Urine sample-derived cerebral organoids suitable for studying neurodevelopment and pharmacological responses

Victor J.T. Lin¹, Jiangnan Hu¹, Ashwini Zolekar¹, Liang-Jun Yan¹, Yu-Chieh Wang¹*

¹ Department of Pharmaceutical Sciences, UNT System College of Pharmacy, University of North Texas Health Science Center, Fort Worth, TX, USA

*To whom correspondence should be addressed:

Yu-Chieh Wang, Ph.D. Department of Pharmaceutical Sciences The University of North Texas Health Science Center 3500 Camp Bowie Boulevard, RES-340F Fort Worth, Texas 76107 Tel: 1-(817) 735-2944 Fax: 1-(817) 735-2603 Email: yu-chieh.wang@unthsc.edu

Running title: UEC-derived hiPSC-developed cerebral organoids

Keywords: urinary epithelial cells, hiPSCs, hESCs, neurodevelopment, cerebral organoids

Supplementary Information:

Materials and Methods

Isolation of UECs from urine samples

Basal urinary cell medium: complete KFSM/EPM (1:1)

Complete Keratinocyte-SFM (KFSM, 500 ml) was obtained from Thermo Fisher Scientific (Carlsbad, CA), containing 25 mg bovine pituitary extract (BPE), 2.5 μ g epidermal growth factor (EGF), 0.03 μ g/ml cholera toxin (MilliporeSigma, Burlington, MA) and penicillin-streptomycin (200 units/ml-200 μ g/ml; Thermo Fisher Scientific, Carlsbad, CA). EPM (500 ml) was composed of 337.5 ml Dulbecco's modified eagle medium (DMEM) with high glucose (Thermo Fisher Scientific, Carlsbad, CA), 112.5 ml Ham's F-12 nutrient mix (Thermo Fisher Scientific, Carlsbad, CA), 50 ml heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Carlsbad, CA).

Working urinary cell medium:

The working urinary cell medium contained 1x GlutaMax, $0.4 \mu g/ml$ hydrocortisone, $5 \mu g/ml$ insulin, 2.5 $\mu g/ml$ transferrin, 20 $\mu g/ml$ triiodythyronine, 10 ng/ml EGF, 1 $\mu g/ml$ adenine. GlutaMax, insulin and EGF were obtained from Thermo Fisher Scientific (Carlsbad, CA); Hydrocortisone, insulin, transferrin, triiodythyronine and adenine were obtained from MilliporeSigma (Burlington, MA).

The isolated cells were maintained in the working medium with medium changing performed every three days. The 10x DMEM with high glucose for spiking into urine samples was prepared by dissolving a pack of the medium power (Thermo Fisher Scientific, Carlsbad, CA) for reconstitution of 1000 ml DMEM with high glucose in 100 ml sterile deionized water.

Cerebral organoid formation

hPSC medium:

The hPSC medium consisted of 20% KnockOut serum replacement, 1% non-essential amino acids (NEAA), 1% GlutaMax, 0.18% 2-mercaptoethanol, 25 *ng*/ml FGF2 in DMEM/F12. All the components were obtained from Thermo Fisher Scientific (Carlsbad, CA).

Neural induction medium:

Neural induction medium containing SB431542 and IWR-1 was composed of 10 μ M SB431542 and 3 μ M *endo*-IWR-1, 1% N2 supplement and 1 μ g/ml heparin in the FGF2-deficient hPSC medium. Neural induction medium containing LDN193189 and A83-01 was composed of 100 *n*M LDN193189 and 500 *n*M A83-01 in the FGF2-deficient hPSC medium.

Differentiation medium I, II and III:

Differentiation medium I contained 1% N2 supplement, 0.025% insulin, 1% GlutaMax, 1% NEAA, penicillin-streptomycin (200 units/ml-200 μ g/ml), 0.05% 2-mercaptoethanol in the NeurobasalTM medium. Differentiation medium II contained 1% N2 supplement, 2% B27 supplement without vitamin A, 0.025% insulin, 1% GlutaMax, 1% NEAA, penicillin-streptomycin (200 units/ml-200 μ g/ml), 0.05% 2-mercaptoethanol in the NeurobasalTM medium. Differentiation medium II contained 1% N2 supplement with vitamin A, 0.025% insulin, 1% GlutaMax, 1% NEAA, penicillin-streptomycin (200 units/ml-200 μ g/ml), 0.05% 2-mercaptoethanol in the NeurobasalTM medium. Differentiation medium III contained 1% N2 supplement, 2% B27 supplement with vitamin A, 0.025% insulin, 1% GlutaMax, 1% NEAA, penicillin-streptomycin (200 units/ml-200 μ g/ml), 0.05% 2-mercaptoethanol in the NeurobasalTM medium. All the components of the differentiation media were obtained from Thermo Fisher Scientific (Carlsbad, CA).

Rhombencephalic conversion in cerebral organoids

Conversion medium I, II and III:

Conversion medium I contained 10 μ M SB431542, 50 *ng*/ml FGF2, 4 μ M CHIR99021 (Bio-Techne, Minneapolis, MN), and 0.5 *n*M retinoic acid (Bio-Techne, Minneapolis, MN) in the hPSC medium. Conversion medium II contained 100 *ng*/ml FGF19, 4 μ M CHIR99021, and 0.5 *n*M retinoic acid in the FGF2-deficient hPSC medium. Conversion medium III contained 1% N2 supplement, 4 μ M CHIR99021, 0.5 *n*M retinoic acid in the FGF2-deficient hPSC medium.

The time points for the use of these conversion media were annotated in the cerebral organoid protocols illustrated in Fig. **S4B**.

Immunofluorescence Staining

For the staining of pluripotency biomarkers in hPSCs and their differentiated derivatives, cells were plated into 24-well plates, fixed and permeabilized and incubated with primary antibodies against specific pluripotency biomarkers and fluorophore-conjugated secondary antibodies (Thermo Fisher Scientific, Carlsbad, CA). Organoid samples were fixed using 4% paraformaldehyde in PBS at at 4°C for overnight, rinsed with PBS, transferred into 15% sucrose

in PBS at 4°C for 48 hours, and transferred subsequently into 30% sucrose in PBS at 4°C for 48 hours. The samples were frozen embedding in a tissue-freezing matrix (O.C.T. compound; Thermo Fisher Scientific, Carlsbad, CA) at -80°C. For the staining of frozen sections of orgnoid and tissue samples, sections on slides were rinsed in phosphate-buffered saline (PBS) for three times to remove the tissue-freezing matrix, blocked with 5% donkey serum in PBS, and reacted with a primary antibody against a specific antigen at 4°C for overnight. After thorough washing with PBS containing 0.2% Tween-20 (PBST; Millipore Sigma, St. Louis, MO), the tissue sections were reacted with a flourescence-conjugated secondary antibody (Thermo Fisher Scientific, Carlsbad, CA) at room temperature for 1 hour, washed with PBST, and counterstained using a DAPI solution. If the biotinylated primary antibody was used, flourescence-conjugated streptavidin (Thermo Fisher Scientific, Carlsbad, CA) was subsequently used to reacted with the section. The stained organoid and tissue sections were mounted with cover slips using a Fluoromount-G medium (Southern Biotech , Birmingham, AL).

Gene Expression Analysis by qRT-PCR and Microarrays

Total RNA was isolated from cell samples using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Carlsbad, CA). The quality of each RNA samples was determined using an Agilent 2200 Tape Station system (Agilent, Santa Clara, CA) for RNA integrity analysis. Samples with RIN^e numbers above 7 were chosen to move forward with global gene expression profiling. The iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA) was used to generate the cDNA of total RNA samples. The cDNA samples were used for qRT-PCR assays. Global gene expression profiling was performed using HT-12v4 Human Gene Expression Bead Chips and an iScan array scanning system (Illumina, Hayward, CA), according to the manufacturer's instructions. The gene expression omnibus (GEO). Data were filtered for detection *p* value <0.01 in GenomeStudio (Illumina, Hayward, CA), and normalized using the LUMI package with RSN (Robust spline normalization) algorithm in R. Qlucore Omics Explorer 3.0 was used to perform differential gene expression analysis, hierarchical clustering, and plot generation. The ontology analysis of differentially expressed genes was performed using the PANTHER 14.1 (www.pantherdb.org).

In vivo Studies

Eight-week-old male NCG (NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/NjuCrl) mice (Charles River, Wilmington, MA) were group-housed under conditions of constant photoperiod (12 hours light: 12 hours dark) with ad libitum access to sterilized food and water. Cerebral organoids developed from UEC001i-009 hiPSCs were used in the animal study. Each isoflurane-anesthetized mouse was placed stably in a stereotactic frame with its body temperature maintained at 37 °C using a heating pad. Small incisions were made at the skin above the skull that covers the mouse cerebral cortex and cerebellum. Craniotomy to create a circular window of ~3mm diameter at the skull above each transplatation site was performed. Subsequently, the underlying dura mater was removed to access the brain tissue and create a small cavity to house the cerebral organoids at each transplantation region by aspiration with a blunt-end needle. The cavities were created unilaterally at the retrosplenial cortex and cerebellum in the same animal. Cerebral organoids without Matrigel embedding at the day 15 of development from the same batch of organoid production were inserted into the cavities. Each transplantation site received 3 organoids at similar size and without noticeable pigmentation. Each transplanted region was covered with a 3-mm cover slip that was mounted to skull using adhesive glue to create a cranial window. The surgical wound was closed with sutures. After the surgery, the mice were allowed to recover in a temperature-controlled cage before returning to their regular cages. The genneral condition of the mice was monitored on a daily basis throughout the entire study.

At terminal sacrifice, mice were anesthetized with a lethal dose of isoflurane and perfused transcardially with PBS (pH 7.2–7.5) followed by PBS containing 4% paraformaldehyde (PFA) freshly prepared. The whole animal brain was harvested and further fixed in PBS containing 4% PFA overnight. After fixation, the brain was transferred into 30% sucrose in PBS for 48 hours and subsequently frozen embedding in the O.C.T. compound at -80°C prior to cryosection.



scale bar: 100 µm

Supplemental Figure 1. The differential responses of UEC-derived hiPSCs to different protocols for cerebral organoid formation. The morphological representations at the indicated time points of the cerebral organoids that were developed from UEC001i-009, UEC001i-010 and UEC715i-501 hiPSCs in response to inhibition of TGF β and WNT signaling (SB431542 + IWR-1) or inhibition of TGF β and BMP signaling (LDN193189 + A83-01). Within two weeks after the induction of neuroectodermal commitment by two different appraoches, disintegration occurred in cell spheres with the treatment of SB431542 and IWR-1. The organoids developed from the cells with the treatment of LDN193189 and A83-01 remained intact and continued to grow.





Supplemental Figure 2. Neurogenesis followed by astrogliogenesis was observed in cerebral organoids developed from UEC-derived hiPSCs. (A) Immunofluorescence staining of TUBB3-positive neuronal cells and GFAP-positive astroglia were performed in the frozen sections of cerebral organoids collected on day 40 and day 80 of development from UEC001i-009 hiPSCs. Compared with the day-80 organoids, the day-40 organoids had none or a limited number of GFAP-positive cells. (B) The expression of TUBB3 and GFAP was also detected by western blotting in the day-40 and day-80 organoids. The low expression of GFAP detected in the day-40 organoids was concordant with the result from immunofluorescence staining.



Supplemental Figure 3. The distinct gene expression profiles in UEC-derived hiPSCs and WA09 hESCs were revealed by global gene expression profiling. (A) The volcano plot of expression difference (average expression in UEC-derived hiPSC samples – average expression in WA09 hESC samples) vs. significance. (B) The expression of the selected genes that were upregulated or downregulated in the analyzed UEC-derived hiPSC samples was validated by qRT-PCR analysis (*n*=3).

Cerebellar organoid formation:





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Hindbrain conversion in cerebral organoids:

Sample collection time points: day 21, 28, 35 (Without conversion) Sample collection time points: day 21, 28, 35 (Conversion started at day 6) Supplemental Figure 4. The procedures to generate cerebellar organoids from hPSCs and to convert the prosencephalic fate of cerebral organoids into a rhombencephalic fate. (A) *Left panel:* A schematic illustration of the procedure used in this study to generate cerebellar organoids UECs from hPSCs. *Right panel:* The rhombencephalic features of cerebellar organoids developed from UEC001i-009 hiPSCs were indicated by the downregulation of FOXG1 and the upregulation of GBX2, HOXA2, and HOXB4 along with the organoid development. The expression of the *FOXG1*, *GBX2*, *HOXA2*, and *HOXB4* genes in organoids samples (*n*=4) collected at the indicated time points was determined using qRT-PCR analysis. (B) A schematic illustration of the procedures for rhombencephalic conversion in cerebral organoids generated by three different approaches of neural induction in hPSCs. Pink highlight: the procedure used for conversion started at day 13 of organoid development. SCOK: STEMdiffTM Cerebral Organoid Kit. SB: SB431542. CHIR: CHIR99021.

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Supplemental Figure 5. Cells in cerebral organoids developed from UEC-derived hiPSCs significantly lost their cellular pluripotency within two weeks at the beginning of organoid development. (A) A schematic illustration of the procedure for generating cerebral organoids that were used in the expression analysis of POU5F1 and FOXG1. (B) The expression of POU5F1 and FOXG1 was detected by qRT-PCR (*left panel*) and western blotting (*right panel*) in organoid samples collected at the indicated time points (n=3, *p<0.05, t-test).

Supplemental Table S1. Major similarities and differences between ESC- and UEC-derived COs			
ESC-derived COs	UEC-derived COs		
The challenge of hPSC sources			
High	Low to moderate		
The variation of neuroepithelial sphere formation in response to different protocols			
Unnoticed to low	Clearly noticed variations among protocols		
Cell propensity for apoptosis during development			
Low	Moderate to high (could be overcome by protocol optimization)		
Cell plasticity permitting conversion between prosencephalic and rhombencephalic fates			
Present	Present		
The overall capacity of normal development			
High	Good		
Utility in studies required different genetic backgrounds and/or a personalized setting			
Limited	High		

Supplemental Table S2. The list of cultured cells used in the study				
Sample Name	Registry Name ^a	Note ^b		
Human embryonic stem cells				
WA09	WA09	Obtained from the WiCell Stem Cell Bank; feeder cell-free culture on Matrigel, passaged using EDTA-based hPSC passaging solution		
Induced pluripotent stem cells from human Urinary Epithelial Cells (UECs)				
UEC715i-501	N/A	Sendai virus-mediated reprogramming in the UECs of a healthy male donor (#715); provided by Dr. Jeanne Loring from The Scripps Research Institute; feeder cell-free culture on Matrigel, passaged using EDTA-based hPSC passaging solution		
UEC001i-003	N/A	Retrovirus-mediated reprogramming in the UECs of a healthy male donor (#001); feeder cell-free culture on Matrigel, passaged using EDTA-based hPSC passaging solution		
UEC001i-009	N/A	Retrovirus-mediated reprogramming in the UECs of a healthy male donor (#001); feeder cell-free culture on Matrigel, passaged using EDTA-based hPSC passaging solution		
UEC001i-010	N/A	Retrovirus-mediated reprogramming in the UECs of a healthy male donor (#001); feeder cell-free culture on Matrigel, passaged using EDTA-based hPSC passaging solution		

a. Name of cell line submitted to University of Massachusetts (UMass) International Stem Cell Registry

b. Somatic cell type, reprogramming method, culture condition, source of cells

N/A: not applicable

Supplemental Table S3. The list of primary antibodies and lectin used in the study				
Antibody/Lectin Name	Catalog Number	Sources		
Antibodies used in IHC or fluorescend	ce staining			
TRA-1-81	09-0011	Stemgent		
POU5F1	2840	Cell Signaling Technology		
NANOG	MABD24	Millipore Sigma		
TUBB3	MRB-435P	Biolegend (formerly Covance)		
Smooth Muscle Actin (SMA)	MAB1420	R&D Systems		
SOX17	AF1924	R&D Systems		
CSPG4	ab83178	Abcam		
Brachyury	sc-17745	Santa Cruz Biotechnology		
SOX2	AB5603	Millipore Sigma		
TBR2	AB2283	Millipore Sigma		
TBR1	ab31940	Abcam		
Phospho-VIM	D076-3	MBL International		
SATB2	ab34735	Abcam		
BCL11B (CTIP2)	ab18465	Abcam		
HOPX	HPA030180	Millipore Sigma		
DCX2	sc-8066	Santa Cruz Biotechnology		
GFAP	ab7260	Abcam		
OTX2	MAB10289	Abnova		
FOXG1	ab18259	Abcam		
Human Nuclear Antigen	MAB1281B	Millipore Sigma		
GBX2	114-182	Abnova		
HOXA2	PA5-68986	Thermo Fisher Scientific		
HOXB4	PA5-23366	Thermo Fisher Scientific		
MKI67	ab16667	Abcam		
Antibodies used in immunoblotting				
GFAP	ab7260	Abcam		
TUBB3	MRB-435P	Biolegend (formerly Covance)		
FOXG1	ab18259	Abcam		
BAX	2772	Cell Signaling Technology		
BCL-XL	2764	Cell Signaling Technology		
CASP3	14220	Cell Signaling Technology		
JARID1D	A301-751A	Bethyl Laboratories		
ACTIN	08691001	MP Biomedicals		
POU5F1	2840	Cell Signaling Technology		
NANOG	MABD24	Millipore Sigma		
CHCHD2	HPA027407	Millipore Sigma		
DUSP1	HPA069577	Millipore Sigma		
Phospho-AKT Substrate	9614	Cell Signaling Technology		
Phospho-AKT (Ser473)	4060	Cell Signaling Technology		
AKT	2920	Cell Signaling Technology		
TBR2	AB2283	Millipore Sigma		
RBFOX3	MAB377	Cell Signaling Technology		
HOPX	PA5-49449	Thermo Fisher Scientific		
SOX2	3579	Cell Signaling Technology		
MKI67	ab16667	Abcam		
Lectin used in fluorescence staining				
UEA-I	FL-1061	Vector Laboratories		