9 months], donor and possible interaction effects [treatment x time], [treatment x donor] and [time x donor]) were entered into the model. When the observation was measured only once (e.g. germ cell differentiation at 9

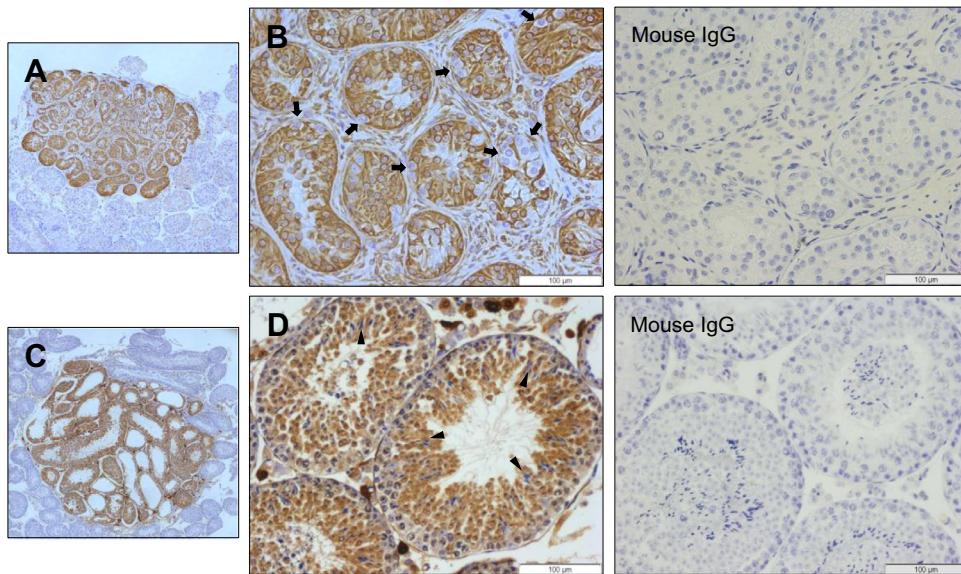


FIGURE 2 Intratesticular testis tissue transplant retrieval at 4 months post-transplantation. (A) Localization of human testis tissue transplant (vimentin⁺) within the mouse testis (vimentin⁻) by vimentin immunostaining; (B) Magnified image of the recovered human testis tissue transplant (arrows indicate germ cells). Mouse IgG isotype control as negative control (human prepubertal testis tissue); (C) Localization of mouse testis tissue transplant (green fluorescent protein [GFP]⁺) in Swiss Nu/Nu mouse testis (GFP) by GFP immunostaining; and (D) magnification of the recovered mouse testis tissue transplant (arrowheads indicate spermatozoa). Mouse IgG isotype control as negative control (mouse adult testis tissue).

months), covariates related to time were not included. A random effect for the mouse was added in which the effect was measured multiple times. For estimating the degrees of freedom, Satterthwaite's approximation (Satterthwaite, 1946) was used for calculating an estimated test statistic for the covariates in the linear mixed-effects model. A multiple testing correction was carried out using Benjamini and Hochberg False Discovery Rate based on the number of statistical models used in this study (Benjamini and Hochberg, 1995; Walters, 2016). A Mann-Whitney test was carried out on the spermatogonial survival data during explant culture. To meet the assumptions of the statistical models (homogeneity of variance and normality of residuals), transformation was applied. All *P*-values were reported two-sided and *P* < 0.05 was considered statistically significant. Graphs were prepared using GraphPad Prism 6 (La Jolla, CA, USA). Results were visualized using bar charts, representing the mean values and standard deviations of the complete dataset in which multiple repetitive measurements were present.

RESULTS

Transplant recovery

As shown in TABLE 2, at 4 months post-transplantation, all transplants (15/15) could be recovered (100% recovery rate) for donor 1, whereas, for donor 2,

the recovery rate was 83% (5/6). Nine months post-transplantation, 80% (12/15) of the transplants could be recovered for donor 1 and 75% (6/8) for donor 2 (FIGURE 2A and FIGURE 2B). For donor 3, none of the transplants could be recovered at any time point and thus no further analysis was conducted. Overall, the recovery rate at 4 months was 92% (11/12) for the VEGF-treated transplants and 100% (9/9) for the control, whereas, at 9 months, the recovery rate was 91% (10/11) and 67% (8/12) for VEGF-treated and control transplants, respectively. No statistical difference was observed between recovery of VEGF-treated transplants and controls at any time point. For the GFP⁺ mouse transplants, all transplants (15/15) could be recovered (100% recovery rate) at 4 months, whereas, at 9 months, the recovery rate was 93% (14/15) (TABLE 2 and FIGURE 2C and FIGURE 2D). Two mice dedicated to donor 1 and one mouse dedicated to donor 2 died before the time of analysis, and those transplants were not included.

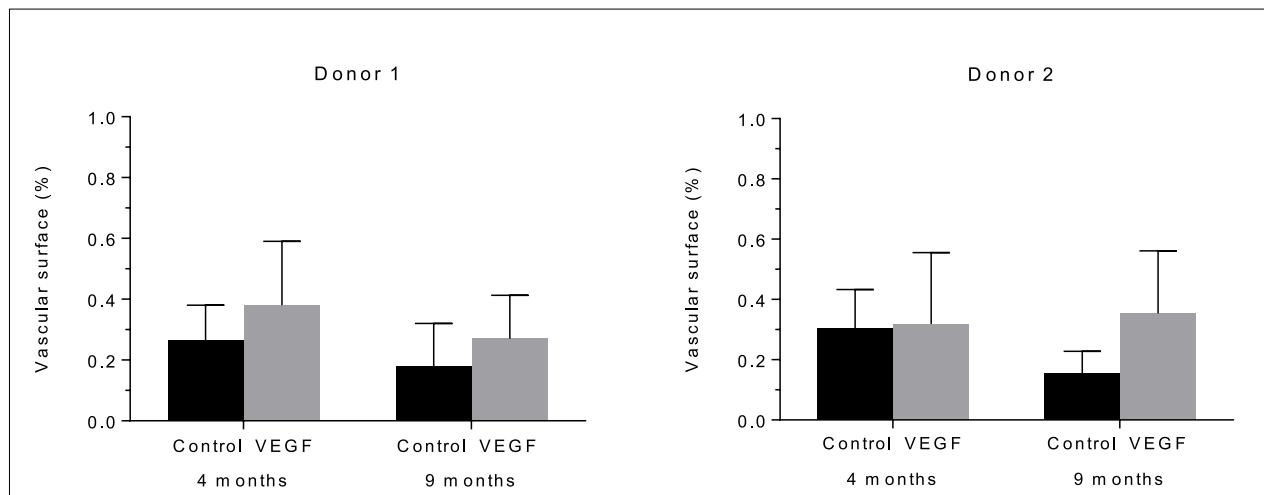
Effect of VEGF on transplant vascularization

For donor 1, the vascular surface at 4 months post-transplantation was $0.38 \pm 0.21\%$ in the VEGF-treated transplants and $0.26 \pm 0.11\%$ in the controls, whereas, at 9 months post-transplantation, it was $0.27 \pm 0.14\%$ and $0.18 \pm 0.14\%$, respectively. For

donor 2, the vascular surface at 4 months was $0.32 \pm 0.23\%$ in the VEGF-treated transplants and $0.30 \pm 0.13\%$ in the controls, whereas, at 9 months, it was $0.35 \pm 0.20\%$ and $0.16 \pm 0.07\%$ respectively. The VEGF-treated transplants exhibited an increased vascular surface (*P* = 0.004) compared with the controls. Overall, the vascular surface was significantly reduced (*P* = 0.004) regardless of the treatment during the transplantation period. No statistically significant difference between donor 1 and donor 2 was observed (FIGURE 3A, FIGURE 3C and FIGURE 3D).

The vessel density for donor 1 was $1.93 \pm 0.67 \times 10^{-5} \mu\text{m}^{-2}$ for the VEGF-treated transplants and $1.74 \pm 0.63 \times 10^{-5} \mu\text{m}^{-2}$ for the controls at 4 months post-transplantation and $1.55 \pm 0.43 \times 10^{-5} \mu\text{m}^{-2}$ and $1.05 \pm 0.29 \times 10^{-5} \mu\text{m}^{-2}$ at 9 months, respectively. For donor 2, the vessel density was $1.96 \pm 1.67 \times 10^{-5} \mu\text{m}^{-2}$ in the treated transplants and $1.94 \pm 0.79 \times 10^{-5} \mu\text{m}^{-2}$ in the controls at 4 months and $1.76 \pm 0.75 \times 10^{-5} \mu\text{m}^{-2}$ and $1.07 \pm 0.32 \times 10^{-5} \mu\text{m}^{-2}$ at 9 months respectively. The VEGF-treated transplants showed an increased vessel density (*P* = 0.011) compared with controls, whereas the vessel density was significantly reduced (*P* = 0.001) between 4 and 9 months post-transplantation, irrespective of

A



B

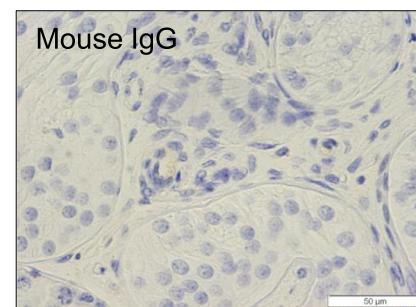
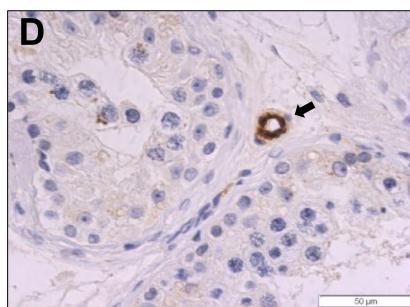
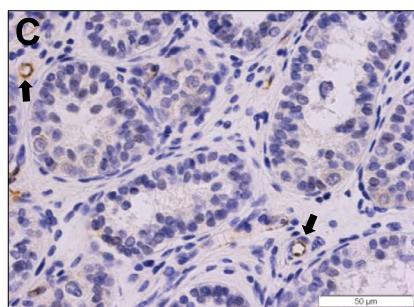
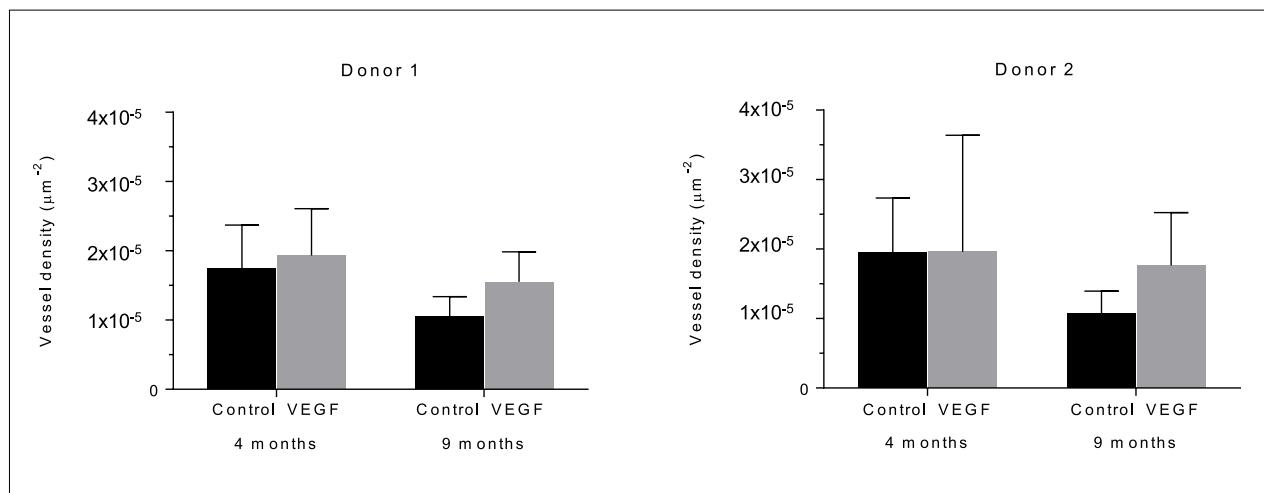


FIGURE 3 Effect of VEGF on transplant vascularization. (A) Vascular surface (%) in transplants from donor 1 and donor 2 at 4 and 9 months post-transplantation. Main effect: treatment- positive ($P = 0.004$); main effect: time- negative ($P = 0.004$); main effect: donor- not significant; (B) Vessel density (μm^{-2}) in transplants from donor 1 and donor 2 at 4 and 9 months post-transplantation. Main effect: treatment- positive ($P = 0.011$); main effect: time- negative ($P = 0.001$); main effect: donor- not significant; (C) Representative immunostaining for the detection of blood vessels by von Willebrand factor (VWF) antibody in VEGF-treated transplant from donor 2 at 4 months post-transplantation; and (D) in human adult testis tissue serving as control for immunostaining. Arrows indicate vessels stained positive for VWF. Mouse IgG isotype control as negative control (human adult testis tissue). Data shown as mean \pm SD.

treatment and donor. Similar to the vessel surface, the vessel density was not statistically different between donors (FIGURE 3B).

Effect of VEGF on seminiferous tubule integrity

For donor 1, the percentage of intact tubules at 4 months after transplantation

was $79 \pm 13\%$ in the VEGF-treated group and $76 \pm 14\%$ in the control group, whereas, at 9 months, it was $74 \pm 12\%$ and $68 \pm 11\%$, respectively. For donor

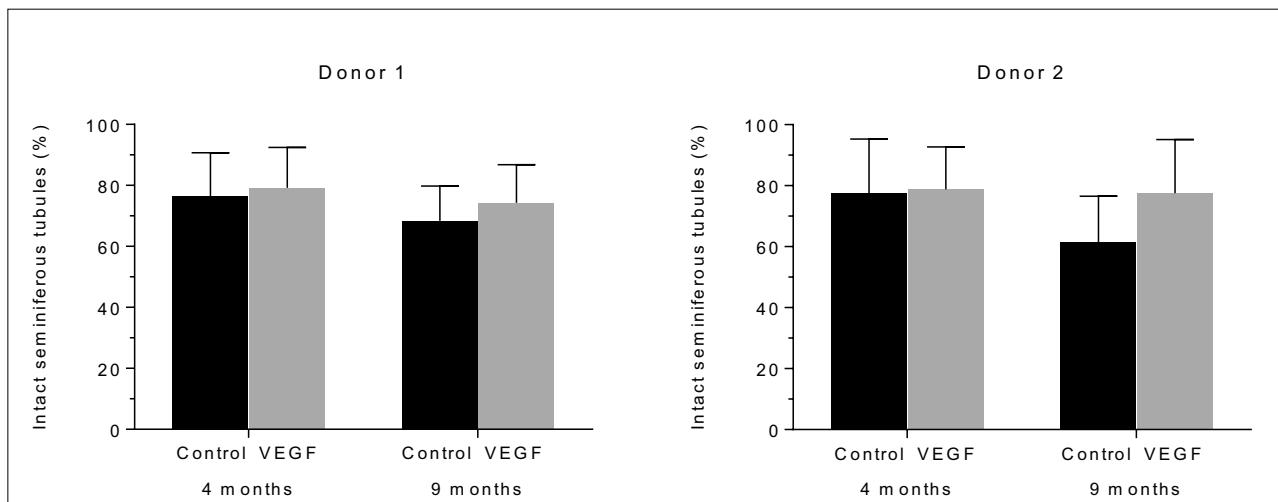


FIGURE 4 Effect of VEGF on seminiferous tubule integrity. Percentage of intact seminiferous tubules in transplants from donor 1 and donor 2 at 4 and 9 months post-transplantation. Main effect: treatment- not significant; main effect: time- negative ($P < 0.001$); main effect: donor- not significant; interaction effect: treatment x time- positive ($P = 0.039$); interaction effect: treatment x donor- not significant. Data shown as mean \pm SD.

2, the percentage of intact tubules was $79 \pm 14\%$ in the VEGF-treated group and $78 \pm 18\%$ in the control group at 4 months post-transplantation and $78 \pm 19\%$ and $61 \pm 15\%$ at 9 months post-transplantation, respectively. During the transplantation period, a deterioration of the seminiferous tubule integrity was apparent for all transplants ($P < 0.001$). However, VEGF treatment demonstrated a significant positive effect on the integrity of seminiferous tubules in time ($P = 0.039$). Again, no difference was observed between the two donors (FIGURE 4A).

Effect of VEGF on spermatogonial survival

Evaluation of the effect of VEGF on the survival of spermatogonia during explant culture was only possible for donor 1. After 5 days in culture, the number of MAGE-A4⁺ cells was 7.5 ± 5.8 cells/mm² in the VEGF-treated fragments and 3.4 ± 1.3 MAGE-A4⁺ cells/mm² in the control fragments (FIGURE 5A). Also, $32 \pm 19\%$ of the seminiferous tubules were positive for MAGE-A4 cells in the VEGF-treated fragments and $16 \pm 11\%$ in the controls (FIGURE 5B). Overall, the treatment with VEGF did not significantly improve the survival of MAGE-A4⁺ cells during culture.

At 4 months post-transplantation, VEGF-treated transplants from donor 1 contained 0.4 ± 0.9 MAGE-A4⁺ cells/mm² and the controls 0.5 ± 0.8 MAGE-A4⁺ cells/mm², whereas, at 9 months post-transplantation, transplants

contained 2.5 ± 2.5 and 1.6 ± 1.9 MAGE-A4⁺ cells/mm², respectively. For donor 2, VEGF-treated transplants contained 18.8 ± 13.2 MAGE-A4⁺ cells/mm² and the controls 13.7 ± 8.3 MAGE-A4⁺ cells/mm² at 4 months and 43.4 ± 23.4 and 28.7 ± 9.2 MAGE-A4⁺ cells/mm² at 9 months, respectively. Overall, the number of spermatogonia was increased over time ($P < 0.001$) irrespective of treatment and donor. Whereas, for the VEGF-treated transplants, the increase was even higher over time ($P = 0.020$). For donor 2, the increase in the number of spermatogonia due to the treatment and over time was even greater ($P < 0.001$ and $P < 0.002$, respectively). The number of spermatogonia between the two donors, however, differed significantly ($P < 0.001$) (FIGURE 5B).

The percentage of MAGE-A4⁺ tubules for donor 1 was $2.39 \pm 3.22\%$ in the VEGF-treated transplants and $3.22 \pm 4.27\%$ in the controls at 4 months and $8.16 \pm 6.74\%$ and $7.11 \pm 11.54\%$ at 9 months, respectively. For donor 2, at 4 months post-transplantation, $53.32 \pm 24.46\%$ of the tubules in VEGF-treated transplants contained spermatogonia and $46.70 \pm 21.90\%$ of the tubules in the controls, whereas, at 9 months, the percentages were $79.08 \pm 23.55\%$ and $73.37 \pm 16.55\%$, respectively. During the transplantation period, the percentage of MAGE-A4⁺ tubules increased over time ($P < 0.001$) in both groups and for both donors. The percentage increase of MAGE-A4⁺ tubules in the VEGF-treated transplants

from donor 2, however, was even greater than for donor 1 ($P = 0.035$). Overall, a significant difference in the percentage of MAGE-A4⁺ tubules was observed between the two donors ($P < 0.001$) (FIGURE 5B).

Effect of VEGF on the differentiation of human spermatogonia

Histological analysis of the ITT from both donors at the time the biopsy was taken revealed that spermatogonia were the most advanced germ cell type present (FIGURE 6A). At 4 months post-transplantation, spermatogonia were still the most advanced germ cells detected in the transplants from both donors, irrespective of treatment. At 9 months post-transplantation, spermatocytes were present in both VEGF-treated and control transplants (FIGURE 6A). More specifically, for donor 1, the percentage of tubules containing spermatocytes was $0.16 \pm 0.54\%$ in the VEGF-treated transplants and $0.07 \pm 0.38\%$ in the control group, whereas the percentage of tubules with spermatogonia was $7.72 \pm 6.01\%$ and $7.04 \pm 11.50\%$, respectively. The percentages of tubules lacking germ cells were similar ($92.12 \pm 6.13\%$ versus $92.88 \pm 11.54\%$). For donor 2, the percentage of tubules containing spermatocytes was $6.79 \pm 6.89\%$ in the VEGF-treated transplants and $3.36 \pm 4.43\%$ in the control group, whereas the percentage of tubules containing spermatogonia was $79.35 \pm 10.53\%$ and $70.46 \pm 18.34\%$, respectively. The percentages of tubules without germ cells was $13.87 \pm 12.70\%$ in the treated

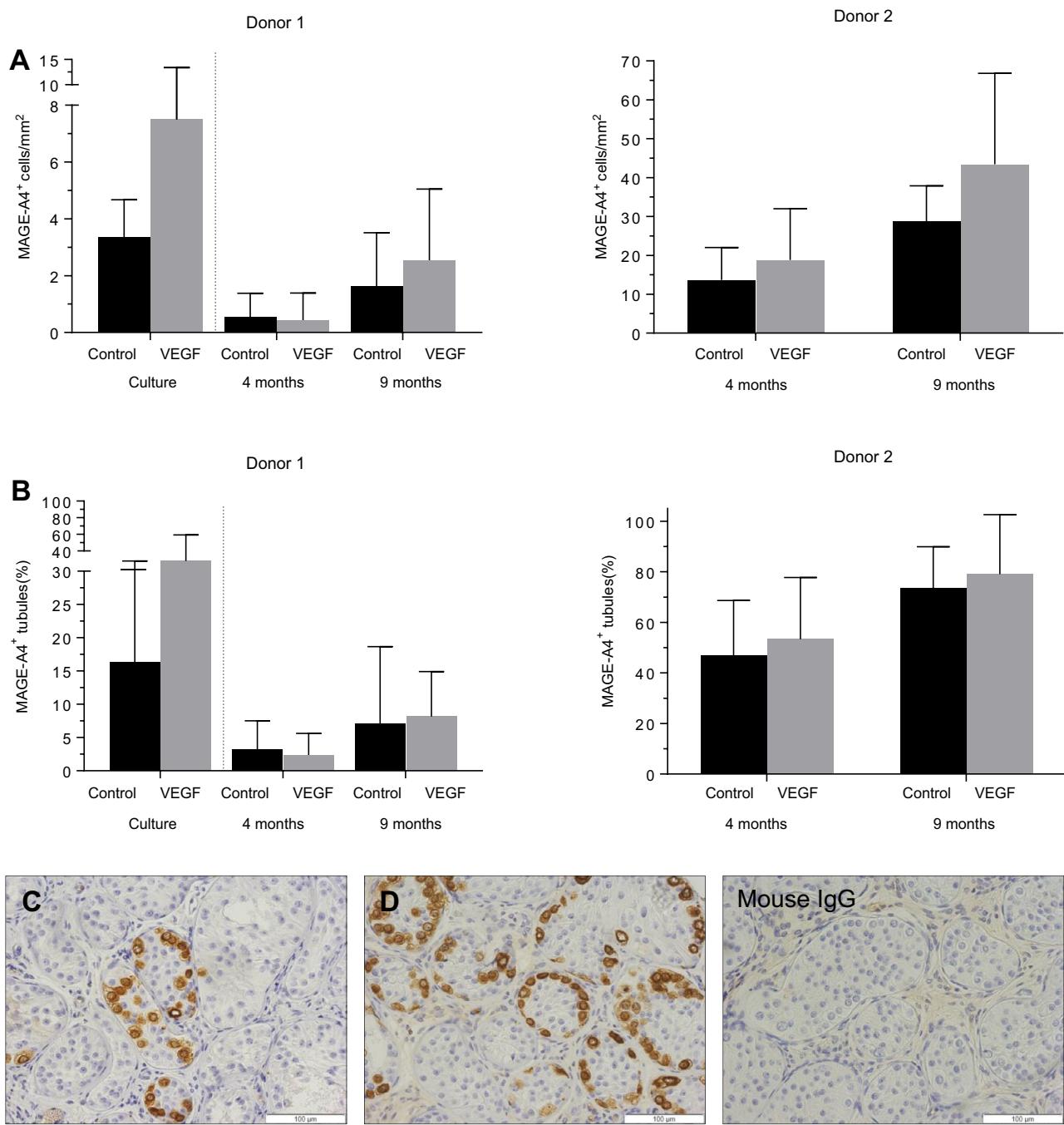


FIGURE 5 Effect of VEGF on spermatogonial survival and proliferation. (A) Number of MAGE-A4⁺ cells/mm² in testis tissue fragments after culture for 5 days and in transplants from donor 1 and in transplants from donor 2 at 4 and 9 months post-transplantation. Main effect: treatment- not significant; main effect: time- positive ($P < 0.001$); main effect: donor- significant ($P < 0.001$); interaction effect- treatment x time- positive ($P = 0.020$); interaction effect: treatment x donor- significant ($P < 0.001$); interaction effect: time x donor- significant ($P = 0.002$); (B) percentage of MAGE-A4⁺ tubules in testis tissue fragments after culture and in transplants from donor 1 and in transplants from donor 2 at 4 and 9 months post-transplantation. Main effect: treatment- not significant; main effect: time- positive ($P < 0.001$); main effect: donor- significant ($P < 0.001$); interaction effect: treatment x donor- significant ($P = 0.035$); (C) testicular tissue containing MAGE-A4⁺ and MAGE-A4⁺ tubules from donor 1 and (D) from donor 2, in VEGF-treated transplants at 9 months post-transplantation. Mouse immunoglobulin IgG isotype control as negative control (human prepubertal testis tissue). Data shown as mean \pm SD.

transplants and $26.18 \pm 18.92\%$ in the control group. In general, the distribution of tubules containing spermatocytes, spermatogonia and no germ cells differed significantly between donor 1 and donor 2 ($P < 0.001$). Adjusting for this variable,

VEGF-treated transplants from donor 2 had a higher percentage of tubules containing spermatocytes ($P < 0.001$) and a higher number of spermatogonia ($P = 0.019$). Additionally, within the VEGF-treated transplants, the percentage

of tubules without germ cells was decreased to a greater extent in donor 2 than in donor 1 ($P = 0.001$) (FIGURE 6B).

Meiotic activity was confirmed by immunostaining for the meiotic

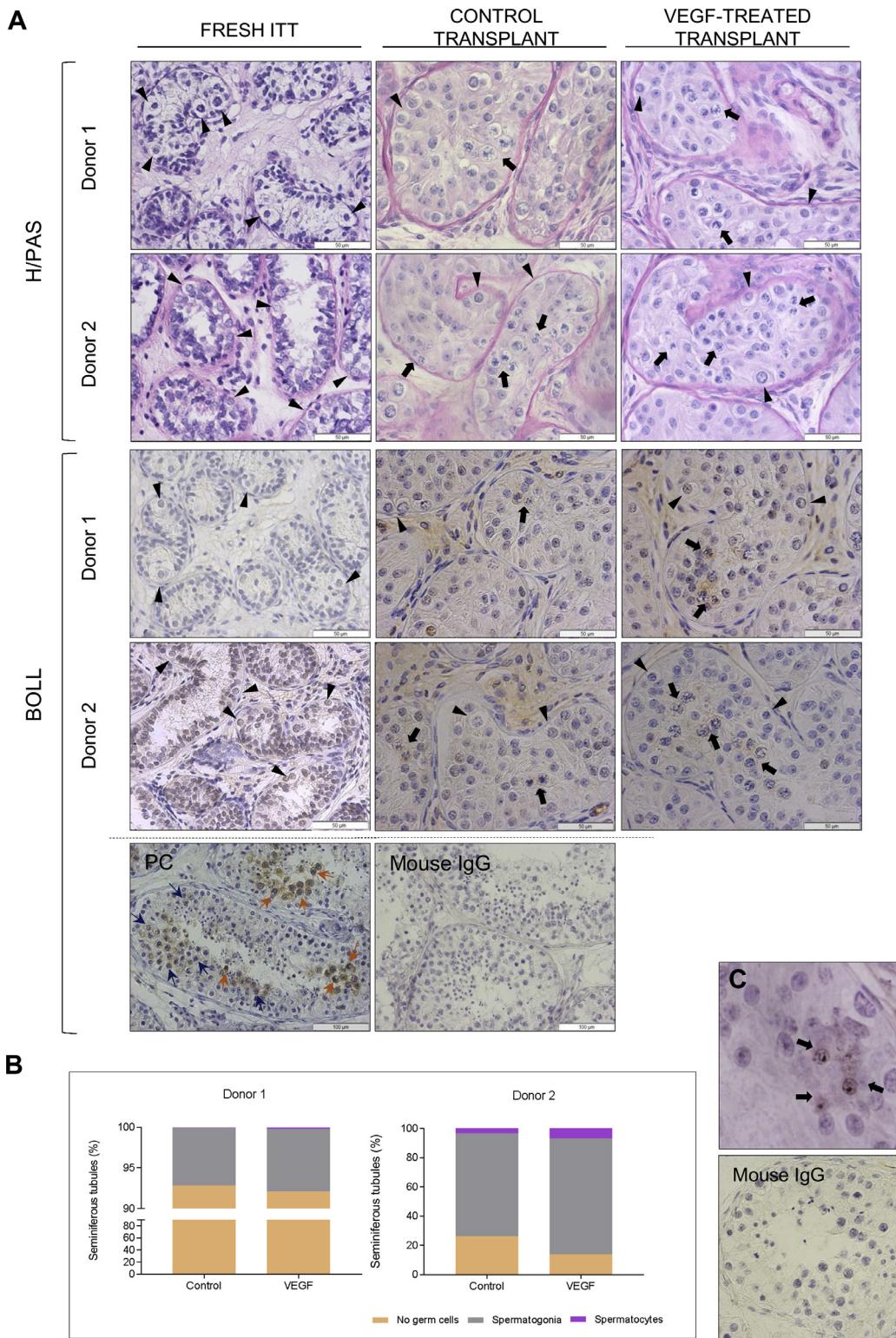


FIGURE 6 Effect of VEGF on the differentiation of human spermatogonia. (A) Spermatogonia were the most advanced germ cells present in fresh immature testis tissue from both donors. At 9 months post-transplantation, spermatocytes were morphologically identified on H/PAS stained sections in both VEGF-treated and control transplants. BOLL expression, although faint, confirmed the meiotic activity in the xenotransplants. Arrowheads indicate spermatogonia; arrows indicate spermatocytes. PC, positive control for BOLL antibody (human adult testis tissue). Orange arrows indicate late spermatocytes with strong BOLL expression; blue arrows indicate early spermatocytes with faint BOLL expression. Mouse IgG isotype control as negative control (human adult testis tissue); (B) Distribution of the different cell types in transplants from donor 1 and donor 2 at 9 months post-transplantation. (1) Modelling 'No germ cells'. Main effect: treatment- not significant; main effect: donor- significant ($P < 0.001$); interaction effect: treatment x donor- significant ($P = 0.001$); (2) Modelling 'Spermatogonia'. Main effect: treatment- not significant; main effect: donor- significant ($P < 0.001$); interaction effect: treatment x donor- significant ($P = 0.019$); (3) Modelling 'Spermatocytes'. Main effect: treatment- not significant; main effect: donor- significant ($P < 0.001$); interaction effect: treatment x donor- significant ($P < 0.001$); (C) Pachytene spermatocytes identified by the typical pattern of γ H2XA expression in VEGF-treated transplants from donor 2 at 9 months. Mouse IgG isotype control as negative control (human adult testis tissue).

marker BOLL (FIGURE 6A). Expression of BOLL, although faint, was detected in both VEGF-treated and control transplants. The presence of pachytene spermatocytes as the most advanced germ cell stage was confirmed by the typical pattern of γ H2XA expression during spermatogenesis (FIGURE 6C).

All mouse transplants showed complete spermatogenesis at 4 and 9 months post-transplantation (FIGURE 2C and FIGURE 2D).

Niche maturation

The expression of markers for Sertoli cell maturation and functionality (AMH, AR and INHA) did not differ significantly between VEGF-treated transplants and controls from both donors. In the transplants recovered after 4 and 9 months, AMH was expressed by Sertoli cells. At 9 months, some neighbouring tubules demonstrated different intensities of AMH expression. Tubules containing more spermatogonia and spermatocytes exhibited lower intensity of AMH compared with tubules with less spermatogonia or without spermatocytes indicating a different level of maturation. INHA expression was observed in all Sertoli cells. AR was observed in most Sertoli cells and in the interstitium (FIGURE 7). At the time of the biopsy (fresh tissue) compared with at 9 months, the expression of AMH was more intense whereas AR was observed in fewer Sertoli cells. INHA expression in Sertoli cells was denser.

DISCUSSION

In this study, the effect of VEGF on human prepubertal testis tissue xenotransplants was evaluated. After pre-treatment of ITT with VEGF, an overall enhancement of vascularization was observed, improvement of seminiferous tubule integrity, increase in the number of spermatogonia and the number of seminiferous tubules containing spermatogonia. The results were slightly variable between donors based on the time the testicular biopsy was taken (donor age, before or after the gonadotoxic treatment). Overall, during the xenotransplantation period, a deterioration of the vascular surface and seminiferous tubule integrity, an increase of the number of spermatogonia, and the number of tubules containing spermatogonia was observed in the transplants from both donors irrespective of treatment.

Our data show that VEGF had a profound effect on the vascular surface and vessel density of the transplants from both donor 1 and donor 2. In a previous study, mouse testis transplants supplemented with alginate-VEGF nanoparticles showed a significantly higher vascular surface at 5 and 21 days post-transplantation (Poels *et al.*, 2016). Furthermore, the blockage of the VEGF/VEGFR2 pathway by daily exogenous administration of the SU5416 antagonist led to disruption of angiogenesis and a substantial reduction of the number of blood vessels in regenerated mouse testis tissue (Tian *et al.*, 2016). Other studies, however, reported no considerable differences in the formation of blood vessels in the presence of exogenous recombinant mouse VEGF-164 in bovine xenotransplants 6 or 8 weeks after transplantation (Schmidt *et al.*, 2006; Caires *et al.*, 2009) or recombinant human VEGF-165 in de-novo formed testis tissues from piglets after 3 months (Dores and Dobrinski, 2014). Assessment of seminiferous tubule integrity revealed a considerable increase of intact seminiferous tubules in the VEGF-treated transplants over time. The more efficient vascularization of these transplants compared with the controls may have resulted in the improved preservation of intact tubules and seminiferous epithelium, a prerequisite for the establishment of the microenvironment for SSC maintenance, germ cell survival, proliferation and further differentiation (Oatley and Brinster, 2012). A tendency for better tubular integrity has been reported in mouse testis tissue transplants when tissue was embedded in hydrogel loaded with VEGF nanoparticles. The beneficial effect, however, was transient as it was present at 5 but not at 21 days post-transplantation (Poels *et al.*, 2016).

Vascular endothelial growth factor has been proposed as a regulator of germ cell survival and proliferation. The data obtained show that pre-treatment of human ITT with VEGF resulted in better spermatogonial survival in time. Previously, culture of bovine testis tissue with VEGF stimulated the expression of genes that prevent germ cell death (BCL family) (Caires *et al.*, 2009). Additionally, an upregulation of the germ cell specific marker MVH was observed in VEGF-treated mouse testis tissue after ectopic

transplantation, and an upregulation of c-Fos and Cyclin D1, which are involved in germ cell proliferation, has been reported *in-vitro* 24 and 48 h after VEGF stimulation (Tian *et al.*, 2016). In our study, no significant difference was observed in germ cell survival between the VEGF-treated fragments and the controls after 5 days in culture. During transplantation of ITT, it is known that a substantial number of spermatogonia is lost irrespective of the transplantation site or the cryopreservation protocol (Wyns *et al.*, 2007; Van Saen *et al.*, 2009; Jahnukainen *et al.*, 2012). The data we obtained from donor 1 demonstrated a lower number of spermatogonia after transplantation compared with their number just after culture, regardless of treatment.

The notable differences observed in the number of spermatogonia between the two donors could be due to their age difference, as spermatogonial quantity in human prepubertal testes is age related (Masliukaite *et al.*, 2016). Additionally, treatment with carboplatin before the time of the testis biopsy for donor 1 could have contributed to the low germ cell number. Carboplatin is a platinum agent commonly used to treat childhood cancer and is considered to cause azoospermia with moderate to high risk (Trottman *et al.*, 2007). During the study, none of the transplanted ITT fragments from donor 3 were retrieved at any time point. Donor 3 was diagnosed with aplastic anaemia and had treatment with cyclosporine. Cyclosporine is a calcineurin inhibitor and acts as immunosuppressant (Northrop *et al.*, 1994; Rao and Hogan, 1997). In cases of solid organ transplants in patients treated with cyclosporine, endothelial cell damage and impairment of their functionality has been observed (Rodrigues-Diez *et al.*, 2016). Moreover, cyclosporine prevented the migration of primary endothelial cells and angiogenesis induced by exogenous VEGF (Hernández *et al.*, 2001) and had a toxic effect on vascular smooth muscle cells (Amador *et al.*, 2015). Therefore, we hypothesize that the treatment of donor 3 with cyclosporine led to destruction of the function and migratory capacity of endothelial cells, resulting in reduced revascularization capability of the grafts and sequential loss. The above cases stress the importance of the timing a testicular biopsy should be taken from a prepubertal boy, as

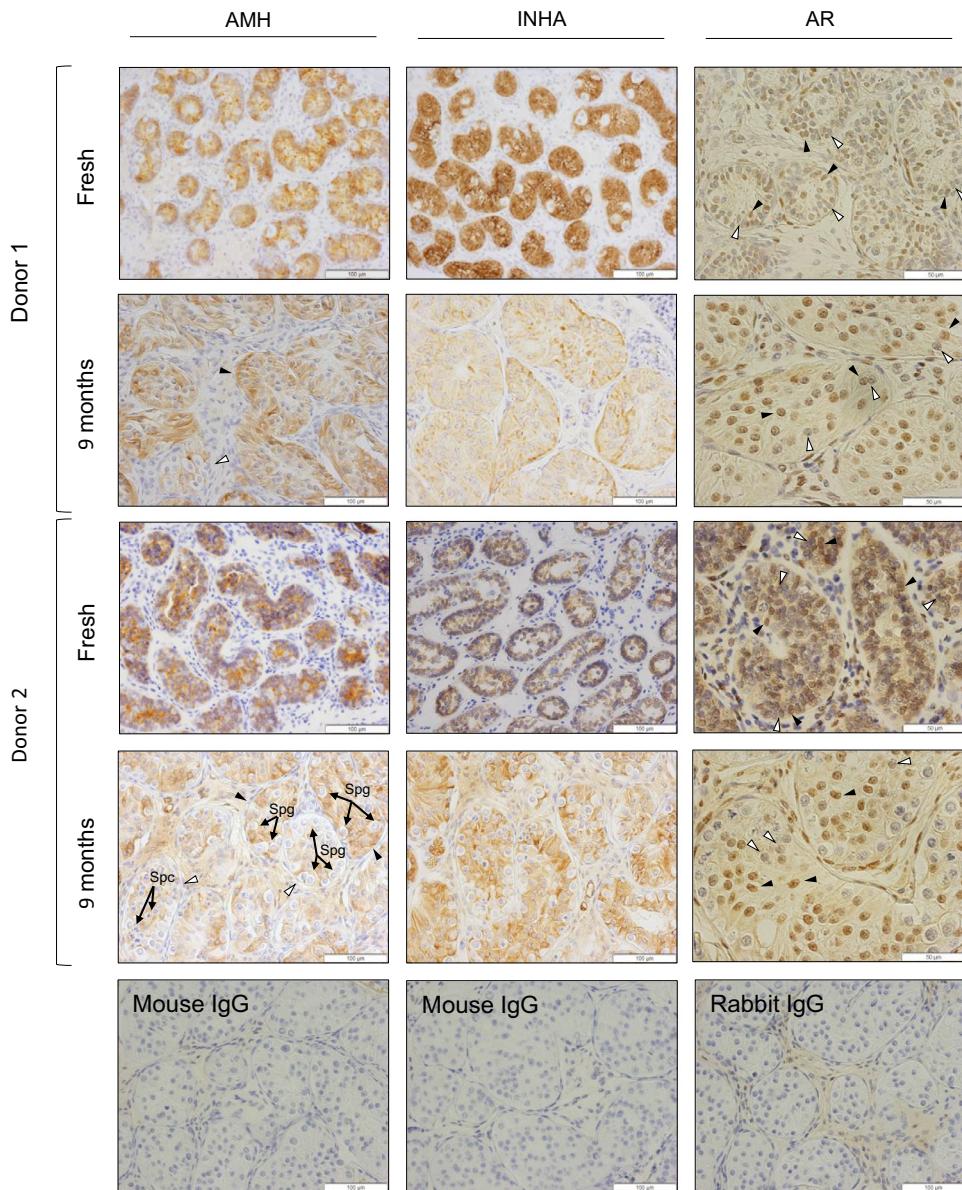


FIGURE 7 Sertoli cell maturation and functionality in fresh testis tissue and transplants treated with VEGF at 9 months post-transplantation. Anti-Müllerian hormone (AMH) expression (cytoplasmatic localization) was detected in both fresh tissue and xenotransplants, regardless of treatment. Neighbouring tubules showed different intensities of AMH expression, representing different levels of maturation. White arrowheads indicate seminiferous tubules faintly stained for AMH; black arrowheads indicate tubules stained stronger for AMH; arrows indicate the presence of spermatogonia (Spg) or spermatocytes (Spc). Inhibin A (INHA) was expressed (cytoplasmatic localization) in both fresh tissue and xenotransplants at 9 months post-transplantation, although less dense in the latter, confirming Sertoli cell functionality. Androgen receptor (AR) was expressed (nuclear localization) in some Sertoli cells in the fresh tissue, whereas, at 9 months after transplantation, AR was expressed in most Sertoli cells. White arrowheads indicate Sertoli cells negative for AR; black arrowheads indicate Sertoli cells positive for AR. Mouse IgG isotype controls as negative controls (human prepubertal testis tissue) for AMH and INHA and rabbit IgG isotype control as negative control (human prepubertal testis tissue) for AR.

previous treatment with gonadotoxic regimens may compromise the viability of the transplants, quality, or both, and the number of germ cells in a future ITT auto-transplantation.

In the present study, pachytene spermatocytes were observed at 9 months post-transplantation in the transplants from both donors, regardless of treatment. The expression pattern of

γ H2AX, that in human adult seminiferous epithelium follows a similar distribution to that found in rodents (Blanco-Rodriguez, 2009), with the staining of sex body evident at pachytene stage, confirmed their presence. Meiotic activity of the germ cells in the transplants was confirmed by immunostaining for the meiotic marker BOLL. The expression was localized at the cytoplasm of spermatocytes but also at the nucleus

of spermatogonia, as described before (Van Saen *et al.*, 2011), but not in all germ cells that morphologically resembled spermatocytes. The faint expression is possibly reflecting the initiation of the protein expression. Interestingly, VEGF-treated transplants from donor 2 contained more seminiferous tubules with spermatocytes compared with controls. Although it has been suggested that VEGF may

promote germ cell differentiation (Schmidt *et al.*, 2006), the observed increase could also be due to the higher number of tubules with spermatogonia in the VEGF-treated transplants and their further differentiation. Complete spermatogenesis in human ITT xenotransplants has not yet been reported. Spermatogenic cells up to pachytene spermatocytes were observed in ITT transplanted into the scrotum (Wyns *et al.*, 2008) or under the dorsal skin of immunodeficient mice (Sato *et al.*, 2010). Previous studies in our laboratory have demonstrated differentiation up to secondary spermatocytes in ITT transplanted into the mouse testicular parenchyma (Van Saen *et al.*, 2011; 2013). ITT xenotransplants from both human and common marmoset have been characterized by spermatogenic arrest at pachytene stage (Wistuba *et al.*, 2004; Wyns *et al.*, 2008; Sato *et al.*, 2010; Van Saen *et al.*, 2011). Although accelerated maturation of primate testis tissue xenotransplants in mice has been reported (Honaramooz *et al.*, 2004; Liu *et al.*, 2016), the short lifespan of immunodeficient mice that we are currently using as animal model for the xenotransplantation experiments could be a limiting factor for successful completion of spermatogenesis. Humans have a longer prepubertal period, as they do not become capable of producing mature sperm until around the age of 12 years, compared with the other species that have been successfully used so far. Also, although the functionality of Sertoli cells in human ITT xenotransplants was confirmed at 9 months post-transplantation, the expression of AMH indicates the not fully matured status. Initiation of spermatogenesis is advanced after xenotransplantation; however, the time required for full maturation might be longer than the lifespan of a mouse.

Further research is necessary to improve germ cell differentiation in human prepubertal xenotransplants. Initiation and maintenance of spermatogenesis is highly dependent on interactions between hormones of the hypothalamic–pituitary–gonadal axis (Sofikitis *et al.*, 2008). Although exogenous gonadotrophin administration in equine and monkey xenotransplants resulted in post-meiotic differentiation (Rathi *et al.*, 2006; 2008), subcutaneous administration of recombinant human FSH did not result in the complete spermatogenesis in human ITT

xenotransplants (Van Saen *et al.*, 2013). Hypoxia-induced VEGF production is known to stimulate testosterone release in Leydig cells through binding to VEGFR-1 and VEGFR-2 (Ergun *et al.*, 1997; Hwang *et al.*, 2007). Although the distinct role of FSH and testosterone in spermatogenesis has been a matter of controversy (Vigier *et al.*, 2004), the presence of testosterone is critical for male fertility, and spermatogenesis does not proceed beyond meiosis in the absence of testosterone or the androgen receptor (Sharpe, 1994). The effect of exogenous testosterone on human ITT xenotransplants has been studied but the short evaluation point (5 days) did not allow any observations to be made on the potential differentiation of spermatogonia (Poels *et al.*, 2014).

VEGF is one of the numerous paracrine factors secreted by mesenchymal stem cells (MSC), together with other angiogenic and proliferative factors, anti-inflammatory cytokines and anti-apoptotic molecules. The ability of MSCs to migrate to sites of tissue injury promoting the survival and recovery of injured cells, repair and protection of damaged tissue makes them ideal candidates for regenerative medicine (Mackenzie and Flake, 2001; Wu *et al.*, 2003; Uccelli *et al.*, 2008; Feng *et al.*, 2011). Co-transplantation of ITT with MSCs could be an alternative strategy to improve the efficacy of the transplantation procedure in future experiments.

Unfortunately, studies using human ITT are limited owing to the scarcity of the tissue for research purposes. Moreover, the small volume of each biopsy does not permit the simultaneous evaluation of other factors or conditions and the heterogeneity in the age of the donors and the treatment they may have received before testicular biopsy limits the size of a study.

In conclusion, the findings from the present study suggest that culture of human ITT with 100 ng/ml of recombinant human VEGF-165 for 5 days improves vascularization, seminiferous tubule integrity and spermatogonial survival during long-term xenotransplantation in testis tissue from boys who did not receive cyclosporine treatment before the biopsy. To the best of our knowledge, this is the first study investigating the

potential effect of VEGF on human ITT xenotransplants and, although further studies are required, VEGF may be considered as a potential candidate for improving the long-term efficiency of ITT transplantation.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.rbmo.2019.02.012](https://doi.org/10.1016/j.rbmo.2019.02.012).

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