Engineering of human brain organoids with a functional vascular-like system

Bilal Cakir
Yale University https://orcid.org/0000-0003-4610-0317
Yangfei Xiang
Yale University https://orcid.org/0000-0002-6256-6312
In-Hyun Park (inhyun.park@yale.edu)
Yale University

Method Article

Keywords: Cortical organoids, vascularization, ETV2, hCO, scRNA-seq, hESC, hiPSC, endothelial cells.

Posted Date: October 7th, 2019

DOI: https://doi.org/10.21203/rs.2.13464/v1

Version of Record: A version of this preprint was published at Nature Methods on October 7th, 2019. See the published version at https://doi.org/10.1038/s41592-019-0586-5.

Abstract

Here, we engineered human embryonic stem cells (hESCs) to ectopically express human ETS variant 2 (ETV2) to create in vitro vasculature in cortical organoids (hCOs), namely vhCOs (vascularized hCOs). ETV2-expressing cells in hCOs contributed to forming a complex vascular-like network in hCOs. This protocol describes hESC maintenance, the generation of inducible ETV2 expressing hESCs, and the generation of cortical organoid with functional vascular-like network.

Introduction

The hCOs have provided an exclusive opportunity to study the initial brain development and neurologic disorders [1]. Although the advent of 3D brain organoids has opened innovative avenues, this technology suffers from several limitations: the absence of endogenous blood vessels, the generation of microglia, and the stratification of the six distinct cortical layers [2]. Remarkably, the absence of a vascularized brain system is critical to organoids, and millimeter-scale organoids under long-term culture consistently exhibit apoptotic cell death at the inner-most regions [3,4]. Recently, it has been shown that human brain organoids transplanted onto the cortex of the mouse brain induced the outgrowth of murine vessels into the human tissue, which increased cell survival and maturation [5]. Beyond this, there is no systematic approach to produce robust vascularization in brain organoids, which limits their application to the study of normal or pathogenic development *in vitro.* In this protocol, we describe the generation of cortical organoids with functional vascular-like networks. This platform can be used for studying human cortical development, disease mechanisms and neurogenesis in the presence of vasculature *in vitro*.

Reagents

REAGENT or RESOURCE

SOURCE

IDENTIFIER

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

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

Procedure

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 ETV2

1. To generate hESCs expressing inducible ETV2, 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 ETV2 (FUW-tetO-ETV2 [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 ETV2 expression

Cortical organoids (hCOs) with vasculature were generated by mixing ETV2-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 ml (7200 parental HES-3 hESC cells and 1800 ETV2 infected hESC cells were combined for each 150 ml). Then, 50 µM ROCK inhibitor Y27632 and 5% (v/v) heat-inactivated FBS (Life Technologies) were added.

7. 150 ml 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 ETV2 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 ml media and adding 150 ml 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 ETV2 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 ETV2 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.

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 ETV2 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.

Time Taken

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.

§ ETV2 virus titer is critical for the generation of vascularization inside the organoid.

Anticipated Results

References

1 Lancaster, M. A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373-379, doi:10.1038/nature12517 (2013).

2 Pasca, S. P. The rise of three-dimensional human brain cultures. *Nature* **553**, 437-445, doi:10.1038/nature25032 (2018).

3 Lancaster, M. A. & Knoblich, J. A. Generation of cerebral organoids from human pluripotent stem cells. *Nat Protoc* **9**, 2329-2340, doi:10.1038/nprot.2014.158 (2014).

4 Yin, X. *et al.* Engineering Stem Cell Organoids. *Cell Stem Cell* **18**, 25-38, doi:10.1016/j.stem.2015.12.005 (2016).

5 Mansour, A. A. *et al.* An in vivo model of functional and vascularized human brain organoids. *Nat Biotechnol* **36**, 432-441, doi:10.1038/nbt.4127 (2018).

6 Lee, S. *et al.* Direct Reprogramming of Human Dermal Fibroblasts Into Endothelial Cells Using ER71/ETV2. *Circ Res* **120**, 848-861, doi:10.1161/CIRCRESAHA.116.309833 (2017).

Acknowledgements

I.-H. P. was partly supported by NIH (GM111667-01, R01AA025080-01, R01CA203011-2), CSCRF (14-SCC-YALE-01, 16-RMB-YALE-04), Kavli Foundation, KRIBB/KRCF research initiative program (NAP-09-3). This work was supported by the College of Medicine, University of Arkansas for Medical Sciences to S.-H. L.

and Core Facilities of the Center for Translational Neuroscience, Award P30 GM110702 from the IDeA program at NIGMS. Computation time was provided by Yale University Biomedical High Performance Computing Center.

Figures

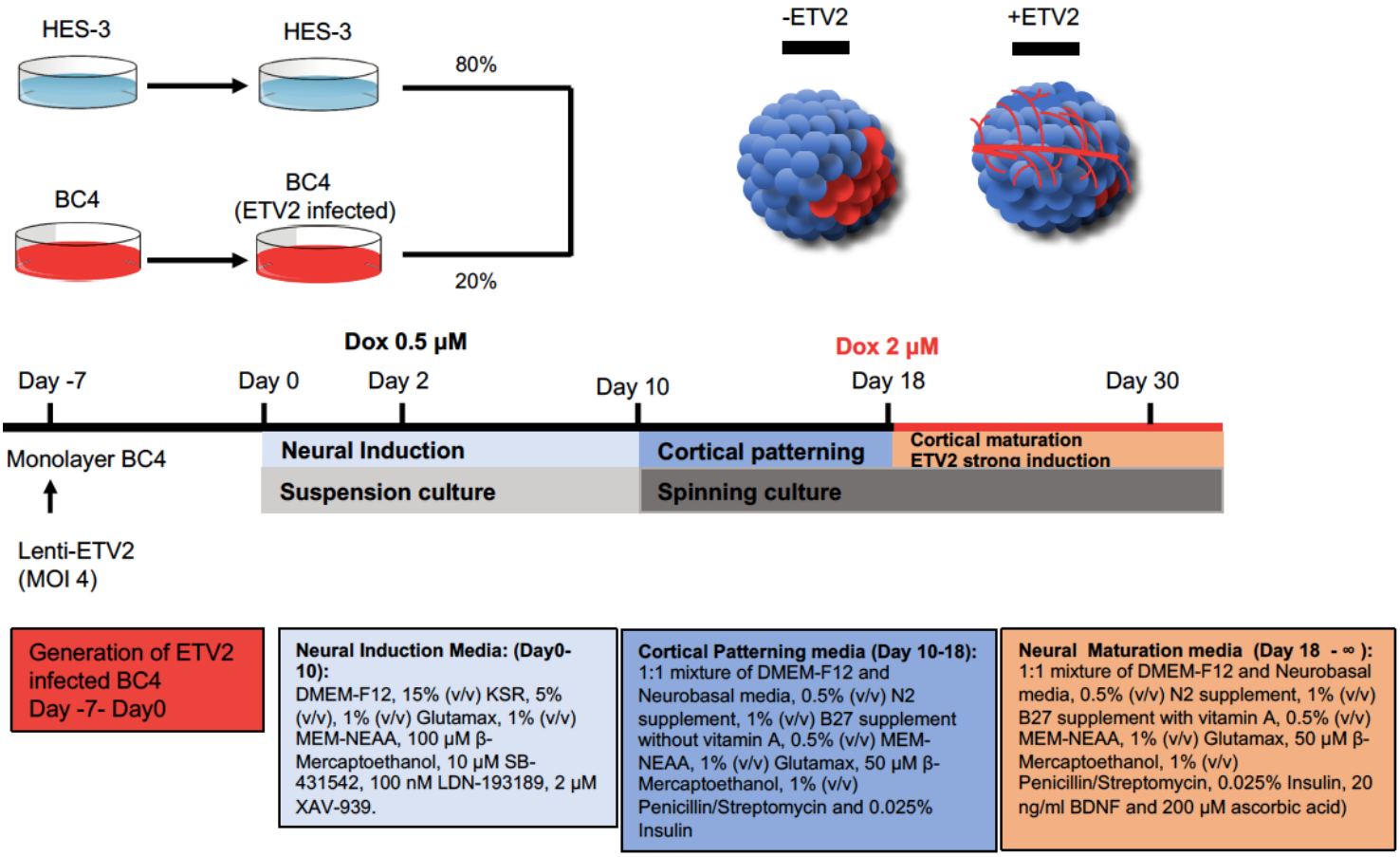

Figure 1

Schematic of ETV2 expression in hCOs. (a) Top, generation of Dox-inducible ETV2 expression in BC4 hESCs, and dosage and timing of ETV2 expression in BC4 mixed with control HES-3 hESC line. Bottom, the depiction of culture protocol for the 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 day 18.