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Generation of human vascularized brain organoids

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Abstract

The aim of this study was to vascularize brain organoids with a patient's own endothelial cells (ECs). Induced pluripotent stem cells (iPSCs) of one UC Davis patient were grown into whole-brain organoids. Simultaneously, iPSCs from the same patient were differentiated into ECs. On day 34, the organoid was re-embedded in Matrigel with 250 000 ECs. Vascularized organoids were grown *in vitro* for 3–5 weeks or transplanted into immunodeficient mice on day 54, and animals were perfused on day 68. Coating of brain organoids on day 34 with ECs led to robust vascularization of the organoid after 3–5 weeks *in vitro* and 2 weeks *in vivo*. Human CD31-positive blood vessels were found inside and in-between rosettes within the center of the organoid after transplantation. Vascularization of brain organoids with a patient's own iPSC-derived ECs is technically feasible.

Keywords

blood vessel; brain organoid; CD31; STEM121; transplantation; vascularization

Introduction

The generation of 3D whole-brain organoids from induced pluripotent stem cells (iPSCs) was described by Lancaster *et al.* [1]. Whole-brain organoids follow an endogenous development program that recapitulates the gene expression program of the fetal neocortex development [2] and epigenetic signatures of the fetal human brain [3].

Whole-brain organoids can be maintained in culture for more than a year; however, their growth typically stalls after a few months [4]. The center of the organoid may turn necrotic. Cells more than 200–400 μm away from the cell surface do not receive enough oxygen and nutrients through diffusion [5]. Therefore, further strategies to preserve the organoid's nutritional needs are in demand. As whole-brain organoids lack blood vessels, vascularization of the brain organoid may further promote its growth and supply with nutrients. It is unknown however, whether coculture with endothelial cells (ECs) will lead to

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Conflicts of interest
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penetration of the blood vessels into the organoid and whether interference of blood vessels will disturb the self-assembly properties of whole-brain organoid.

This study was performed to investigate whether a human whole-brain organoid from a UC Davis patient could be vascularized with the same patient's iPSC-derived ECs. The developing central nervous system does not produce vascular progenitor cells, and so ingression of blood vessels is required for continued central nervous system development and function [6]. Blood vessels invade the fetal brain from the surrounding perineural vascular plexus in a stereotypical pattern [7].

We therefore chose a strategy of revascularization from the outside surrounding matrix as opposed to direct injection into the center of the organoid to recapitulate early fetal vascular brain development.

Materials and methods

Generation nonvascularized brain organoids *in vitro*

Whole-brain organoids were developed with the STEMdiff Cerebral Organoid Kit (catalog #08570; StemCell Technologies, Cambridge, Massachusetts, USA) from passage 26 iPSCs of a UC Davis patient (Dura 6.9-WC iPSC P26) that was published previously [8]. The patient's iPSCs were maintained in culture on Matrigel/mTeSR1 (catalog #85850; StemCell Technologies). On day 0, iPSCs were washed with PBS and detached with Gentle Cell Dissociation Reagent (StemCell Technologies) for 8–10 min at 37°C. Cells were gently resuspended by pipetting and rinsed with 1ml of Embryoid Body (EB) Seeding Medium (StemCell Technologies). The suspension was centrifuged at 300 g and resuspended in 1–2ml of EB Seeding Medium. EB Seeding Medium was used to adjust the concentration of cells to 90 000 cells/ml. Subsequently, 9000 cells/well were added to a 96-well round-bottom ultra-low attachment plate (Corning, Corning, New York, USA). On days 2 and 4, 100 μl of EB Formation Medium were added per well. On day 5, 0.5ml of Induction Medium (StemCell Technologies) was added to each well of a 24-well ultra-low attachment plate and 1–2 EBs were transferred into each well and incubated for 48 h at 37°C. On day 7, Matrigel (Corning) was thawed on ice for 1–2 h. Parafilm (Bemis, Neenah, Wisconsin, USA) in an empty, sterile, 100mm dish, was used as the embedding surface. Using a wide-bore 200-μl pipette tip, 25–50 μl of medium+EB from one well of the 24-well plate was drawn up and transferred to the embedding surface. Excess medium was removed. Using a pipettor with a cold 200-μl standard pipette tip, 15 μl of Matrigel were added dropwise onto each EB. The dish was subsequently placed in an incubator at 37°C for 30min to polymerize Matrigel. Using a 1-ml pipette tip, Expansion Medium (StemCell Technologies) was used to gently wash the Matrigel droplets off the sheet into a well of a six-well ultra-low adherent plate (Corning). Embedded organoids were incubated at 37°C for 3 days. On day 10, the medium was replaced with 3ml/well of Maturation Medium (StemCell Technologies). The plate of organoids was placed on an orbital shaker at 85 rpm in a 37°C incubator. A full Maturation Medium change was performed every 3–4 days.

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Differentiation of induced pluripotent stem cells into endothelial cells

iPSCs of the above-mentioned UC Davis patient from passage 32 (Dura 6.9-WC iPSC P32) were used for the production of ECs [8]. Differentiation of ECs from these iPSCs was progressively induced in three stages. Briefly, iPSCs were manually passaged as small clusters onto human embryonic stem cell-qualified Matrigel-coated culture plates in mouse embryonic fibroblast conditioned human embryonic stem cell medium with an additional 10 ng/ml of fibroblast growth factor 2. Matrigel coating was performed according to the manufacturer's suggestion (Corning). After 1 day, culture medium was switched to StemDiff APEL medium (catalog #05210; StemCell Technologies) with 6 μM of 6-[[2-[[4-(2,4-dichlorophenyl)-5-(5-methyl-1*H*-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl] amino]-3-pyridinecarbonitrile (CHIR99012, catalog #4423; Tocris Bioscience, Avonmouth, UK) for 2 days. Next, the cells were cultured in StemDiff APEL medium (StemCell Technologies) supplemented with 25 ng/ml bone morphogenetic protein 4 (BMP4) (catalog #314-BP; R&D Systems, Minneapolis, Minnesota, USA), 10 ng/ml fibroblast growth factor 2 (catalog #233-FB; R&D Systems), and 50 ng/ml vascular endothelial growth factor (VEGF) (catalog #293-VE; R&D Systems) for 2 days. Cells were lifted with Accutase (catalog #AT104; Innovative Life Technologies, San Diego, California, USA) at day 4 and seeded onto p100 culture dishes at 5×10^3–1×10^4 cells/cm^2 in EC Growth Medium MV2 (ECGM-MV2, catalog #C-22022; PromoCell, Heidelberg, Germany) with an additional 50 ng/ml VEGF. The medium was changed every 2 days for 4–6 days to generate ECs.

Generation of vascularized whole-brain organoids *in vitro*

On day 34 of the STEMDiff Cerebral Organoid Kit protocol, four organoids were re-embedded in 15 μl of Matrigel containing 250 000 ECs (generated from Dura 6.9-WC iPSC P32) using the same technique mentioned above for the generation of nonvascularized brain organoids. The dish was placed in an incubator at 37°C for 30 min to polymerize Matrigel. Matrigel droplets were washed off the sheet into a well of a six-well ultra-low adherent plate and cultured in 1 : 1 Maturation Medium and EC Growth Medium MV2 supplemented with VEGF (final concentration=50 ng/ml). A full medium exchange was performed every 3–4 days. On day 54, vascularized whole-brain organoids were used for transplantation.

Transplantation of vascularized and nonvascularized organoids into immunodeficient NSG mice

All surgical procedures were approved by the UC Davis IACUC (protocol #19596). The IACUC policy on rodent survival surgery was followed (http://safetyservices.ucdavis.edu/iacuc/policies/rodent-survival-surgery). Two 2-month-old male NSG mice (JAX stock 005557, https://www.jax.org/strain/005557; Jackson laboratories, Sacramento, California, USA) were anesthetized with isoflurane (2–3% in oxygen) and placed into a stereotaxic frame. Hair was removed using Nair. The skin was cleaned with alternating applications of betadine and alcohol three times. A small incision (1 cm) was made in the scalp to allow visualization of the skull. A slightly less than 5mm craniotomy was drilled using a small electric drill. The pia of the hemisphere was opened using a 25G needle. An ~2mm×2mm×2mm cavity was created by direct removal of brain tissue. The day 54 vascularized whole-brain organoid was placed onto the raw edges of the resection cavity of

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one mouse, and a day 54 nonvascularized whole-brain organoid was transplanted in the same fashion in the second mouse. The Matrigel embedding was not removed from the organoid before transplantation. The craniotomy flap was reinserted and the incision sutured. Carprofen was administered by subcutaneous injection (5mg/kg) at the time of surgery and again the following day. All postoperative animals were observed daily for 7 days to monitor recovery, behavior, and incision healing.

Histology

Animals were euthanized 2 weeks after the transplantation by CO_2 asphyxiation and underwent transcardiac perfusion with saline followed by formalin. Brains and in-vitro organoids were fixed overnight in formalin, incubated in 30% sucrose for 24–48 h at 4°C, frozen in Optimal Cutting Temperature Compound Embedding Medium (Sakura Finetek, Torrance, California, USA) using dry ice/isopropanol and stored at −80°C. Brains and organoids were subsequently cut into 40 μm wide sections at −20°C in a Cryotome FSE (Thermo Scientific, San Jose, California, USA) and mounted on Superfrost Plus slides (Thermo Scientific). Sections were circled with a Liquid Blocker Super Pap Pen (Daido Sangyo Co., Kawasaki, Japan). Sections were incubated for 20 min in 0.2% Triton X-100 (Roche, Basel, Switzerland) in PBS (Hyclone, Logan, Utah, USA) followed by 30 min of 1% BSA in PBS. Sections were incubated overnight at 4°C with 1 : 200 rabbit anti-human CD31 antibody (catalog #IHC-00055; Bethyl Laboratories, Montgomery, Texas, USA). The following day, the sections were washed three times with PBS and incubated with 1 : 1000 mouse STEM121 antibody (catalog #40410; Takara Bio USA, Mountain View, California, USA) overnight at 4°C. The next day, sections were washed four times with PBS and incubated with 1 : 500 AlexaFluor 488 goat anti-mouse IgG (catalog #A-11001; Invitrogen Molecular Probes, Carlsbad, California, USA) and 1 : 500 AlexaFluor 594 goat anti-rabbit IgG (catalog #R37117; Invitrogen Molecular Probes) at room temperature for 1 h. Sections subsequently underwent three washes in 1% BSA followed by two washes in PBS. Sections were dried for 30 min until a coverslip was placed with VectaShield with DAPI Mounting Medium (Vector Laboratories, Burlingame, California, USA). Whole coronal mouse brain sections were scanned at ×4 magnification and the whole-brain organoid at –20 magnification using the large image stitching tool of NIS-Elements Advanced Research software (Nikon, Tokyo, Japan). Images in ×40 magnification were taken on an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan). Specific staining was verified with PE mouse IgG1 isotype controls (catalog #550617; BD Pharmingen, San Jose, California, USA) at the same concentration as the primary antibody. We also performed control experiments without the primary antibodies.

Results

On the day of transplantation, CD31-positive cells encircled the organoid, and capillaries had grown into its outer layers from the Matrigel embedding (Fig. 1a–d). The iPSC-derived ECs autoassembled to tubular structures in the brain organoid, similar to what we had previously seen with these cells in an in-vitro tube formation assay that did not involve brain organoids [9]. Organoids that had not undergone Matrigel-based coating with ECs did not show positive staining for CD31 2 weeks later which coincides with the length of the

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transplantation experiment (Fig. 1e–h). In contrast, capillaries had further penetrated towards the center in the organoids that had been coated with ECs (Fig. 1i–l). Interestingly, there was robust vascularization of the outer, more organized layers of the organoid, whereas there was only some penetration into the densely cellular STEM121-positive core of the organoid *in vitro* (arrow; Fig. 2). No specific signal was seen in the isotype control staining.

After 54 days *in vitro* and 2 weeks *in vivo*, the vascularized whole-brain organoid had grown to a size of almost 2mm (Fig. 3a). The medial side of the organoid was attached to the mouse brain. The human brain stem cell marker STEM121 confirmed that the organoid consisted of human cells. After exposing the organoid for 20 days *in vitro* and 2 weeks *in vivo* to Matrigel-based coating with ECs derived from the same patient, the whole-brain organoid showed robust vascularization in several regions (Fig. 3b). The nonvascularized whole-brain organoid did not survive 2 weeks after transplantation in NSG mice. On sectioning, only the resection cavity from the surgery was appreciated and no viable human tissue was identified (Fig. 3c).

In some regions, the human vasculature penetrated the center of STEM121-positive rosettes (Fig. 4a), in other regions blood vessels spanned the periphery of rosettes (Fig. 4b). Blood vessels were of human origin as confirmed by the human CD31 antibody which is directed against a human epitope (https://www.bethyl.com/product/pdf/IHC-00055.pdf), and we did not see reactivity with mouse blood vessels in the host mouse brain. Individual capillaries were tubular, exhibited typical capillary morphology, and could be tracked on z-stack imaging without signs of discontinuity within the 40 μm section.

Discussion

Vascularization of cerebral organoids is considered a crucial next step in the bioengineering of complex human brain tissue. Here we present evidence that Matrigel coating of organoids with iPSC-derived ECs from the same patient leads to robust vascularization of the organoid within 5 weeks. Blood vessels did not stay on the periphery of the organoid but instead penetrated its center *in vivo*. *In vitro*, robust vascularization of the outer layer and more organized structures of the organoid was appreciated. Our data strongly supports the presence of vascularization as human CD31-positive cells formed tubular channels with capillary morphology inside the brain organoid and did not show signs of discontinuity on confocal imaging. We could not verify the presence of rodent blood in these capillaries as we perfused brains with saline and formalin for the purpose of fluorescent staining.

Our strategy was to model the developmental perineural vascular plexus [7] by coating the whole-brain organoid with Matrigel-embedded ECs. This approach seemed to recapitulate the physiological processes during development as the cytoarchitecture or self-assembly program of the whole-brain organoid did not seem to be grossly disturbed by the ingrowth of blood vessels. Capillaries were found in the center of rosettes as well as in-between rosettes. The brain organoid may send signals that promote ingrowth of blood vessels from the outside. No growth of blood vessels into the already vascularized mouse brain was appreciated. The driver of vessel ingress may be hypoxia. Hypoxic angiogenesis is

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controlled by activation of downstream genes by hypoxia-inducible factor-1 and angiopoietin-2 [10] and can even occur in adult conditions such as Moyamoya disease [11].

A PubMed search on 28 January 2018 for 'cerebral organoid' or 'brain organoid' did not return publications describing vascularization of brain organoids with the patient's own iPSC-derived ECs. Mansour *et al.* [12] reported at the Society for Neuroscience Meeting 2017 on the generation of vascularized brain organoids after transplantation into mouse brain; however, blood vessels were derived from the rodent. It is interesting that their brain organoids survived *in vivo* after transplantation as we did not see survival of our nonvascularized brain organoids after 2 weeks *in vivo*. However, we only had an $n = 1$ for this condition so further research is warranted. One of the limitations of this study is also that we did not prove connectivity of the human brain organoid capillaries with the rodent host brain or perfusion of the human capillaries with rodent blood.

Conclusion

Here we describe for the first time a protocol for the generation of endogenously vascularized whole-brain organoids. Further research is needed on the optimal timing of vascularization, its influence of organoid growth and survival and to verify functional connectivity of the transplanted human vasculature with the host brain.

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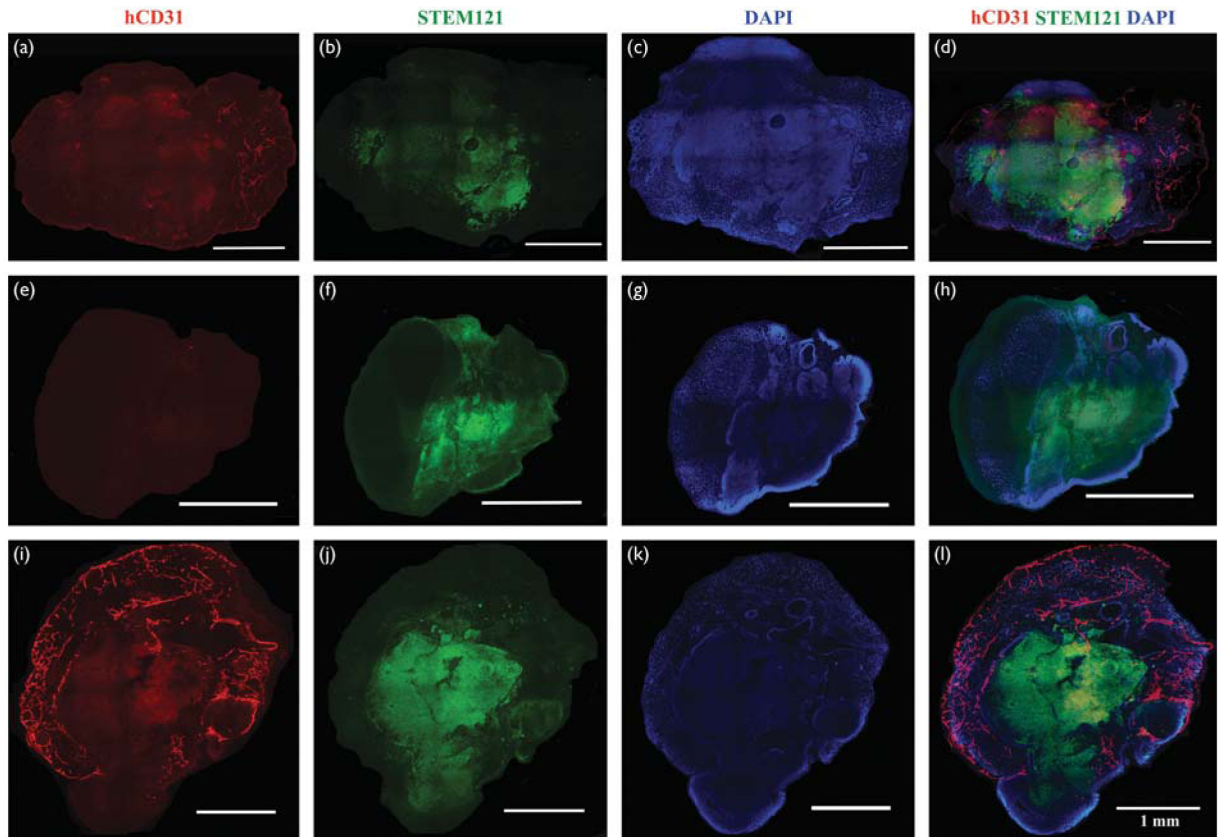

Fig. 1.

At day 54, the day of transplantation, there is some penetration of capillaries into the outer layers of the organoid *in vitro* (a–d). Two weeks later, which coincides with the termination of the in-vivo transplantation experiment, organoids without endothelial coating do not contain any blood vessels *in vitro* (e–h). At the same time, organoids with endothelial coating show robust ingrowth of CD31-positive blood vessels into the organoid *in vitro* (i–l; hCD31, red; STEM121, green; DAPI, blue; all scale bars: 1 mm).

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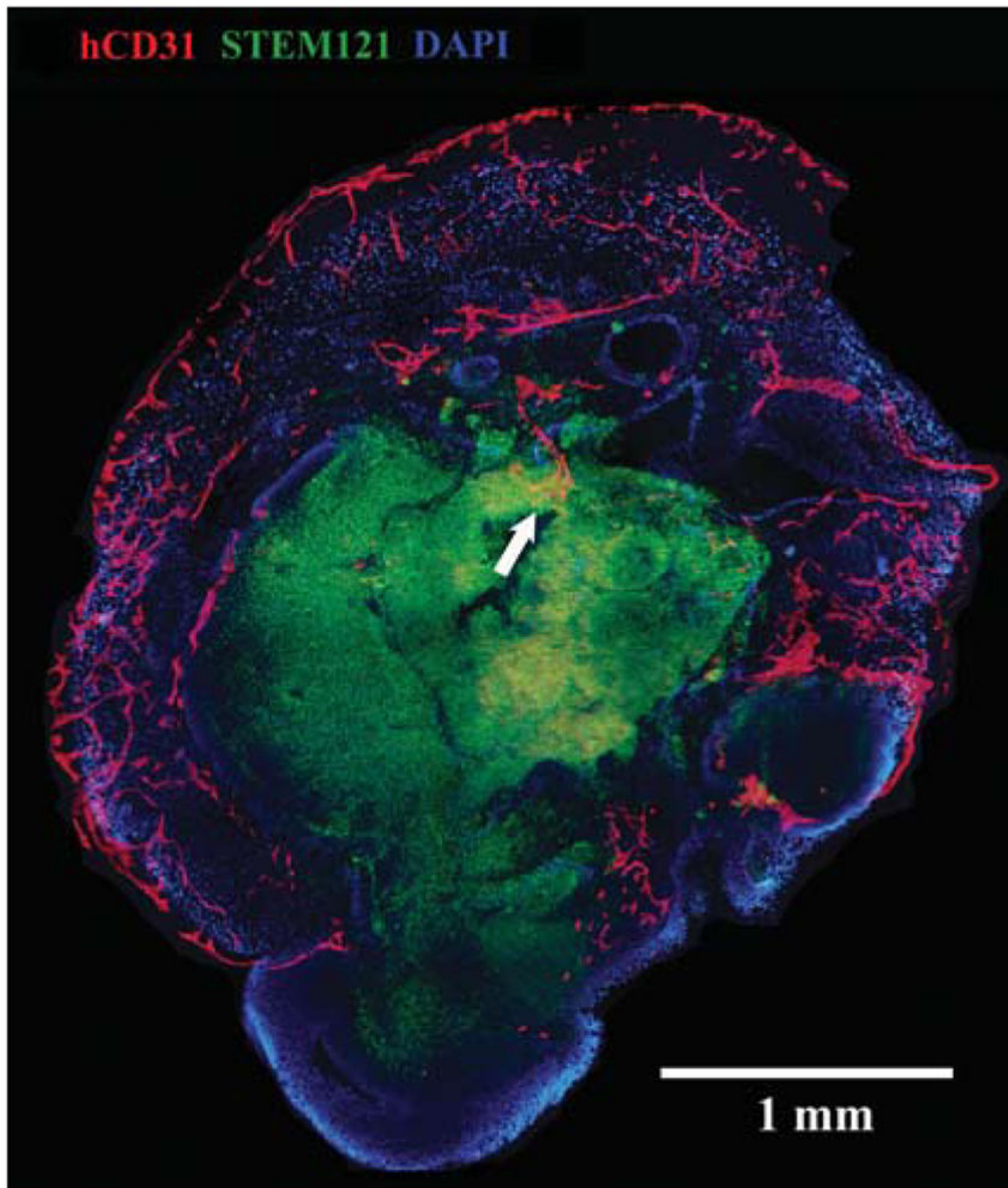

Fig. 2.

Magnification of Fig. 1l. There is robust penetration of the outer, more organized layers of the organoid *in vitro*. There is some ingrowth of blood vessels into the STEM121-positive core of the organoid (white arrow). Some parts of the outer layer of the organoid are not vascularized which may be due to detachment of the Matrigel in affected areas during the incubation (hCD31, red; STEM121, green; DAPI, blue).

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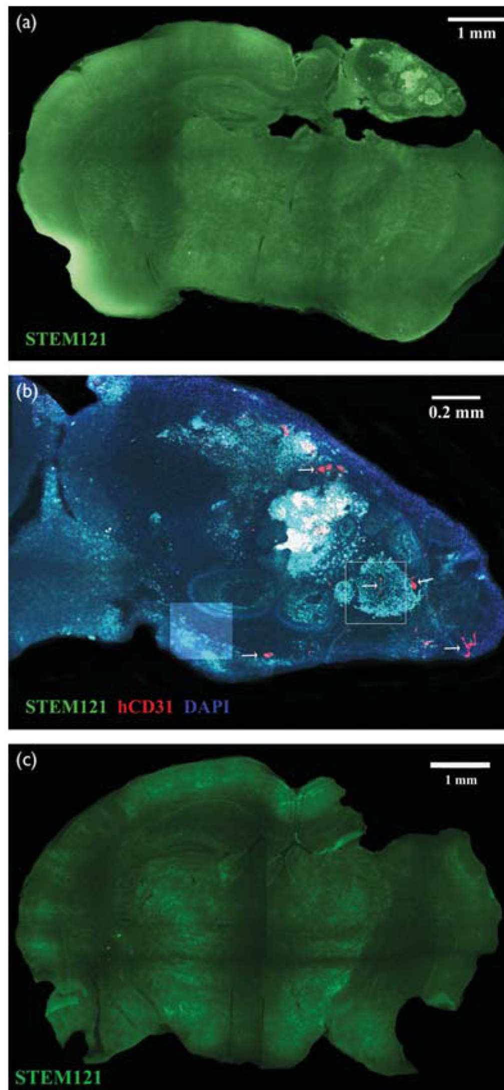

Fig. 3.

A coronal section of the transplanted NSG mouse brain shows that the vascularized organoid measures almost 2 mm in largest diameter 2 weeks after transplantation and 68 days after embryoid body formation. The human brain stem cell marker STEM121 (green) exclusively stains structures inside the organoid (a). Magnified images of the coronal section of the organoid show that human blood vessels (hCD31, red, multiple white arrows to point to examples) have formed in several regions of the human brain organoid (b; STEM121, green; DAPI, blue). Regions of interest are delineated with boxes for Fig. 4a (white frame) and Fig. 4b (no frame). The nonvascularized organoid did not survive after 2 weeks *in vivo* (c; STEM121, green).

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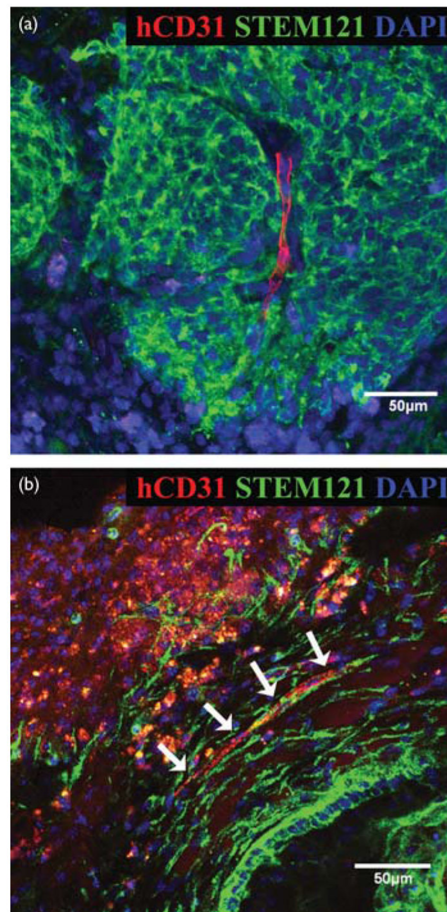

Fig. 4.

A blood vessel has penetrated the center of a rosette inside the organoid *in vivo*. (a) A magnification of the white frame in Fig. 3b. Blood vessels can also be found on the periphery of rosettes (b; hCD31, red, white arrows to delineate a blood vessel; STEM121, green; DAPI, blue). (b) A rotated magnification of the box with no frame in Fig. 3b.