Generation of vascularized human brain organoids

Note: This protocol was not part of the peer review process.

Procedure:

1. Maintenance of hESCs

- Human embryonic stem cells (hESCs) are cultured on Matrigel coated tissue culture plates.
- hESC colonies are passaged every 6 to 7 days when they achieve around 80% confluence.
- All cultures are checked regularly for mycoplasma and chromosomal abnormalities.

2. Generation of hESCs expressing inducible hETV2

1. To generate hESCs expressing inducible hETV2, doxycycline-inducible (BC4) dCAS9-mCherry and rTTA (addgene plasmid # 73497) cassette was introduced into the AAVS1 locus of HES-3 NKX2-1GFP/w.
    a. 2 million HES-3 NKX2-1GFP/w hESCs were electroporated with 8 µg donor plasmid, 1 µg AAVS1 TALEN-L and 1 µg AAVS1 TALEN-R by using Amaxa nucleofector device (AAB-1001, Lonza, program A-023).
    b. Cells were seeded in mTeSR1 plus ROCK inhibitor Y-27632 (10 µM).
    c. After 3 days, G-418 (Thermo Fisher Scientific) was applied for 7 days (400 µg/ml for the first 3 days and 300 µg/ml for the next 4 days) to obtain stable colonies.
    d. A single isogenic colony was picked and expanded. This hESC line was named BC4-hESCs.
2. After generating the tetracycline-inducible lentivirus for hETV2 (FUW-tetO-hETV2 [6]), BC4-hESCs were infected with the virus at multiplicity of infection (MOI) ~ 4.
3. 24 hours later, infected hESCs were washed with 1X PBS three times. Then, fresh mTeSR1 media (Stem Cell Technologies) was added and cells were cultured for 5 more days.

3. Formation of embryoid bodies (EBs) and induction of neural differentiation and hETV2 expression

Cortical organoids (hCOs) with vasculature were generated by mixing hETV2-infected BC4 and non-infected parental HES3 hESCs (differentiation scheme is shown in **Figure 1**).

1. Aspirating the media and washing cells once with 1 ml DMEM-F12 medium was followed by the addition of 1 ml of 1x Accutase.
2. After 10 minutes of incubation at 37 °C, single cell suspension was confirmed under a microscope and transferred into a 15ml-falcon tube containing 5 ml DMEM-F12.
3. Cells were collected by centrifugation for 3 min at 1100 rpm.
4. After aspirating the supernatant, cells were resuspended in 1 ml neural induction medium (DMEM-F12, 15% (v/v) KSR, 1% (v/v) Glutamax, 1% (v/v) MEM-NEAA, 100 µM β-Mercaptoethanol, 10 µM SB-431542, 100 nM LDN-193189, 2 µM XAV-939).
5. Live cells were counted using a hematocytometer.
6. Cells were diluted with neural induction media to a final concentration of 9000 cells/150 µl (7200 parental HES-3 hESC cells and 1800 hETV2 infected hESC cells were combined for each 150 µl). Then, 50 µM ROCK inhibitor Y27632 and 5% (v/v) heat-inactivated FBS (Life Technologies) were added.
7. 150 µl of single cell suspension was added into each well of a U-bottom-ultra-low attachment 96-well plate (Day 0 of differentiation).
8. Basal activation of hETV2 was started on day 2 by adding 0.5 µM dox, whereas FBS and Y27632 were removed on days 2 and 4, respectively.
9. On days 4, 6, and 8, the neural induction media was replenished via removing 125 µl media and adding 150 µl fresh media.

4. Patterning of cortical organoids with functional vascular-like network

1. At day 10, organoids were transferred to ultra-low-attachment 6-well plates in hCO patterning media with minus vitamin A (1:1 mixture of DMEM-F12 and Neurobasal media, 0.5% (v/v) N2 supplement, 1% (v/v) B27 supplement without vitamin A, 0.5% (v/v) MEM-NEAA, 1% (v/v) Glutamax, 50 µM β-Mercaptoethanol, 1% (v/v) Penicillin/Streptomycin and 0.025% Insulin) for spinning culture and the media was changed every other day. Basal activation of hETV2 was preserved by adding 0.5 µM dox.
2. On day 10, 12, 14, and 16, 2.4 ml medium was removed from each well and 2.5 ml hCO patterning media with 0.5 µM dox was added.
3. The spinning culture was started by placing the plate on an orbital shaker at 80 rpm inside the incubator.

4. Maturation of vascularized cortical organoids

1. After day 18, the media was changed to hCO maturation media (1:1 mixture of DMEM-F12 and Neurobasal media, 0.5% (v/v) N2 supplement, 1% (v/v) B27 supplement with vitamin A, 0.5% (v/v) MEM-NEAA, 1% (v/v) Glutamax, 50 µM β-Mercaptoethanol, 1% (v/v) Penicillin/Streptomycin, 0.025% Insulin, 20 ng/ml BDNF and 200 µM ascorbic acid).
2. Final activation of hETV2 expression was performed from day 18 by adding 2 µM dox continuously in the media.
3. Media was changed every 4 days after day 18.

Timing

- Generation of stable cell line: 30 days
- Viral infection and transduction: 7 days
- Suspension of hESC cell colonies to form EB: 10 days
- Neural specification: 8 days
- Neural maturation/ vascular characterization: 12 days

Notes:

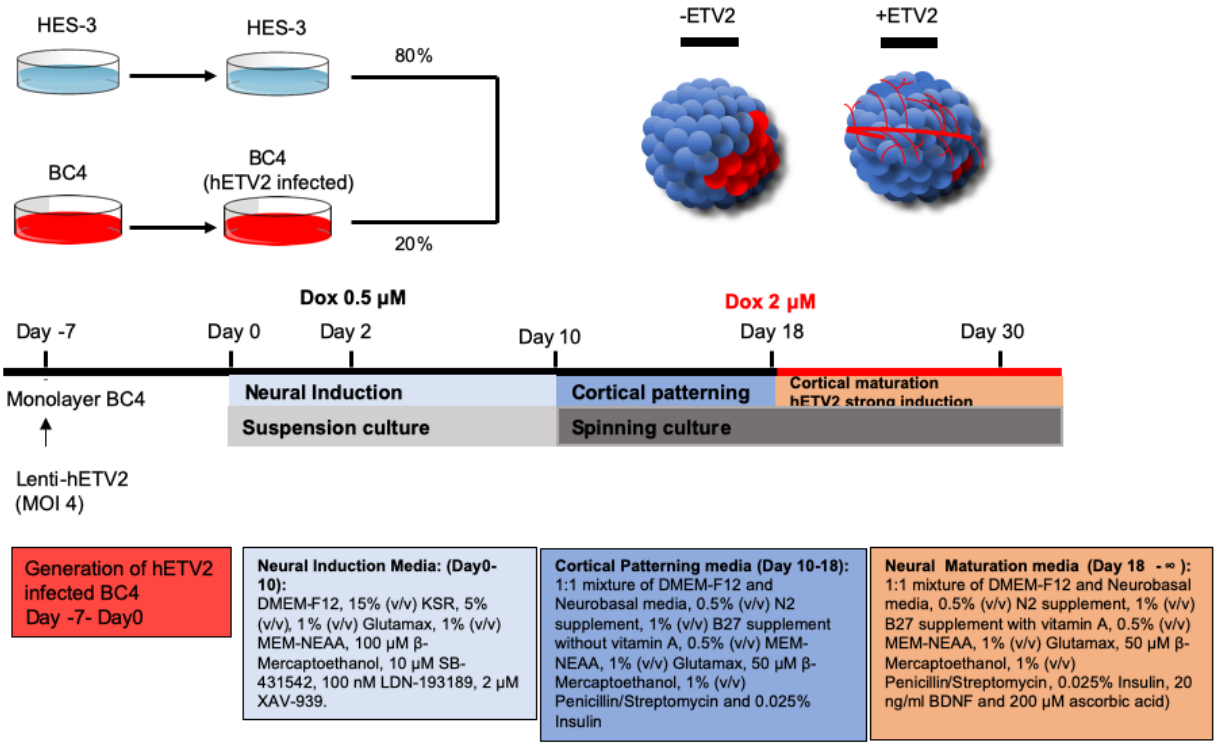
- Differentiated cells may interrupt the neural induction and the following differentiation process.
- The starting size of cell number to generate embryoid bodies can be cell line-dependent and should be preferentially titrated according to specific cell lines to be used.
- Transfer the embryonic bodies cautiously, to avoid damaging the samples.
- During induction and patterning stages, media changes should be performed gently.
- hETV2 virus titer is critical for the generation of vascularization inside the organoid.

Troubleshooting

- **Low efficiency of directed differentiation and vascular network formation:** hESCs colonies should be checked for quality control for a successful organoid generation. Differentiated colonies should be removed before generating a single cell suspension. After the infection of the hETV2 lentivirus, colonies showing differentiation should be removed. Lentivirus titer and quality are critical for the generation of endothelial cells inside the organoid.
- **EB formation:** EBs should form 24 hours after plating single cell suspension. Check the quality of hESC colonies, amount of ROCK inhibitor, and duration of Accutase treatment, if EBs fail to form.

  Failure of long-term development of organoid: Initially, extra growth factors were not added. However, these factors, FGF2 or EGF, may benifit organoid development.

Figure1: Schematic and recipes for the generation of vascularized cortical organoids



Equipment:

U-bottom ultra-low-attachment 96-well plate	Corning	CLS7007-24EA
Ultra-low-attachment 6-well plate	Corning	CLS3471-24EA
Orbital shaker	IKA	KS260

Experimental Models: Cell Lines

HES-3 NKX2-1$^{GFP/w}$	Elefanty lab	https://www.ncbi.nlm.nih.gov/pubmed/21425409

Reagents

Chemicals, Peptides, and Recombinant Proteins		
mTeSR1	Stem Cell Technologies	Cat# 05875
DMEM-F12	Life Technologies	Cat# 11330057
Neurobasal Media	Life Technologies	Cat# 2110349
FBS	Life Technologies	Cat# 10437028
Amino acids, non-essential	Life Technologies	Cat# 11140050
Penicillin/Streptomycin	Life Technologies	Cat# 15140-122
Glutamax	Life Technologies	Ca# 35050
Insulin	Sigma	Ca# I9278
β-Mercaptoethanol	Sigma	Ca# M7522
N2	Life Technologies	Cat# 17502-048
B27	Life Technologies	Cat# 17504-044
B27 supplement without vitamin A	Life Technologies	Cat# 12587010
bFGF	Millipore	Cat# GF003AF
KnockOut Serum Replacement	Life Technologies	Cat# 10828-028
Matrigel	BD	Cat# 354230
Y-27632	Stem Cell Technologies	Cat# 72304
Dispase (100ml)	Stem Cell Technologies	Cat# 07913
Accutase (100ml)	Stem Cell Technologies	Cat# AT104
LDN-193189	Sigma	Cat# SML0559
SB431542	Abcam	Cat# ab120163
XAV939	Sigma	Cat# X3004
BDNF	Prepotech	Cat# 450-02
Ascorbic acid	Sigma	Cat# A92902

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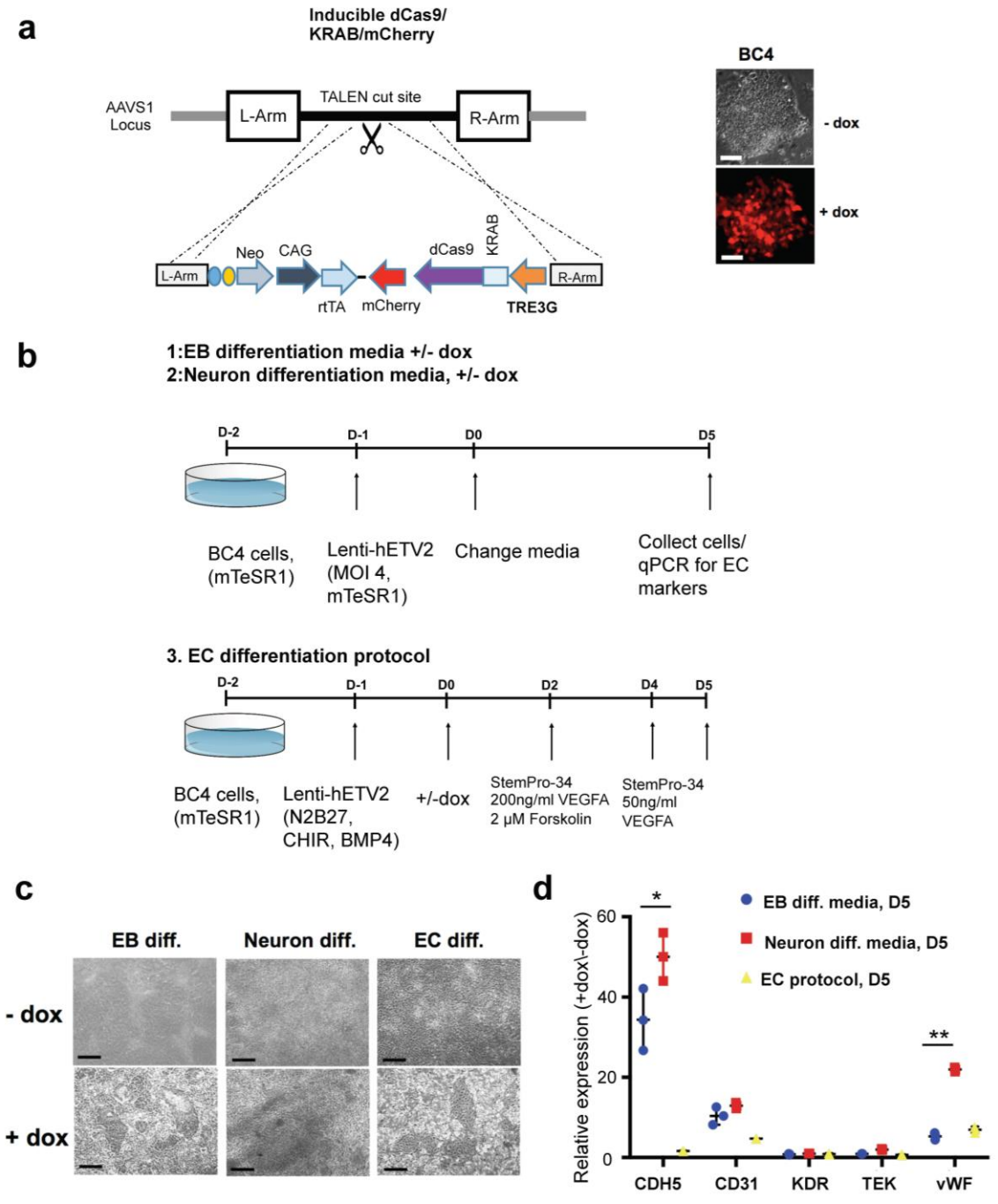
Supplementary Results and Discussion

Supplementary Note 1: Induction of endothelial fate via hETV2 expression

We tested whether the expression of *hETV2* induces EC differentiation in hESC under three conditions. To express *hETV2* in an inducible manner, we introduced doxycycline (dox)-inducible dCAS9-mCherry and rtTA into the AAVS1 locus to generate a rtTA-mCherry$^+$ hESC line (BC4 line, Supplementary Fig. SN1.1. a). Then, we infected BC4 cells with lentivirus expressing TetO-hETV2 to investigate the extent of EC induction under three conditions, 1) EB (embryoid body) differentiation condition, 2) neuron differentiation condition, and 3) EC differentiation condition[1] (Supplementary Fig. SN1.1. b). In all three conditions, hETV2-induction not only changed the morphology of the differentiated cells but also increased the expression of late-stage EC markers such as VE-Cadherin (*CDH5*), platelet and endothelial cell adhesion molecule 1 (*PECAM1*, or *CD31*), and mature EC marker von Willebrand factor (*vWF*) (Supplementary Fig. SN1.1. c, d). Immunostaining revealed that within five days of culture under the EB differentiation condition, over 50% of hETV2-induced cells expressed the EC markers, CDH5 and vWF, while there was a limited number of cells expressing CDH5 and vWF without hETV2 expression (Supplementary Fig. SN1.2. a). Even in the neuronal differentiation condition, hETV2 increased the number of CDH5$^+$ (27 ± 3.5%) and vWF$^+$ (15 ± 0.6%) cells. In the EC differentiation condition without hETV2 expression, about 20% of cells became CDH5$^+$ and vWF$^+$ cells, and hETV2-induction further increased the number of CDH5$^+$ and vWF$^+$ cells (Supplementary Fig. SN1.2. a). We subsequently assessed the efficiency of each differentiation condition by quantifying the proportion of CD31$^+$ cells via flow cytometry. The induction of hETV2 promoted EC differentiation in 25.1%, 45.5% and 32.5% of the cells cultured in EB, neuron and EC differentiation conditions, respectively, assessed by flow cytometry of CD31$^+$ cells (Supplementary Fig. SN1.2. a). We next monitored the real-time endothelial barrier function during EC differentiation with or without *hETV2* induction via electrical cell-substrate impedance sensing (ECIS) (Supplementary Fig. SN1.2. b). The induction of *hETV2* (at day 2) in all differentiation conditions leads to elevated barrier integrity, which was evidenced by increased endothelial resistance that started at day 2 and progressively increased until the endpoint of the assay (Supplementary Fig. SN1.2. b). Moreover, we investigated the source of endothelial cells in monolayer differentiation when a mixture of mCherry$^+$- and control-hESCs were infected with hETV2 lentivirus. All CDH5 positive cells expressed mCherry indicating that the endothelial cells differentiating in these organoids

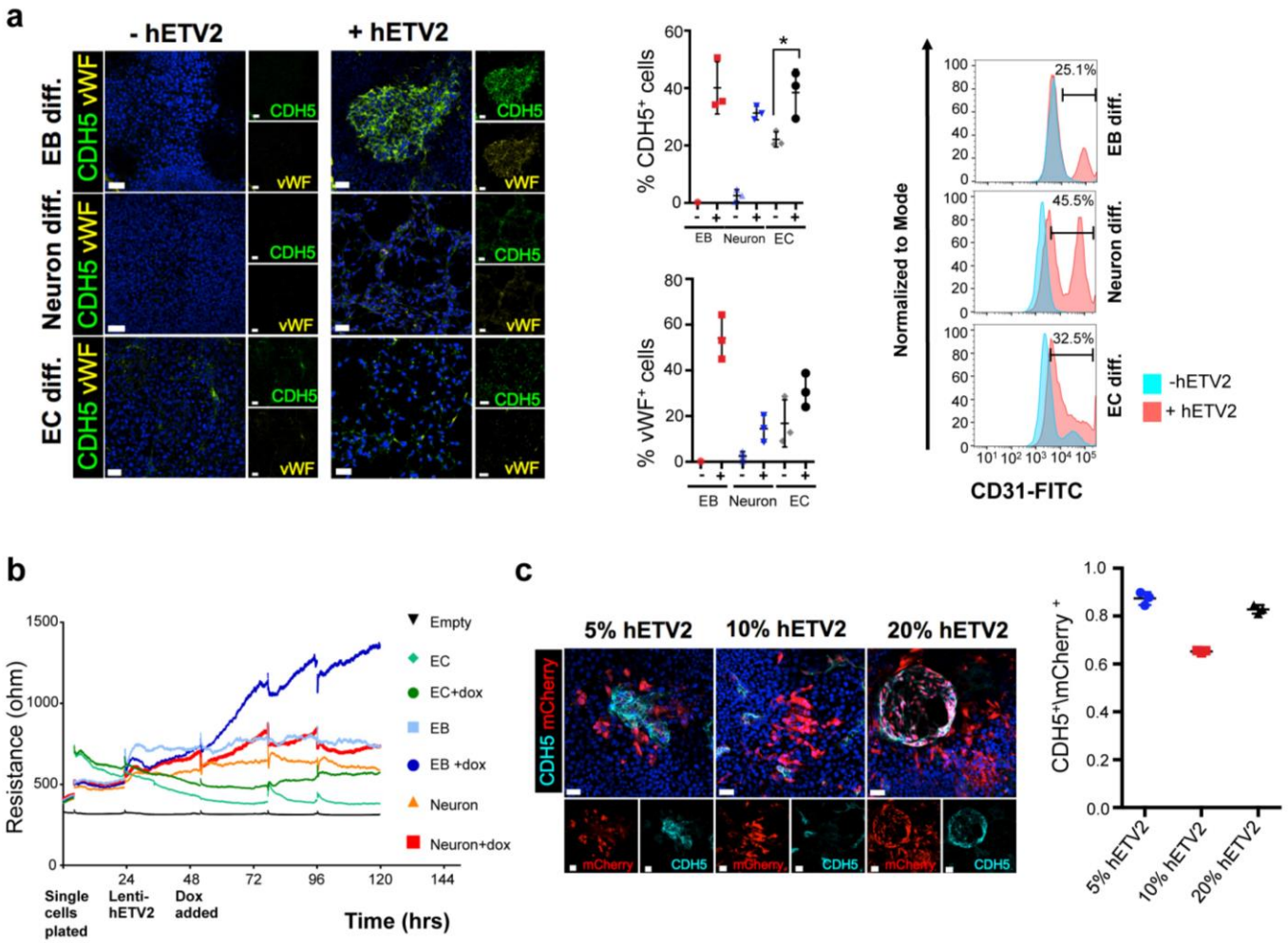
originate from the *hETV2* over-expressing cells (Supplementary Fig. SN1.2. c). These data suggest that overexpression of *hETV2* induces the EC formation regardless of differentiation conditions and in the absence of growth factors (e.g., VEGF) essential for differentiating and maintaining mature endothelial cells in culture [2,3].



Supplementary Figure SN.1.1.

Induction of endothelial fate via *hETV2* expression under different conditions.

(**a**) Left, a construct to target the AAVS1 locus to generate rtTA-mCherry+ hESC line (BC4). Right, expression of mCherry with Doxycycline in BC4 hESCs. (**b**) Top, depiction of infection of hETV2 (MOI 4) under embryonic body (EB) or neuron differentiation media for five days. Bottom, depiction of infection of hETV2 (MOI 4) under endothelial cell (EC) differentiation protocol for five days. (**c**) The hETV2 induced cells under different differentiation conditions displayed different morphology compared to un-induced cells. Data are representative images of 5 samples from three independent experiments. (**d**) Expression of endothelial genes was measured relative to control hESCs and normalized to *β-Actin*. Data represent the mean ± SEM (n=3). One paired t-test was used for comparison (*p=0.017, and **p=0.0000085). The scale bar represents 100 µm in **a**, and **c**.



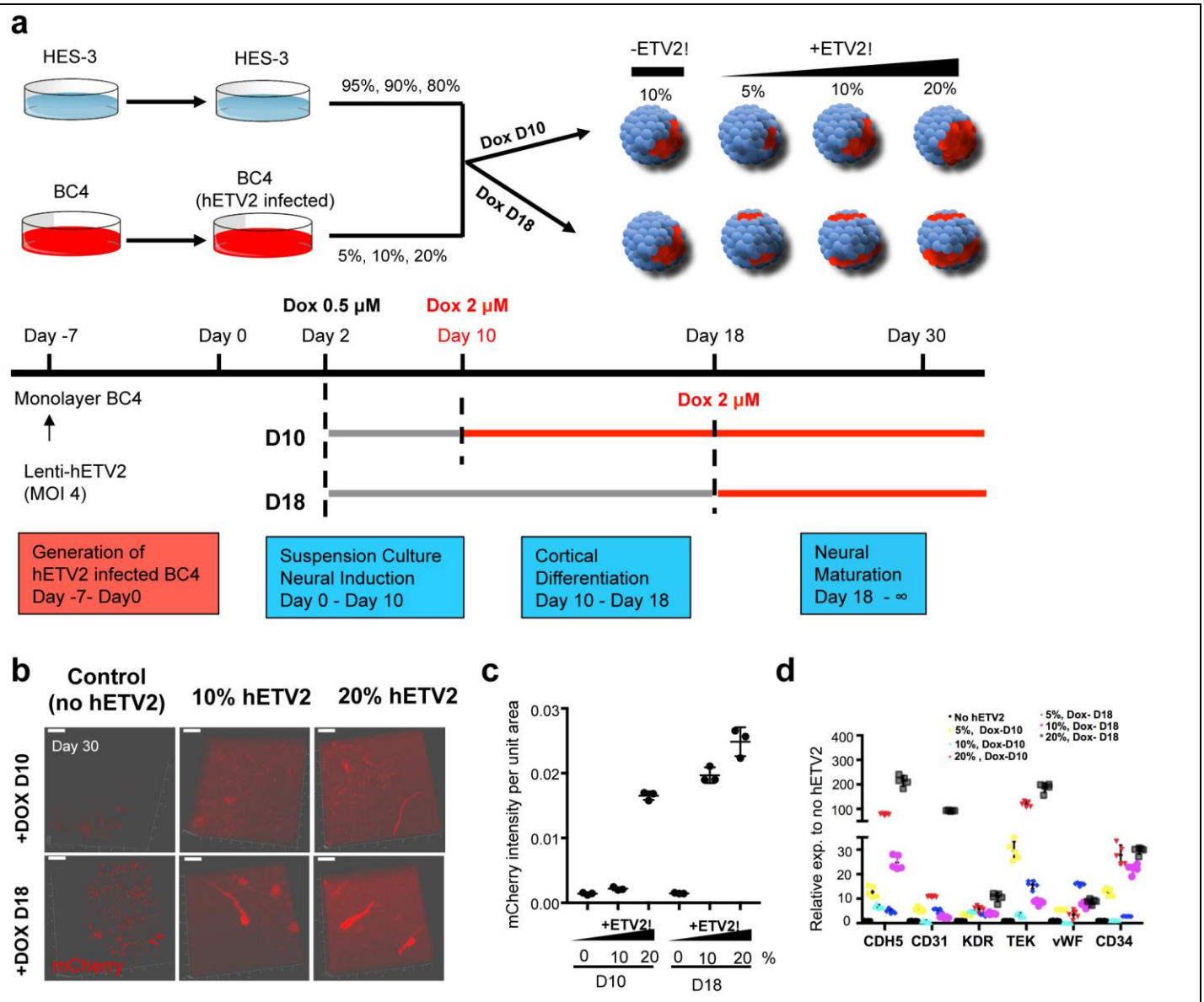
Supplementary Figure SN1.2.

***In vitro* generation of organoids expressing endothelial markers via induction of *hETV2*.**

(**a**) Left, immunostaining for EC markers, CDH5 and vWF, in hESCs differentiated without or with hETV2 induction in EB, neuron, or EC differentiation media. Center, quantification of CDH5 and vWF fluorescence per DAPI fluorescence of hETV2 induced EC cells. Data represent the mean ± SEM. One paired t-test was used for comparison (n=3, *p=0.0014). Right, flow cytometry analysis indicating the expression of CD31 in hESCs converted into EC cells via hETV2 induction under EB, and neuron differentiation media and EC protocol. (**b**) Electric cell-substrate impedance sensing (ECIS) was used to dynamically monitor endothelial resistance change during 3 differentiation conditions in the presence and absence of *hETV2* induction. Data are representative images of two independent experiments. (**c**) Left, immunostaining for mCherry and CDH5 in EC cells generated from hESCs with different ratios of hETV2-induced cells. Right, quantification of $CDH5^+/mCherry^+$ cells indicated that the all endothelial cells are derived from $mCherry^+$ cells. Data represent the mean ± SEM (n=3, three independent experiments). The scale bar represents 50 μm in **a**, and **c**.

Supplementary Note 2: The *hETV2* expression in hCOs.

hETV2-infected BC4 hESCs were mixed with non-infected parental HES3 hESCs in different ratios (5%, 10%, and 20%) to generate hCOs as described previously (Supplementary Fig. SN2.1. a) [4]. Two days after forming hCOs, *hETV2* was induced with a low dosage of dox (0.5 μM) to promote the early phase of reprogramming that is critical for EC maturation. The expression of *hETV2* was then fully induced at either day 10 or day 18 (Supplementary Fig. SN2.1. a, right panel). We performed 3D imaging of live hCOs to characterize the organization of the mCherry^{+} cells that express *hETV2*. At the early stage of organoid formation (day 30), the control hCOs without *hETV2* expression demonstrated a scattered mCherry expression without an organized structure formation. hETV2-expressing hCOs made tube-like structures throughout the inside of the organoids (Supplementary Fig. SN2.1. b). Compared to organoids induced with *hETV2* at day 10, those induced at day 18 contained more mCherry^{+} tubular structures (Supplementary Fig. SN2.1. c). Furthermore, organoids made with 20% hETV2-expressing hESCs had more tubular structures (Supplementary Fig. 3c). Overall, we found that induction with 20% hETV2 cell ratio (D18-20%) hCOs yielded the most robust formation of tubular-like structures compared to other hETV2 hCOs (Supplementary Fig. SN2.1. c). Besides, gene expression analysis showed a direct correlation between the amount of *hETV2* expression in hCOs and the levels of EC gene expression (Supplementary Fig. SN2.1. d). Furthermore, the endothelial progenitor marker *CD34* was also increased in both D10-20% hETV2 and D18-20% hETV2 organoids. D18-20% hETV2 hCOs showed a sharp increase in expression of *CD31* compared to D10-20% hETV2 hCOs, while other EC genes (e.g., *CDH5*, *KDR*, *TEK*, *vWF* and *CD34*) were expressed at similar levels in both conditions (Supplementary Fig. SN2.1. d). Collectively, these results suggest that hCOs with 20% hETV2 induced at day 18 start to form vascular-like structures, identified by EC markers. We named the hCOs with the vascular-like structure, as vascularized hCOs (vhCOs).



Supplementary Figure SN2.1.

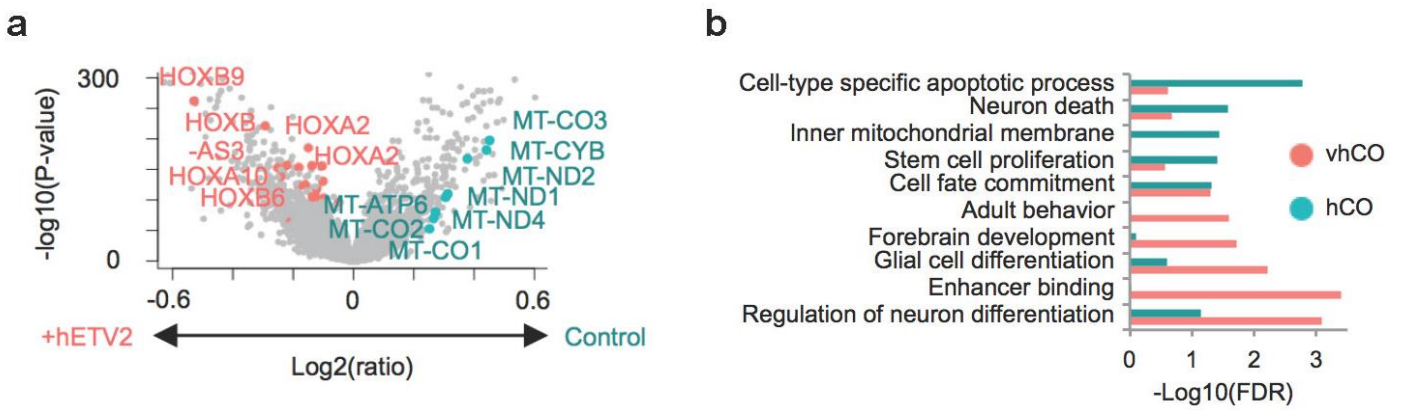
Schematic of *hETV2* expression in hCOs.

(**a**) Top, generation of Dox-inducible hETV2 expression in BC4 hESCs, and dosage and timing of *hETV2* expression in BC4 mixed with control HES-3 hESC line. Bottom, depiction of culture protocol for generation of cortical organoids containing different ratios of hETV2-induced cells. Organoids were developed with a low level of *hETV2* induction beginning at day 2 and higher induction at either at day 10 or day 18. (**b**) Representative images demonstrating mCherry^{+} cells expressing *hETV2* that form tube-like structures. Z stack confocal imaging was performed (~ 40-50 µm). (**c**) Quantification of mCherry fluorescence per unit area (µm2) of control hCOs and vhCOs. The scale bar represents 50 µm. Data represent the mean ± SEM (n=3, three independent experiments). (**d**) Expression of endothelial genes from organoids at day 30 generated by expressing *hETV2* at different dosage and duration. Gene expression was measured relative to control organoids and normalized to *β-Actin*. Data represent the mean ± SEM (n=5, from three independent batches).

Supplementary Note 3: Evaluation of cell clusters from vhCOs.

As observed previously [4,5], we identified unique cell clusters that are distinguishable from neurons and astrocytes and express genes for specific cellular structures (Fig. 2b and c). In particular, proteoglycan-expressing cells (PGC) demonstrated expression of specific proteoglycans (BGN and DCN) (Supplementary Fig. 3c) and a slight enrichment of endothelial gene signatures (Fig. 2c). These same proteoglycans were also highly expressed in endothelial-like cells (Supplementary Fig. 3c). To understand the derivation of these clusters, we constructed a differentiation trajectory from the single-cell transcriptome profile. Interestingly, PGCs were grouped into one branch with endothelial-like cells, suggesting that PGCs may have the potential to differentiate toward the endothelial lineage (Fig. 2h). The other unknown clusters, such as cilium-bearing cells (CBC) and BMP-related cells (BRC), belonged to a branch related glial cell development.

To further understand the effects of vascularization on lineage commitment in brain organoids, we compared transcriptome profiles between vhCOs and control hCOs. We found that vhCOs showed significant up-regulation of *HOX* genes, which are essential for regionalized neuronal identity (Supplementary Fig. SN3.1. a and b). Furthermore, mitochondria transcripts, hypoxic response genes, and apoptotic signaling genes were significantly downregulated in vhCOs (Supplementary Fig. SN3.1. a and b). Overall, these results support our observation that vhCOs attenuate cell damage by improved nutrition or oxygen supplementation to internal organoid regions through the vasculature.



Supplementary Figure SN3.1.

Classification, annotation and evaluation of cell clusters from vhCOs.

(**a**) Volcano plot showing differential expression between total vhCO- and control hCO-derived cells. Mitochondria genes and *HOX* with p<1e-50 were colored by cyan and salmon color, respectively. Two-sided T test was used for comparison. All 25622 genes are shown in gray color. (**b**) GO analysis for differentially-expressed genes between total vhCO- and control hCO-derived cells.

Supplementary Methods

Note 4: Data processing of ScRNA-seq

Briefly, cell clusters were classified into neuronal and non-neuronal clusters by the expression pattern of genes involved in the development of neuronal growth cone (*STMN2*, *GAP43*, and *DCX*) and early neurogenesis (*VIM*, *HES1* and *SOX2*). One cluster was a mixture of STMN2/GAP43/DCX high and VIM/HES1/SOX2 high cells and labeled as intermediate. The neuronal clusters were further separated into excitatory cortical neurons (CN) and inhibitory interneurons (IN) by *vGLUT1*, *GAD1*, and *GAD2* expression. One neuronal cluster lacking these markers was assigned as immature neurons. Among non-neuronal clusters expressing biglycan and decorin, four clusters displayed significant enrichment of “endothelial cell proliferation (GO:0001935)” and were labeled as endothelial-like cells (EN). One of the endothelial clusters showed high expression of cell cycle-related genes and was labeled as endothelial-like progenitors (ENP). Glia cells were assigned from eight non-neuronal clusters with the significance of “glia cell differentiation (GO:0010001)”. Among glia cell clusters, three clusters were labeled as astrocytes (AS) and one cluster as radial glial cells (RGC) by their unique markers. One cluster with high expression of cilium assembly-related genes was defined as cilium- bearing cells (CBC). Three clusters without these markers and cilium-related genes were annotated as glia progenitor cells (GPC).

Two non-glia clusters highly expressed cell cycle genes and were labeled as neuronal progenitor cells (NPC). One non-glia cluster was characterized with genes involved in BMP signaling pathways and was labeled as BMP-related cells (BPC). The one remaining cluster was characterized as “response to unfolded protein (GO:0006986)” and “ERAD signaling (GO:0036503)” and annotated as an unfolded protein-related cells (UPRC). One cluster with high expression of genes involved in epithelial-to-mesenchymal transition was labeled as EMT-related cells (EMT).

The assignment of cell types was verified by enrichment analysis of the cell type-specific gene signatures. The gene signatures for neurons, NPCs, astrocytes, oligodendrocytes, and endothelial cells were obtained from public single-cell RNA-seq of human fetal and adult brains (SRP057196) [6] and processed as described previously [4]. The gene set enrichment analysis (GSEA) was performed by GSEAPY software (v0.9.3) with options ‘‘–max-size 50000–min-size 0 -n 1000’’ to each cell, in which genes were sorted by relative expression to the average of all cells. Doublet frequency was estimated by the co-existence of *TBR1* and *GFAP* genes, which were exclusively expressed in neuron and glial cells [4].

Maturation states of neurons in the vascularized organoids were estimated by comparing the transcriptome profile with the developing human brain (GSE104276) [7]. Differentially-expressed genes between vhCO- and control hCO-derived neurons were identified with 1.25-fold change and $p<0.05$ by two-sided T-test. Then, the enrichment of these genes was calculated in each gestational week by GSEA software (v2.2.2) with 1,000 permutations of gene sets, weighted enrichment statistic and signal-to-noise separation metric.

Differentiation trajectory was predicted by monocle (v2.99.3) in Bioconductor package [8]. Instead of the normalization in monocle tool, we used CCA aligned space from Seurat output as normalized data projection. To construct the trajectory, the dimensionality was reduced by Uniform Manifold Approximation and Projection (UMAP). After partitioning cells into supergroups by approximate graph abstraction, the principal graph was learned by SimplePPT method. We note that most of the cells were classified into one major supergroup. Cells in the other minor supergroup were not used for subsequent analyses. Finally, cells were ordered by Pseudotime according to the graph by selecting cells in NPC clusters as starting points.

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