Improving tissue differentiation in cerebral organoids

Technical Journal Club

Manfredi Carta

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Contents

Cerebral organoids model human brain development and microcephaly

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Human CNS barrier-forming organoids with cerebrospinal fluid production

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

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Introduction

Cerebral organoids model human brain development and microcephaly

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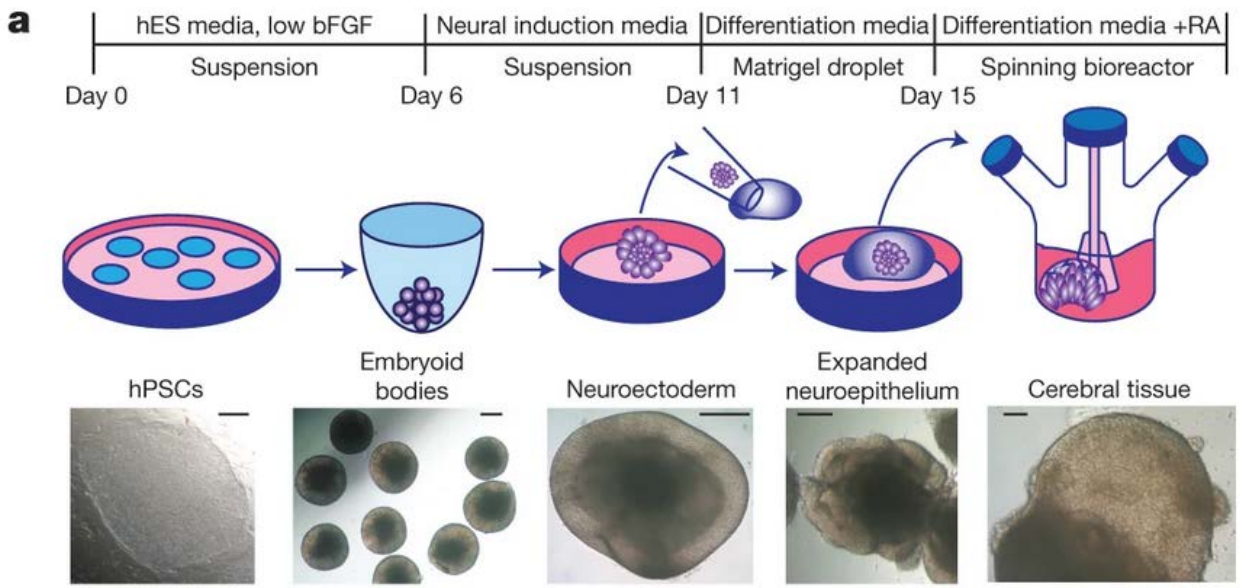
Protocol overview



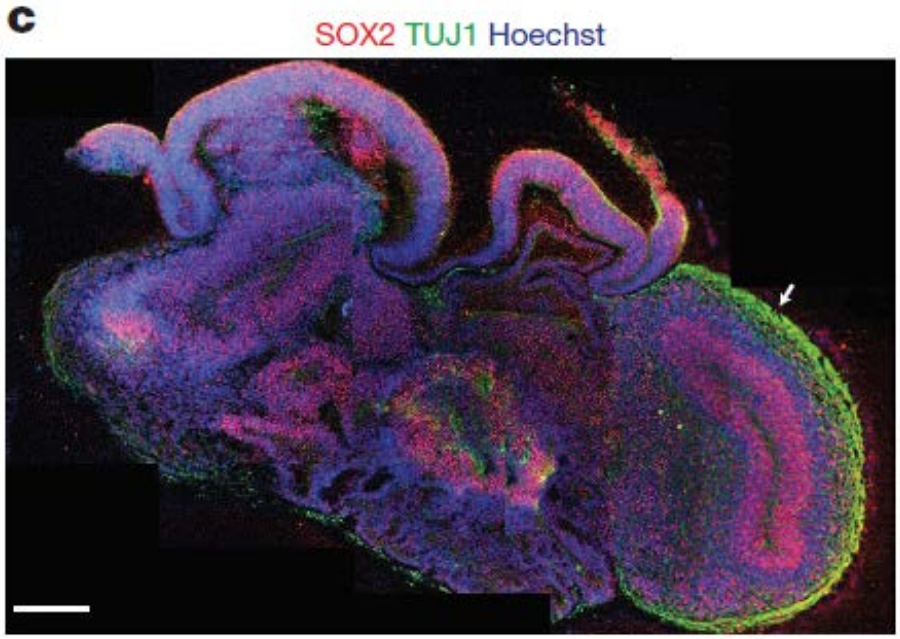
1. hPSCs are incubated in human ES medium with low bFGF4 and ROCK inhibitor → embryoid bodies (= 3D aggregates of hPSCs)
2. Transfer to neural induction medium (DMEM/F12, N2, GluMax, amino acids, heparin)
3. Transfer to Matrigel, grow in differentiation media (DMEM/F12, Neurobasal, B27 w/o Vitamin A, N2, β-ME, insulin, GluMax, amino acids)
4. Add Vitamin A to media, grow in spinning bioreactor

bFGF4: basic fibrobl. growth factor 4
ROCK: Rho-associated protein kinase

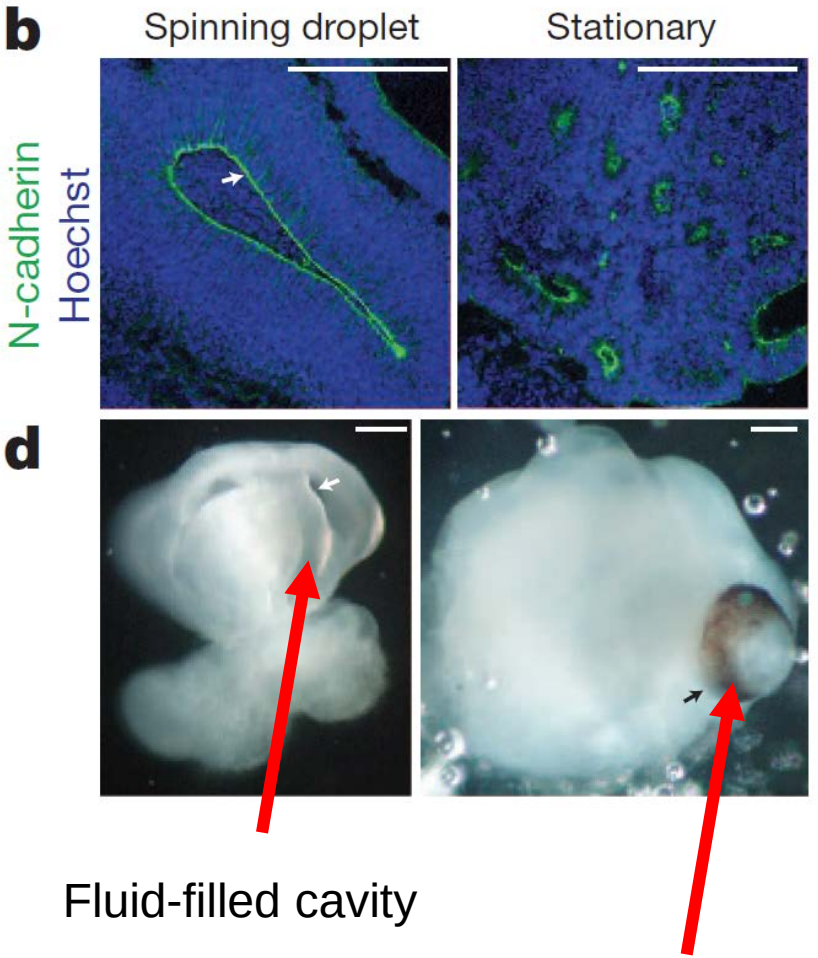
Spinning conditions

Tissues grown in spinning bioreactor

- developed contained large fluid-filled cavities (similar to ventricles)
- apical localisation of neural N-cadherin
- Were larger and more continuous than tissues grown in stationary suspension

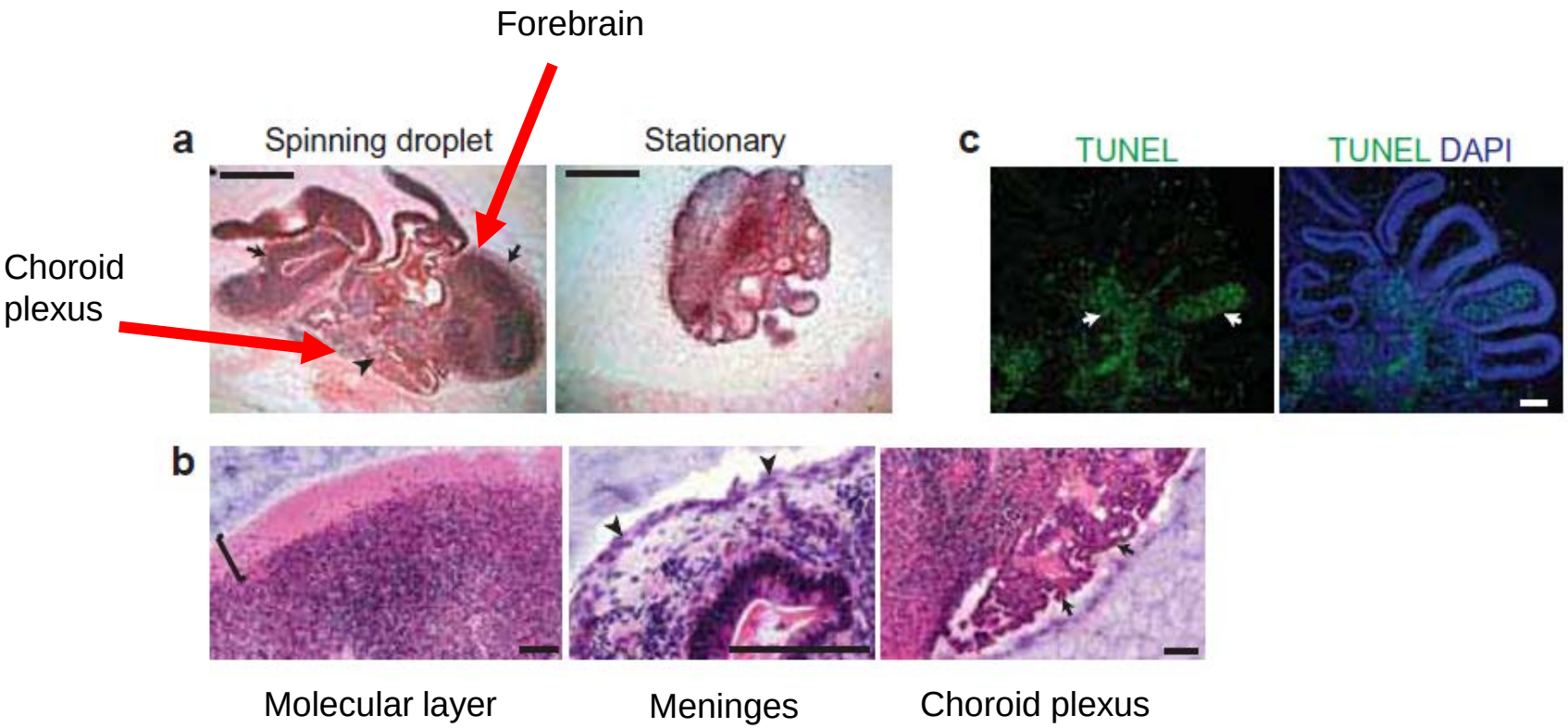


SOX2+: neural progenitors
TUJ1+: neurons



Scale bars: 200µm

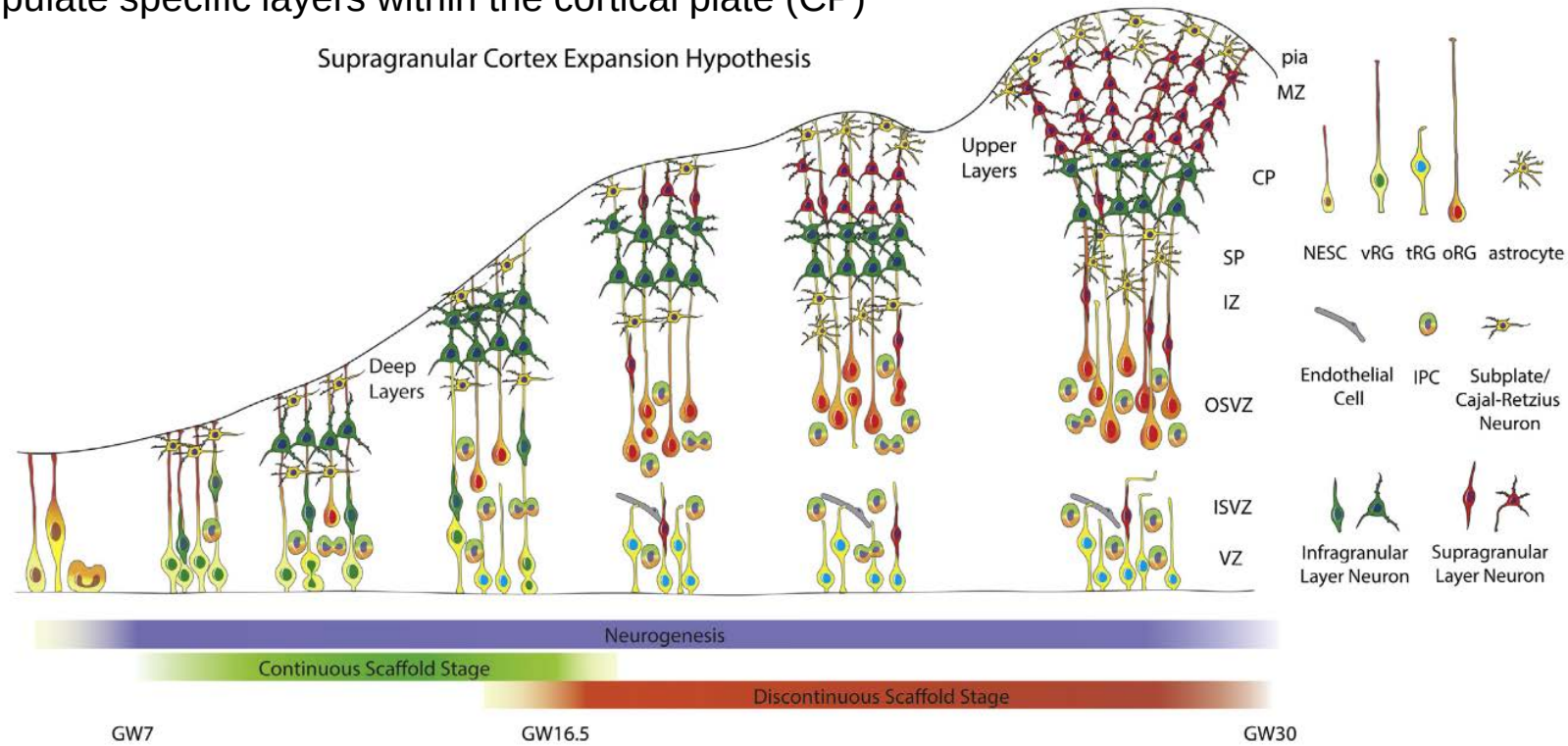
Cerebral organoids: Anatomy



- Spinning conditions: anatomy reminiscent of brain regions
- Organoids lacked vasculature, limited oxygen and nutrient diffusion: cell death in internal areas (TUNEL stain)

Mammalian brain development

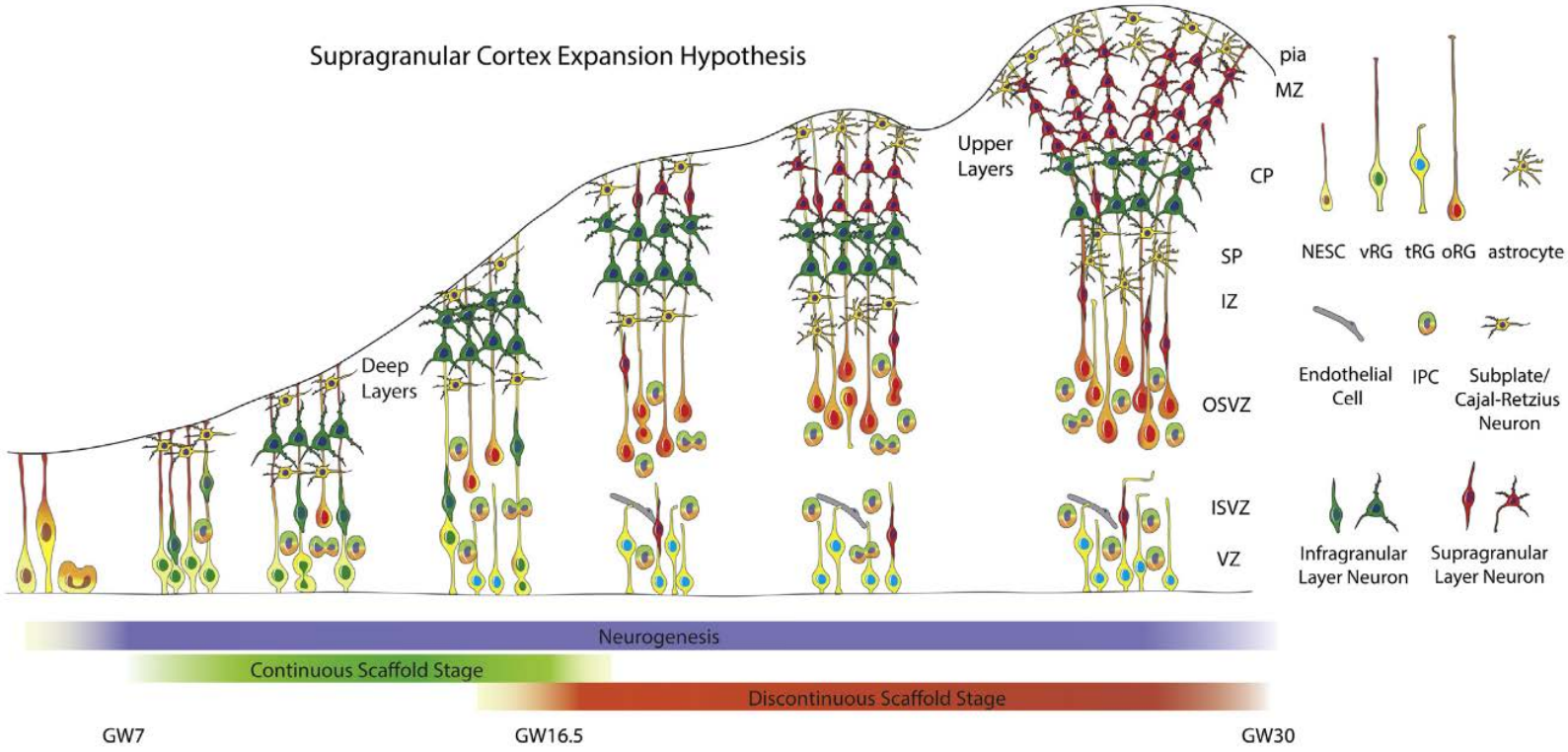
- Neuroepithelium expands to generate radial glial stem cells (RG)
- RG divide at the apical surface within the ventricular zone (VZ), generate neurons and intermediate progenitor cells (IPCs)
- IPCs populate the subventricular zone (SVZ), neurons migrate through intermediate zone (IZ) to populate specific layers within the cortical plate (CP)



MZ: marginal zone, NESC: neuroepithelial stem cell, SP: subplate, tRG: truncated radial glia, vRG ventricular radial glia

Specific to human development

- SVZ is split by inner fibre layer (IFL) into inner SVZ and outer SVZ
- Outer SVZ is populated by IPCs and outer radial glia (oRG)



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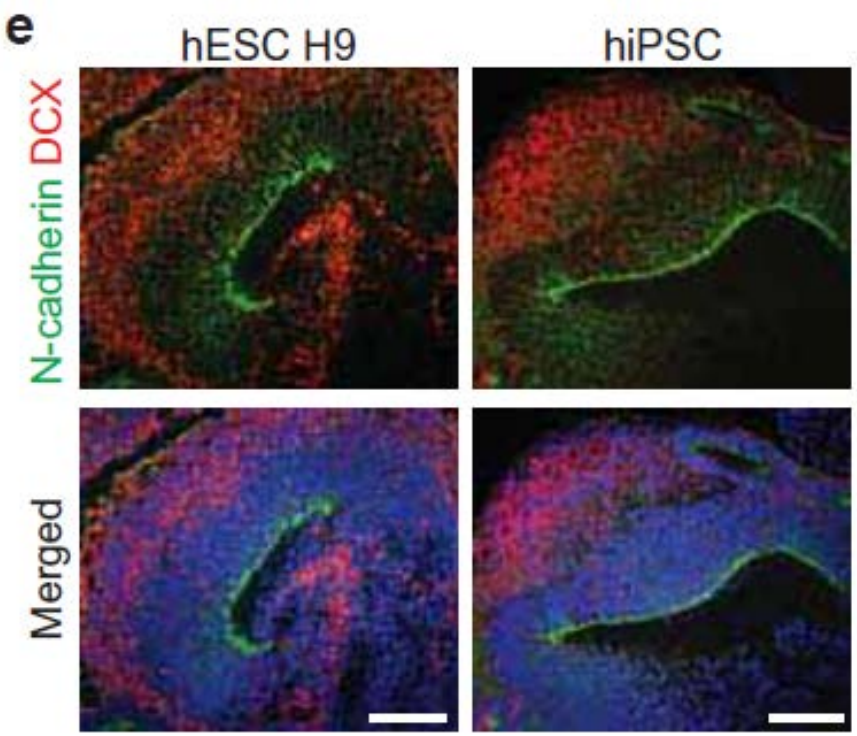
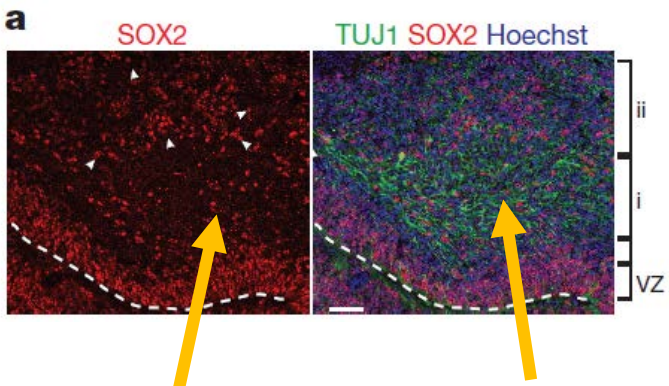
Cerebral organoids: Cellular organisation mimicks brain development





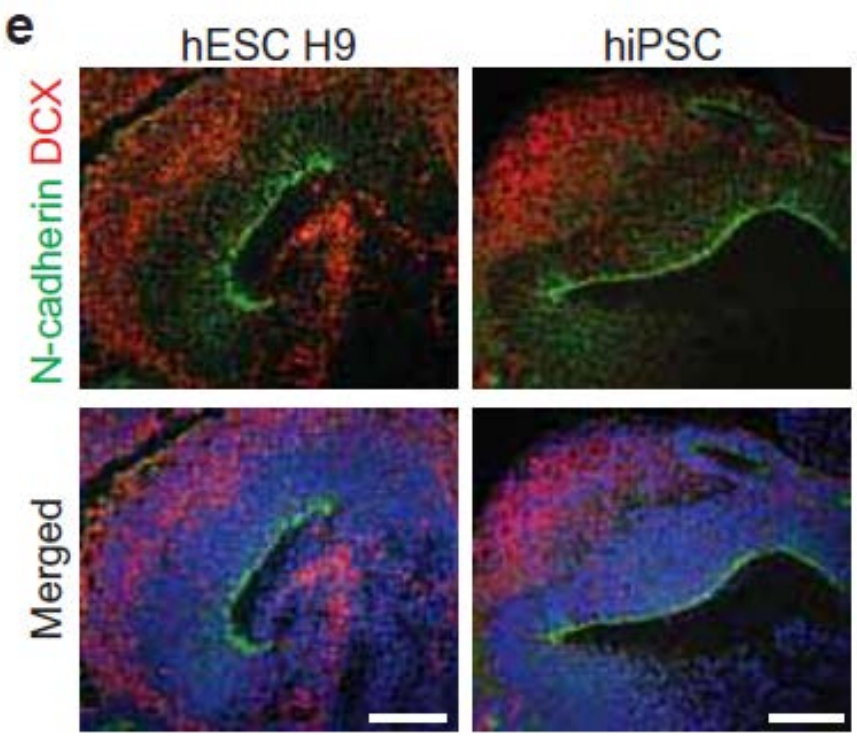
IFL-like zone (i) with TUJ1+ fibres

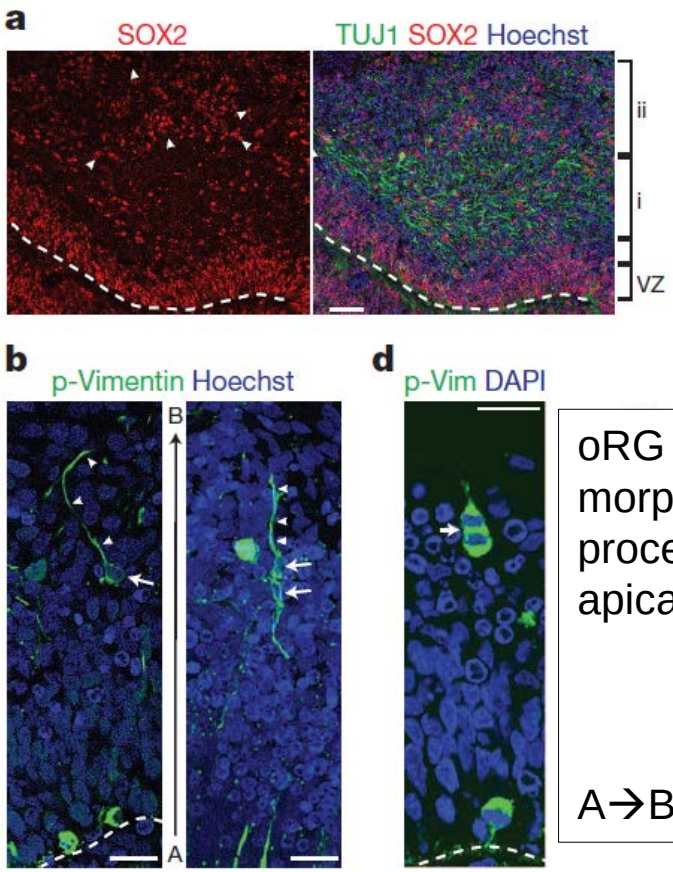
oRG organise in OSVZ-like layer (ii)

Left: Staining for N-cadherin (green) and newborn neurons (DCX, red) reveals similar organisation and intact apical-basal polarity in tissues generated from hESC and human iPS cells

Right: outer radial glia (SOX2+) with typical morphology and behaviour

Cerebral organoids: Cellular organisation mimicks brain development





oRG with typical morphology: basal process, but no apical process

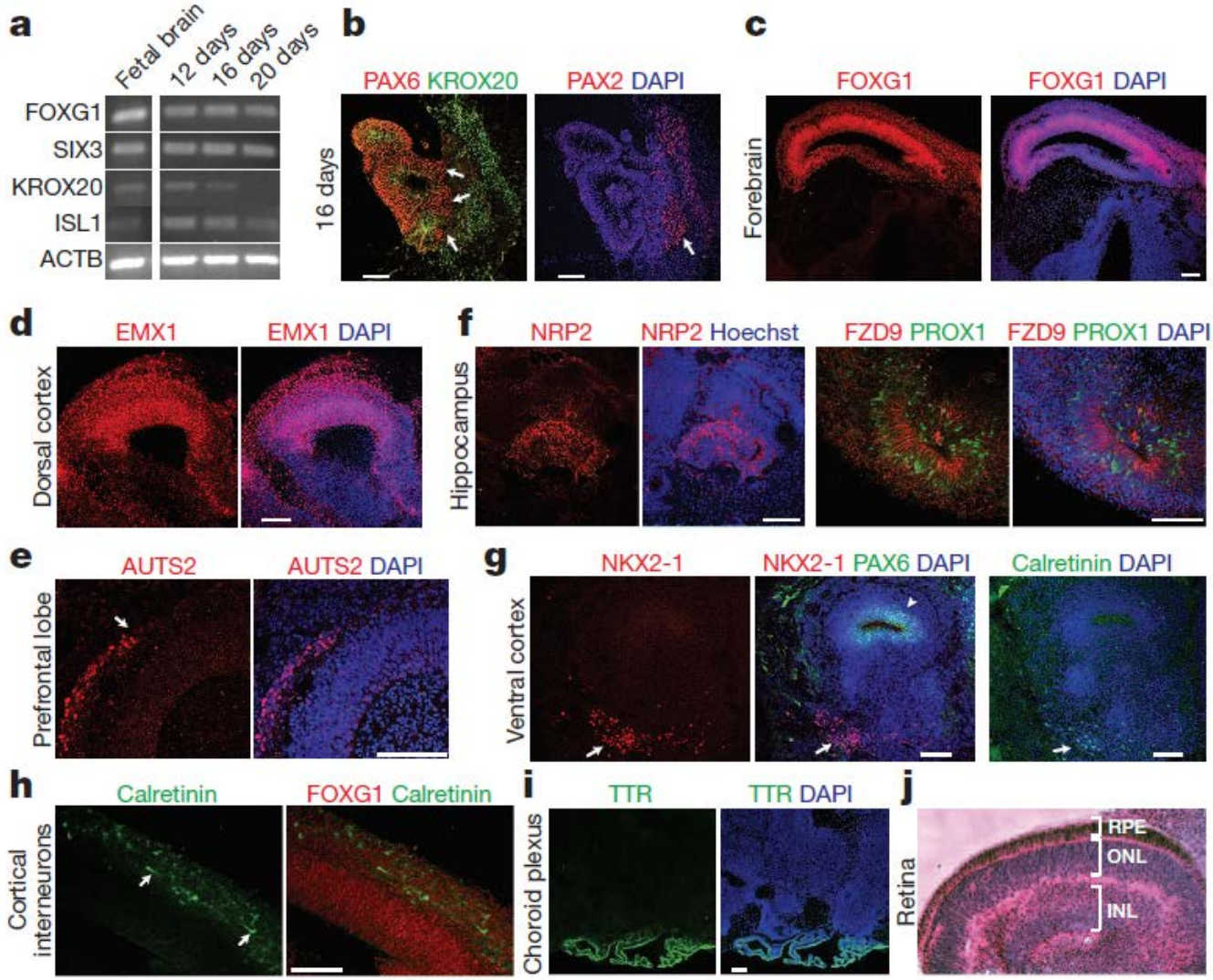
A→B apical-basal

Left: Staining for N-cadherin (green) and newborn neurons (DCX, red) reveals similar organisation and intact apical-basal polarity in tissues generated from hESC and human iPS cells

Right: outer radial glia (SOX2+) with typical morphology and behaviour

Discrete brain regions with expression of specific markers (WB, IHC)

WB of whole organoid



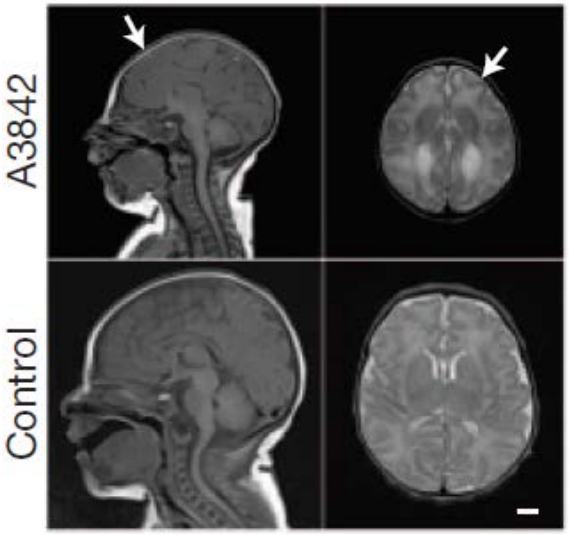
FOXG1, SIX3: forebrain

KROX20, ISL1, PAX2: hindbrain

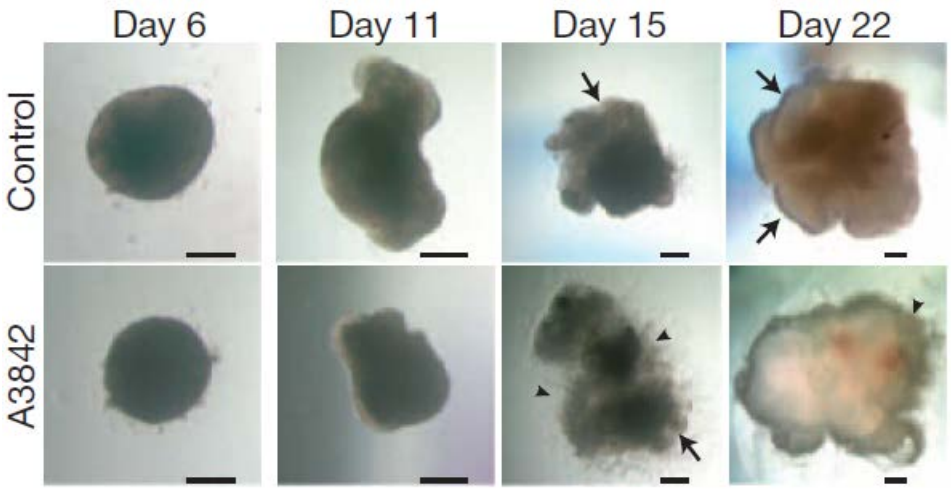
PAX6: cortex, ventr. wall, cerebellum

RPE: retinal pigment epithelium
ONL: outer nuclear layer
INL: inner nuclear layer

Organoid as model for microcephaly

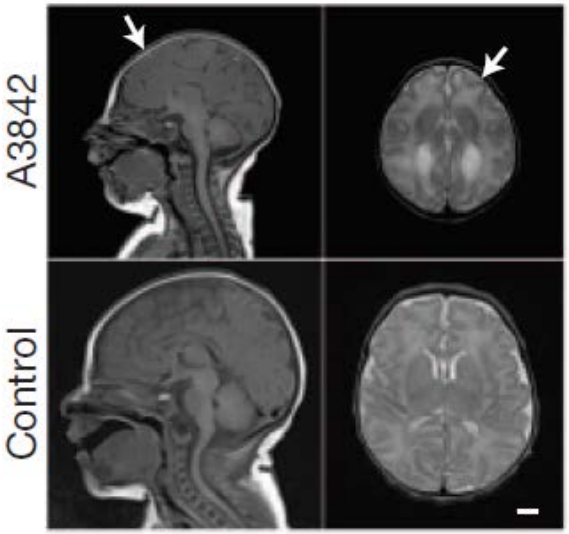


- Fibroblasts obtained from patient with severe microcephaly, exome sequencing showed truncating mutations in *CDK5RAP2* (required for mitotic spindle orientation)
- Lentiviral delivery of reprogramming factors (OCT4, SOX2, MYC and KLF4) → iPS cell clones
- Production of embryoid bodies, grew more slowly than usual → longer culture



- Smaller neuroepithelial tissues, increased neuronal outgrowth

Organoid as model for microcephaly



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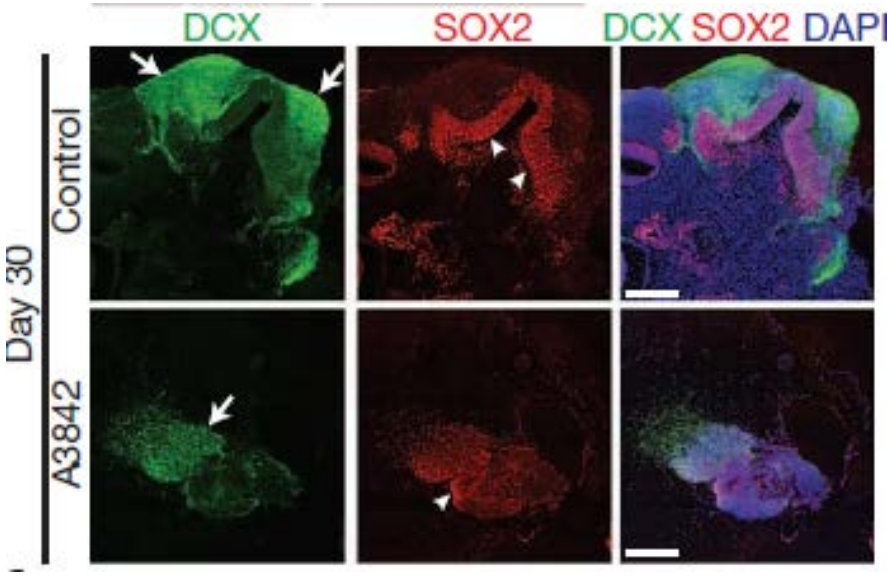
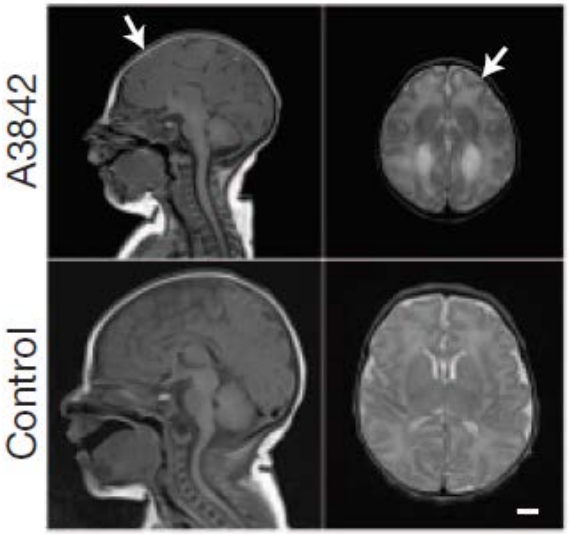


- Smaller neuroepithelial tissues, increased neuronal outgrowth
- Fewer neurons (DCX), smaller progenitor zones (SOX2)

Organoid as model for microcephaly



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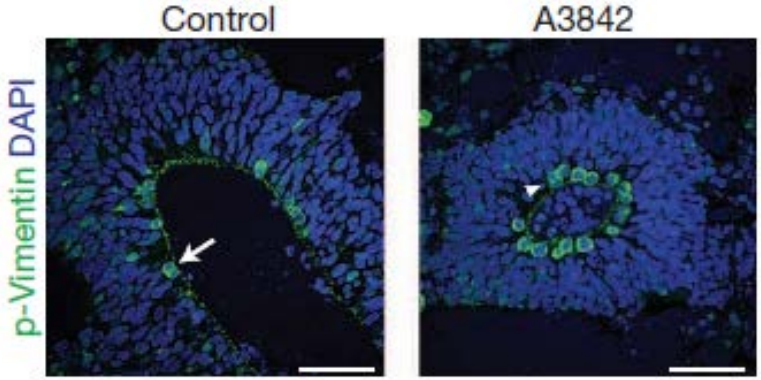

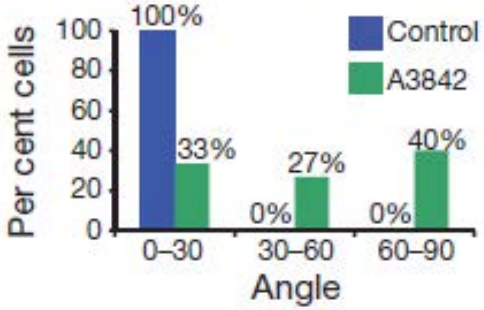

- Smaller neuroepithelial tissues, increased neuronal outgrowth
- Fewer neurons (DCX), smaller progenitor zoines (SOX2)
- Radial glia (Vimentin): horizontal mitosis in control, oblique mitoses in patient (arrowhead)

Summary

- In vitro culture of cerebral organoids from human and mouse pluripotent stem cells
- Many characteristics of human brain development can be recapitulated, including diverse tissues with expected marker gene expression
- A novel model of microcephaly was created by growing organoids from patient cells: RG progenitors failed to expand adequately
  - This cannot be modelled in mice, as RG progenitors probably don’t expand as strongly as in humans

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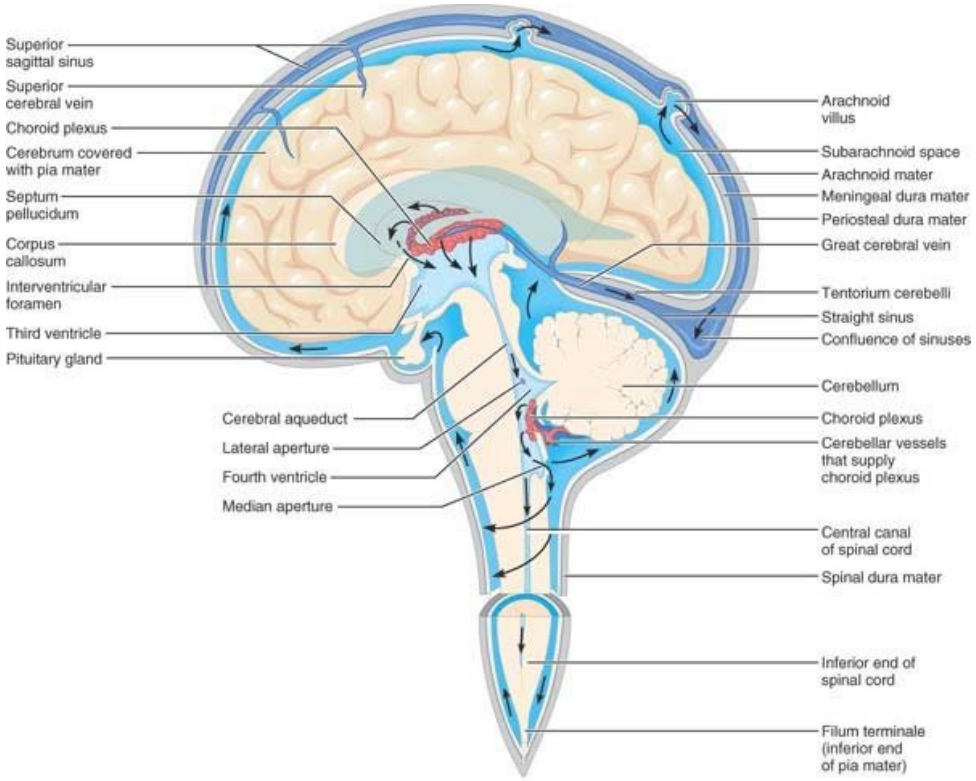
Images: med.utah.edu, Researchgate

Introduction

- **Cerebrospinal fluid** (CSF) is produced by the **choroid plexus** (ChP), which also serves as a protective polarised epithelial barrier, preventing free entry of molecules from the blood: **Blood-CSF barrier** (B-CSF-B)
  - B-CSF-B and BBB prevent entry of most therapeutic molecules

- CSF provides nutrients and signalling molecules and drains toxic byproducts from the brain



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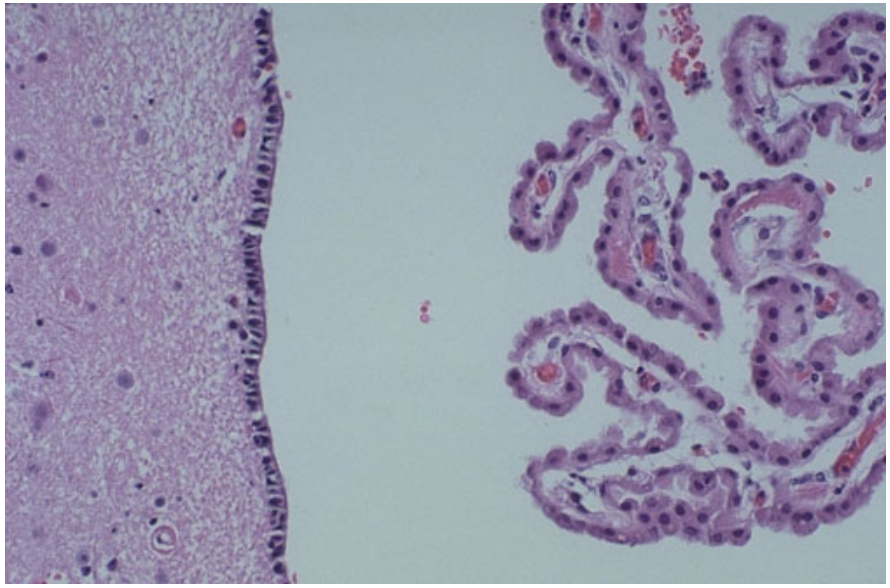
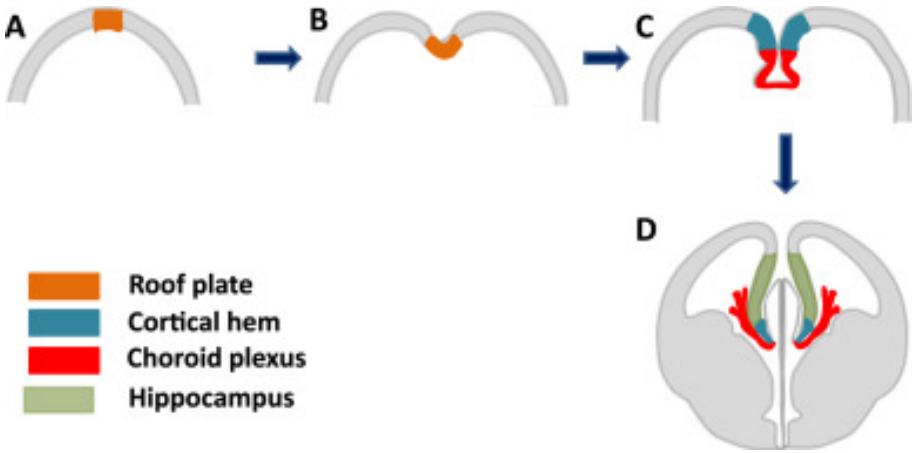


Normal ChP: epithelial layer surrounding stroma with blood vessels
Left: ependymal cells lining cerebral ventricle

Modelling the choroid plexus (1)

- Location deep within brain, surrounded by brain tissue and vasculature, renders ChP difficult to study:
  - What is the cellular makeup?
  - Which CSF molecules are filtered from blood, versus made de novo by ChP?

- During development, the ChP is the most dorsomedial derivative in the telencephalon. Lateral to the ChP is the cortical hem, which divides it from the cortex and regulates development.

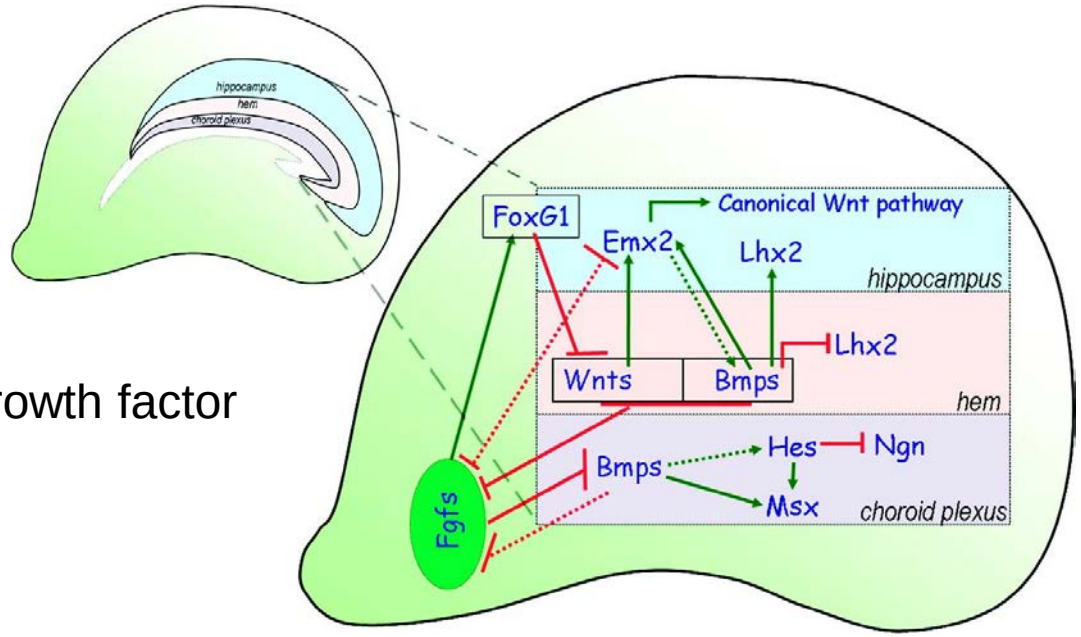


Sakaguchi et al., Nat Commun 2015

Modelling the choroid plexus (2)

- Choroid plexus cells were previously induced from human embryonic stem cells (hESCs) using two factors that are essential for brain dorsal patterning
  1. Bone morphogenetic protein (BMP), secreted by cortical hem and dorsal midline
  2. Wnt, secreted by cortical hem

FGF: fibroblast growth factor



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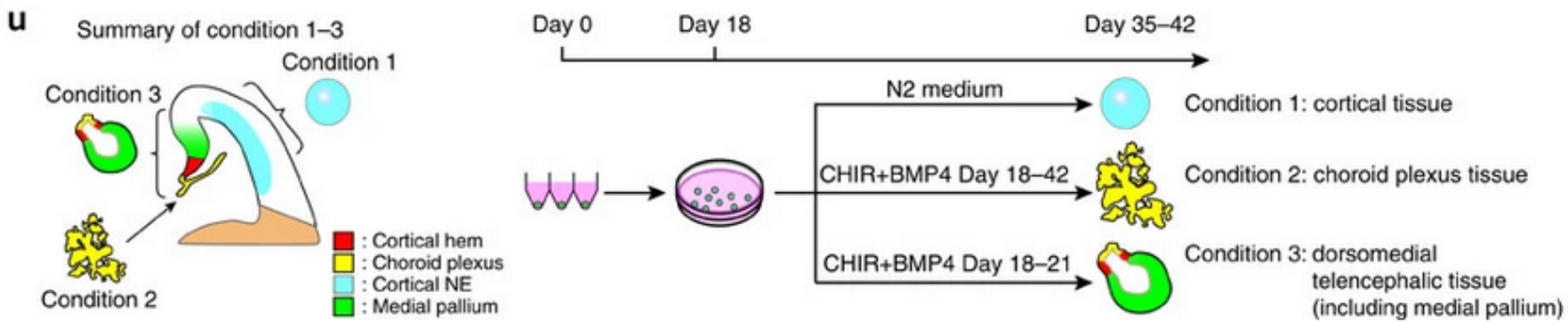
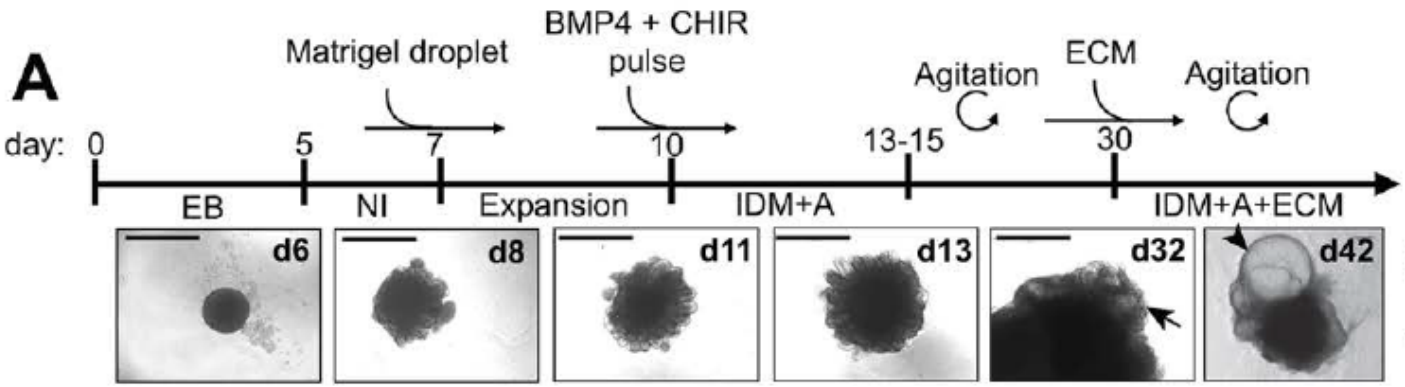


- hESCs were cultured in cortical differentiation medium → cortical tissue
- CHIR (GSK3 inhibitor → activation of Wnt) and BMP4 induce ChP-like tissue or dorsomedial telencephalic tissue, depending on exact timing of treatment

GSK3: Glycogen synthase kinase 3

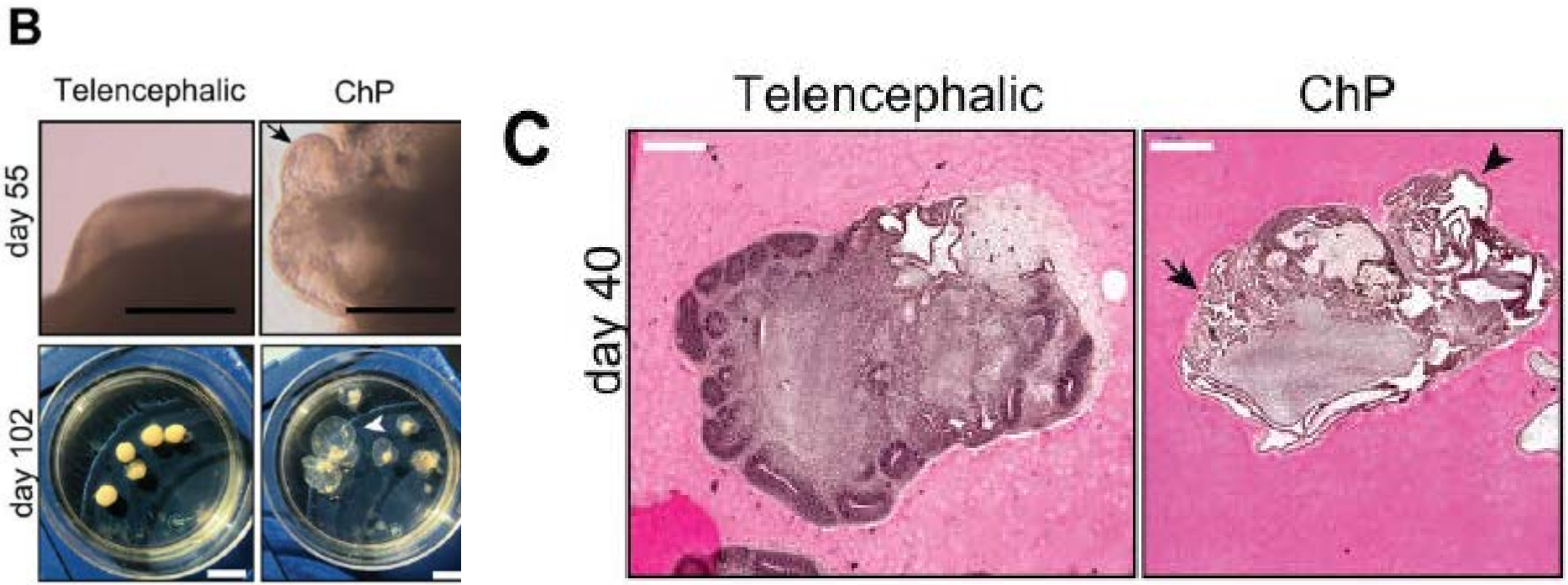
Sakaguchi et al., Nat Commun 2015

ChP organoids: Protocol



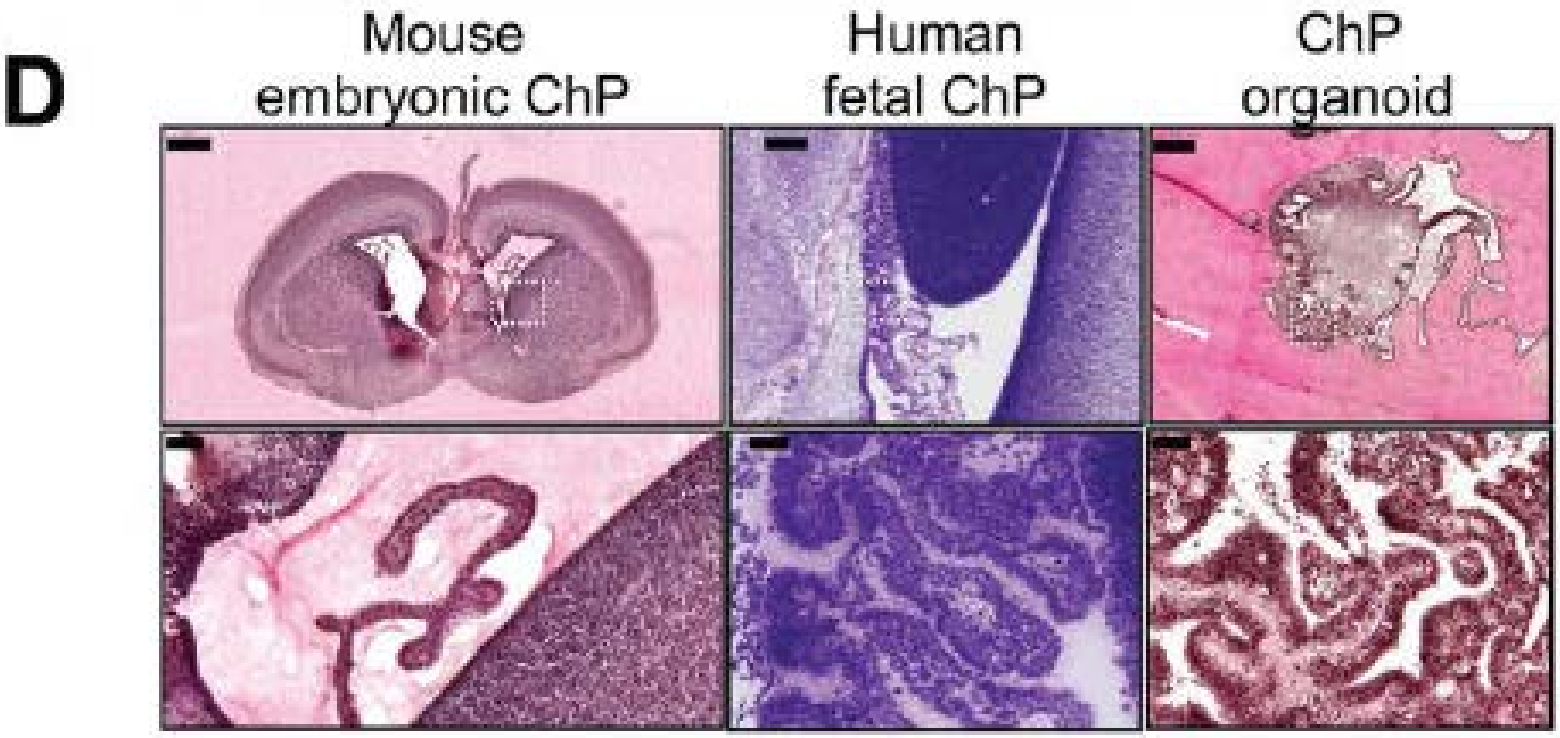
- Start with cerebral organoid protocol, give BMP4 and CHIR pulse
  - No pulse → telencephalic organoids with large, rounded neuroepithelial lobes
  - With pulse → elongated neuroepithelial tissue, similar to elongation of ChP from neuroepithelium in vivo

ChP organoids: Morphology (1)



- Elongated neuroepithelial tissue, similar to elongation of ChP from neuroepithelium in vivo
- ChP organoids contained cuboidal epithelium, fluid-filled cysts and choroid plexus-like morphology

ChP organoids: Morphology (2)



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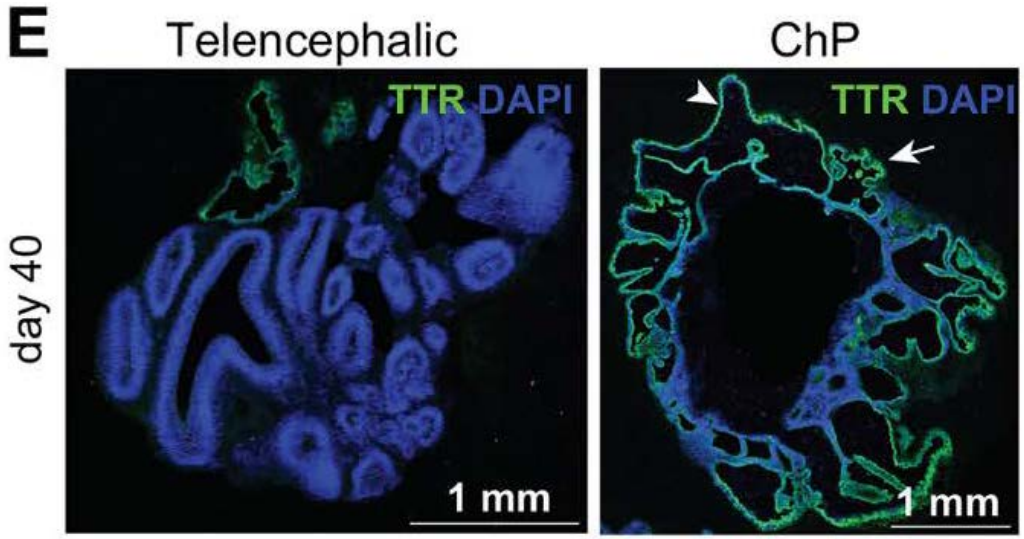
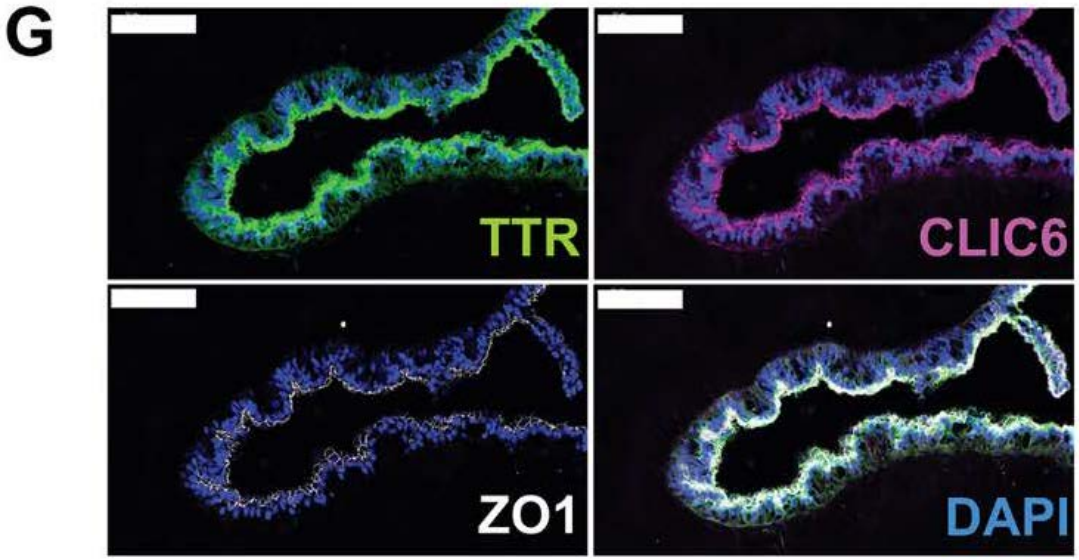
- ChP organoids contained cuboidal epithelium, fluid-filled cysts and choroid plexus-like morphology

ChP organoids: Markers (1)

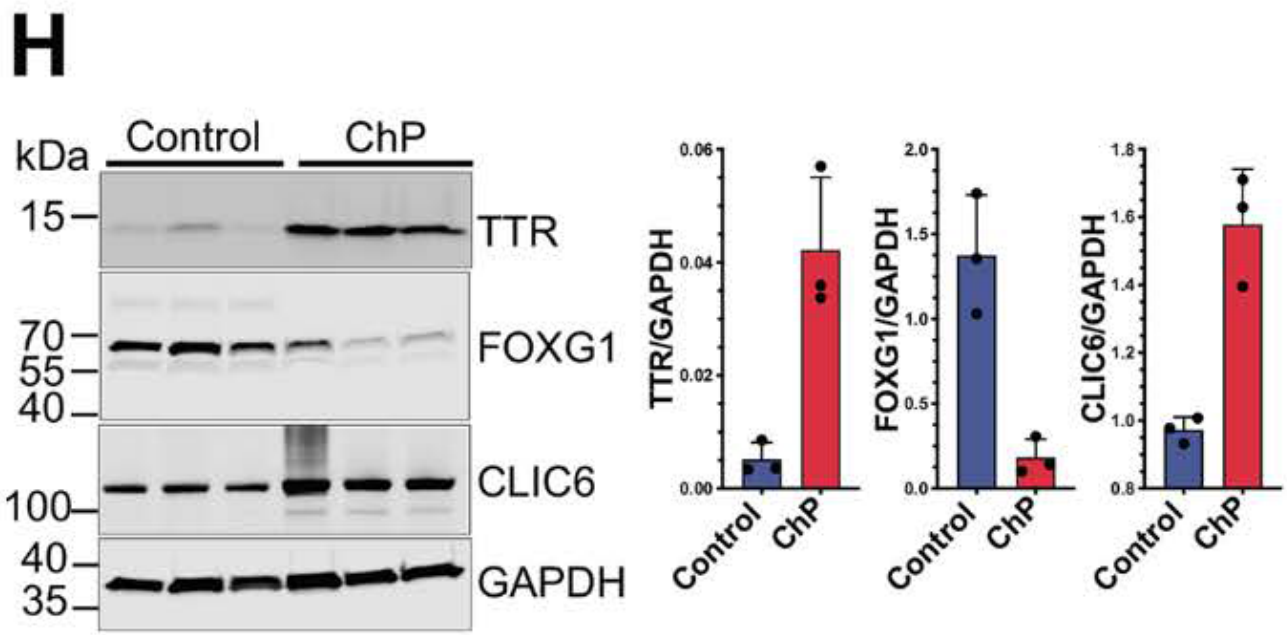
- ChP organoids express ChP markers (TTR, CLIC6) and the tight junction adhesion protein ZO1
- Low expression of neuronal markers (SOX2, DCX, not shown)





TTR: Transthyretin
CLIC6: Chloride intracellular channel 6
ZO1: Zonula occludens 1
SOX2: SRY-Box TF2
DCX: Doublecortin

ChP organoids: Markers (2)



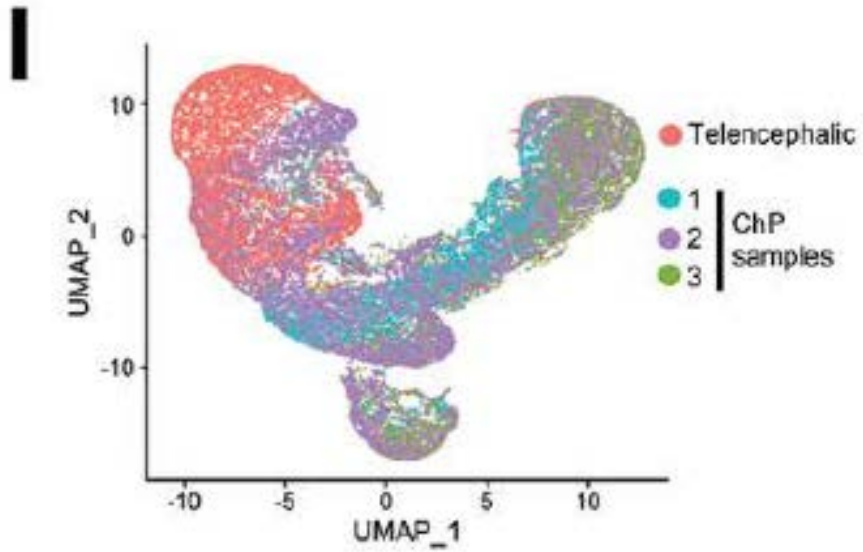
- Confirmation with Western blot and qPCR
- FOXG1: Marker of telencephalon

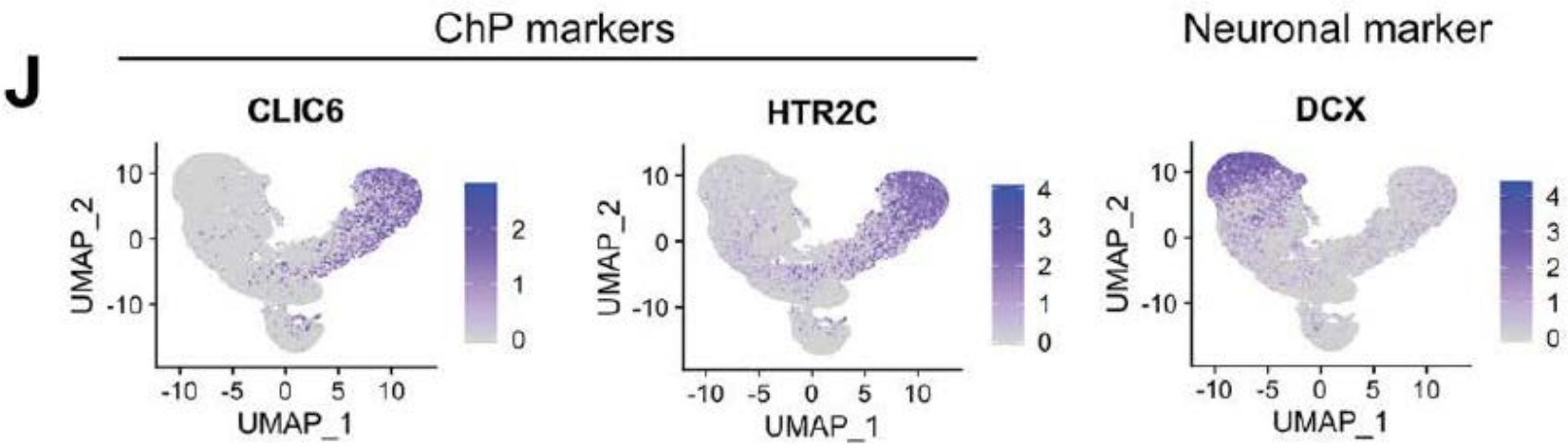
- Summary: reliable generation of ChP tissue in vitro with expected morphological and transcriptional features

TTR: Transthyretin
CLIC6: Chloride intracellular channel 6
FOXG1: Forkhead box 1

scRNA-seq of organoids (10x Chromium)

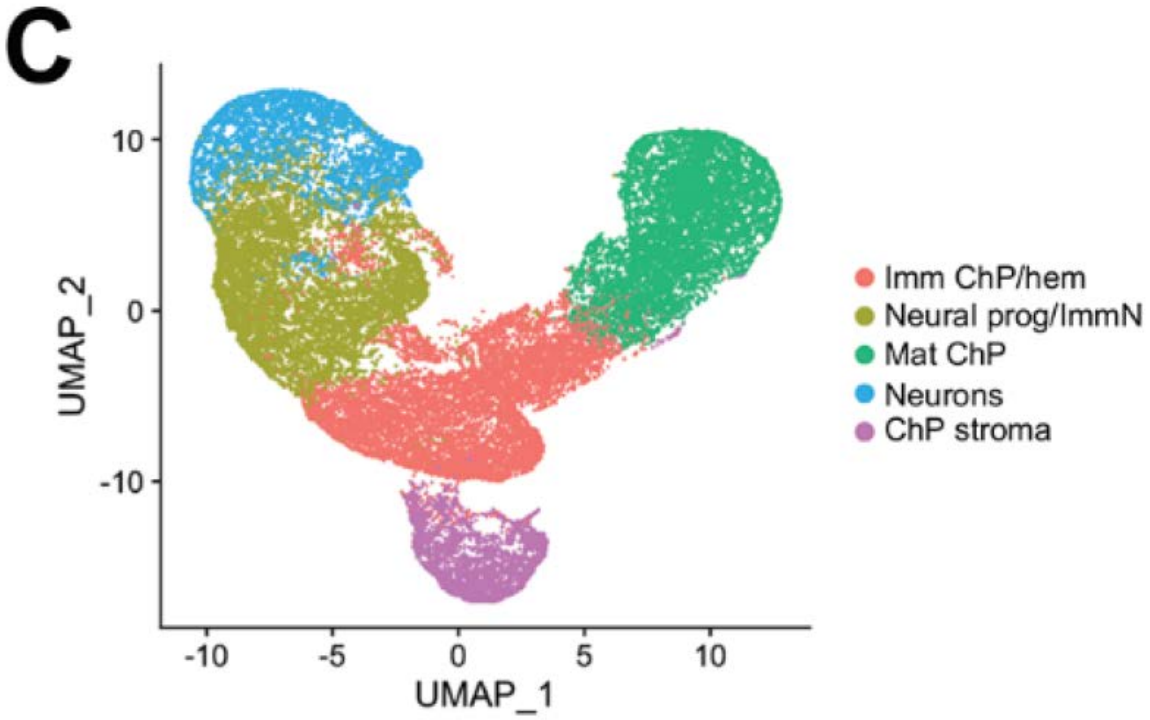
- Batches of ChP organoid cells reliably differ from telencephalic organoid cells
- Expected expression of telencephalic / ChP marker genes





HTR2C: 5-Hydroxytryptamine (Serotonin) Receptor 2C

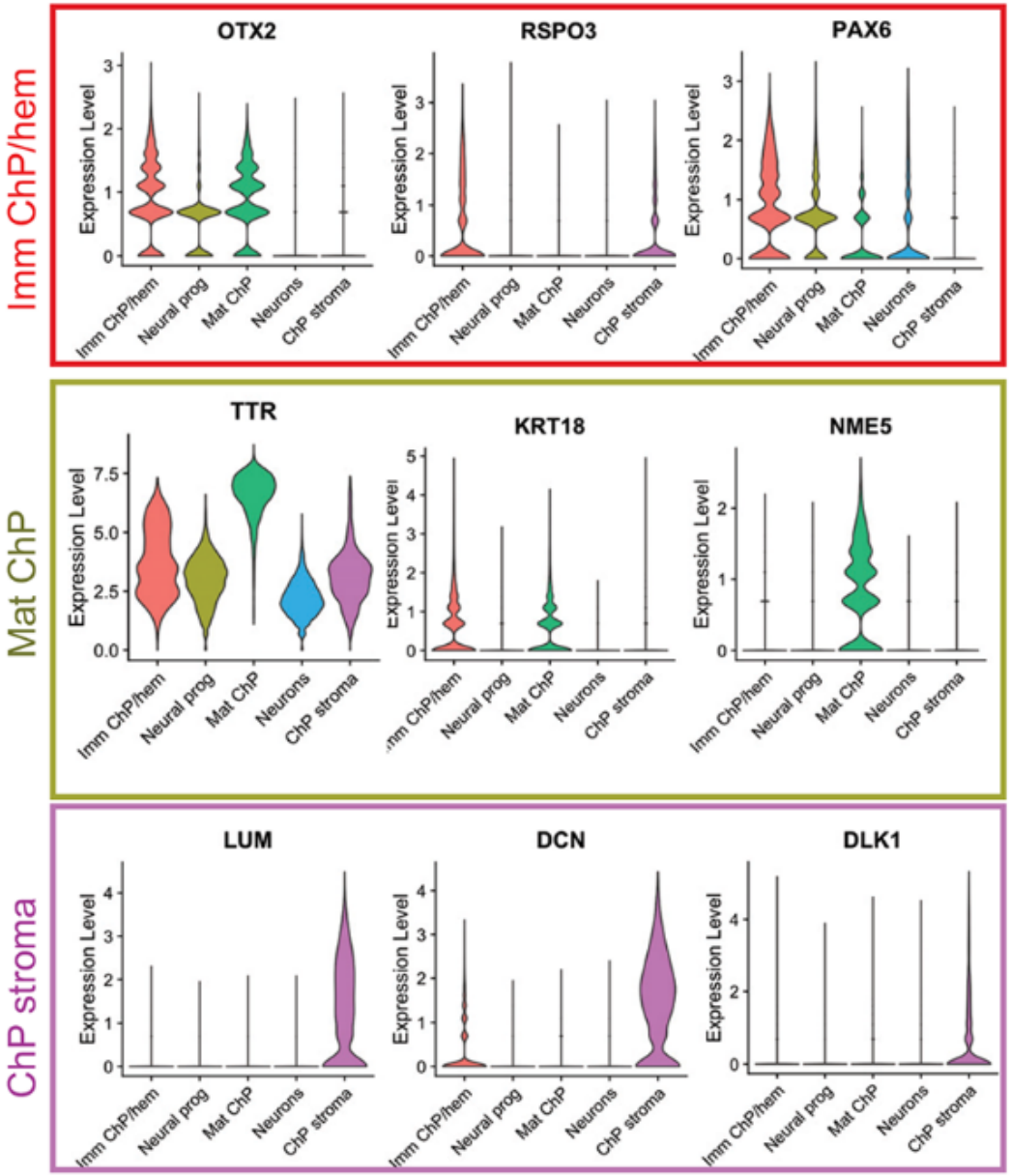
Cluster analysis reveals ChP cellular subtypes



1. Neurons
2. Immature ChP / cortical hem cells
3. Mature ChP
4. ChP stroma

Cluster analysis reveals ChP cellular subtypes

- Immature ChP / cortical hem cells (OTX2, RSPO3, PAX6)
- Mature ChP (TTR, KRT18, NME5)
- ChP stroma (LUM, DCN, DLK1)



OTX2: Orthodenticle homeobox 2

RSPO3: R-spondin-3 precursor

KRT18: Keratin 18

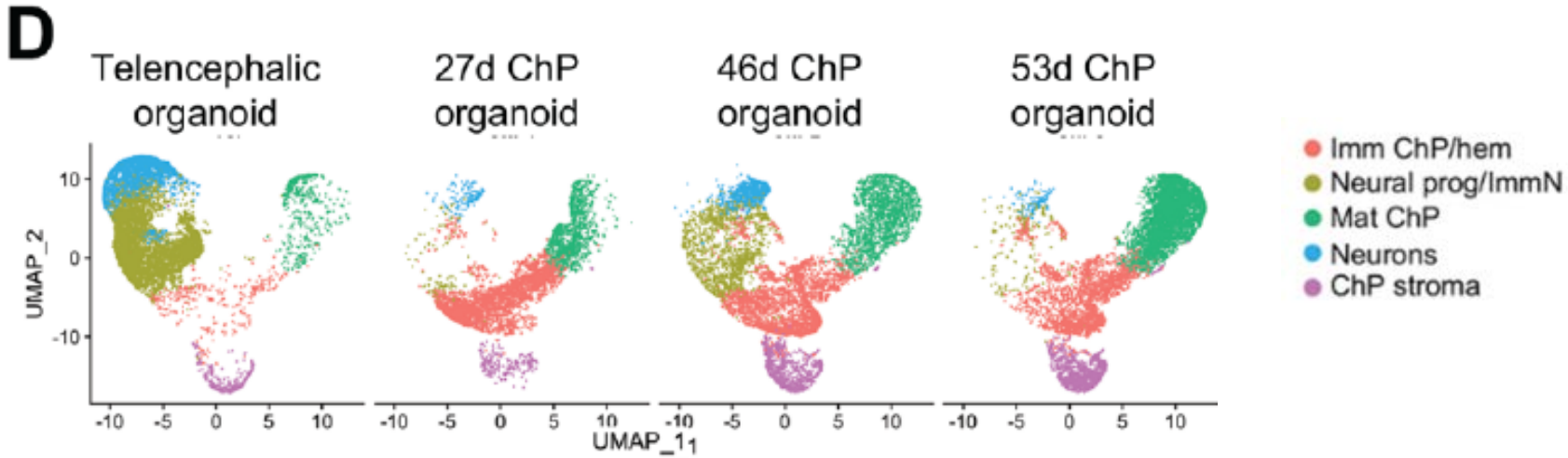
NME5: Nucleoside diphosph. kinase homol. 5

LUM: Lumican

DCN: Decorin

DLK1: Delta-like Notch ligand 1

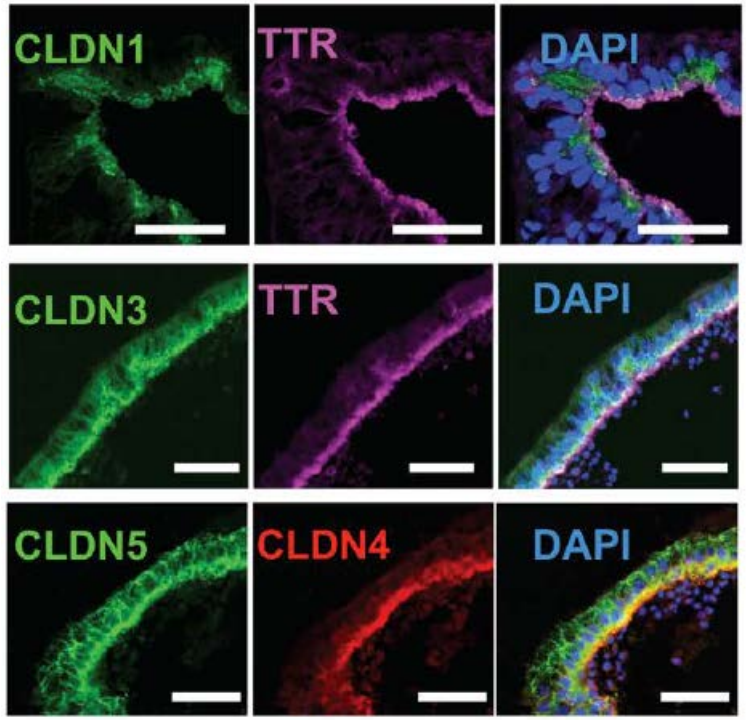
Cluster analysis: Temporal evolution and summary

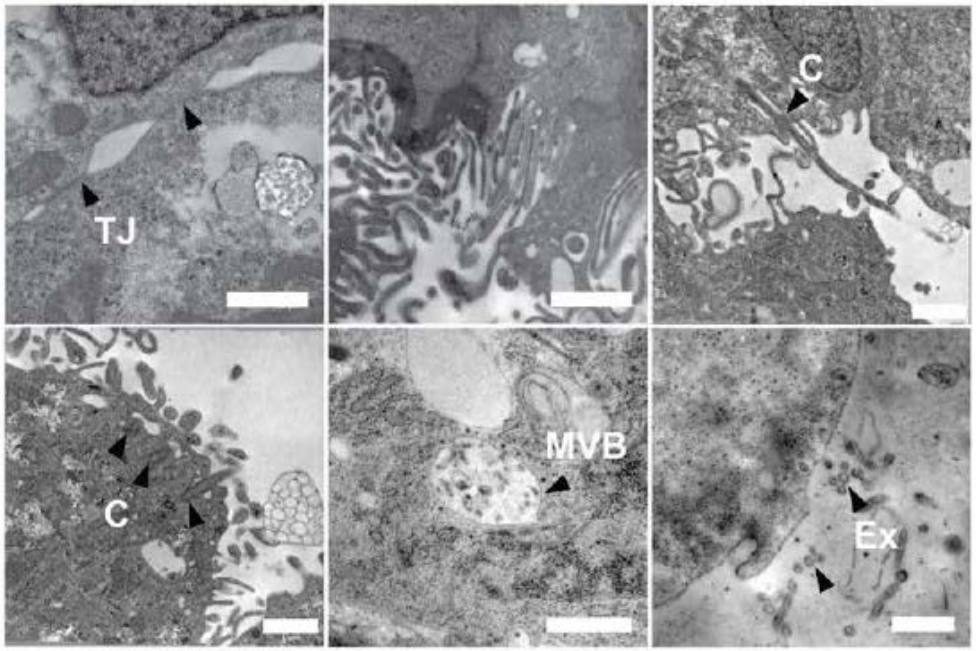


- Organoids contained immature cells expressing early regulatory factors involved in ChP development (OTX2, RSPO3) and neural/ChP progenitor marker PAX6
- Stromal cells with mesenchymal markers (COL1A1, DCN, LUM, DLK1)
- Temporal evolution from progenitors to mature ChP could be recreated in vitro
- Gene expression corresponds to in vivo lateral ventricle ChP (consistent with telencephalic origin, not shown)

COL1A1: Collagen Type 1 A1
DCN: Decorin

Formation of cell-cell junctions

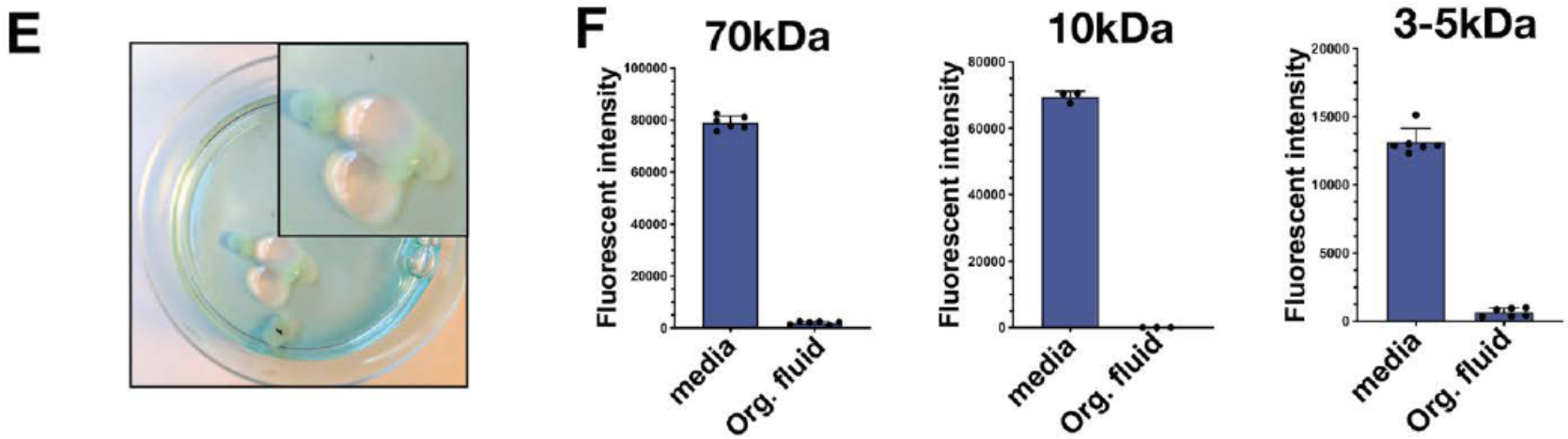




- Expression of Claudins and other tight junction proteins (immunofluorescence, scRNA-seq)

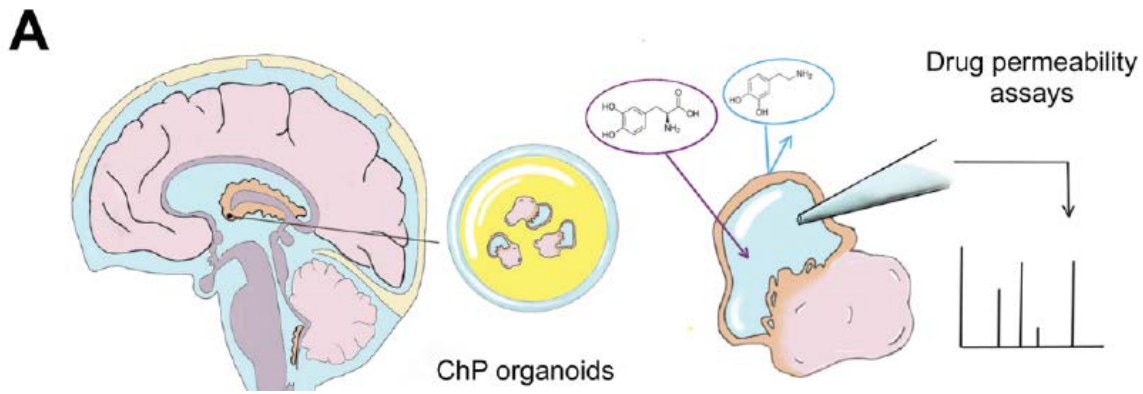
- EM: Tight juntions (TJ), primary cilia (C), microvilli, multi-vesicular bodies (MVB), extracellular vesicles (Ex)

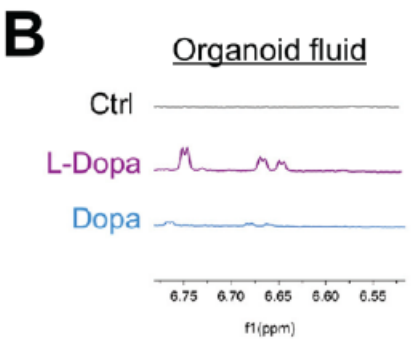
Intact barrier function in organoids

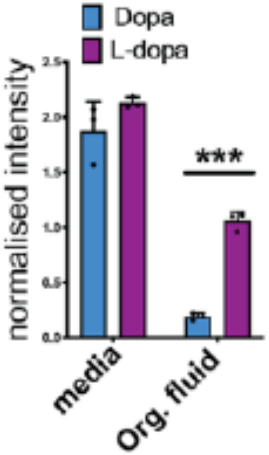


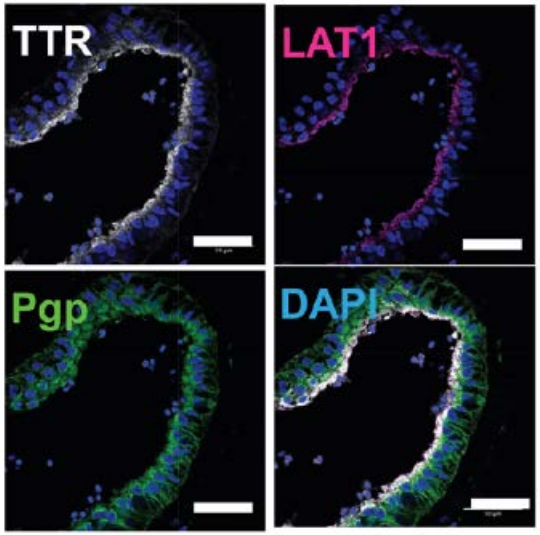
- Alexa647-labelled dextran (several mol. weights) does not diffuse into intra-organoid fluid compartments

ChP-like exclusion / transport of therapeutic molecules



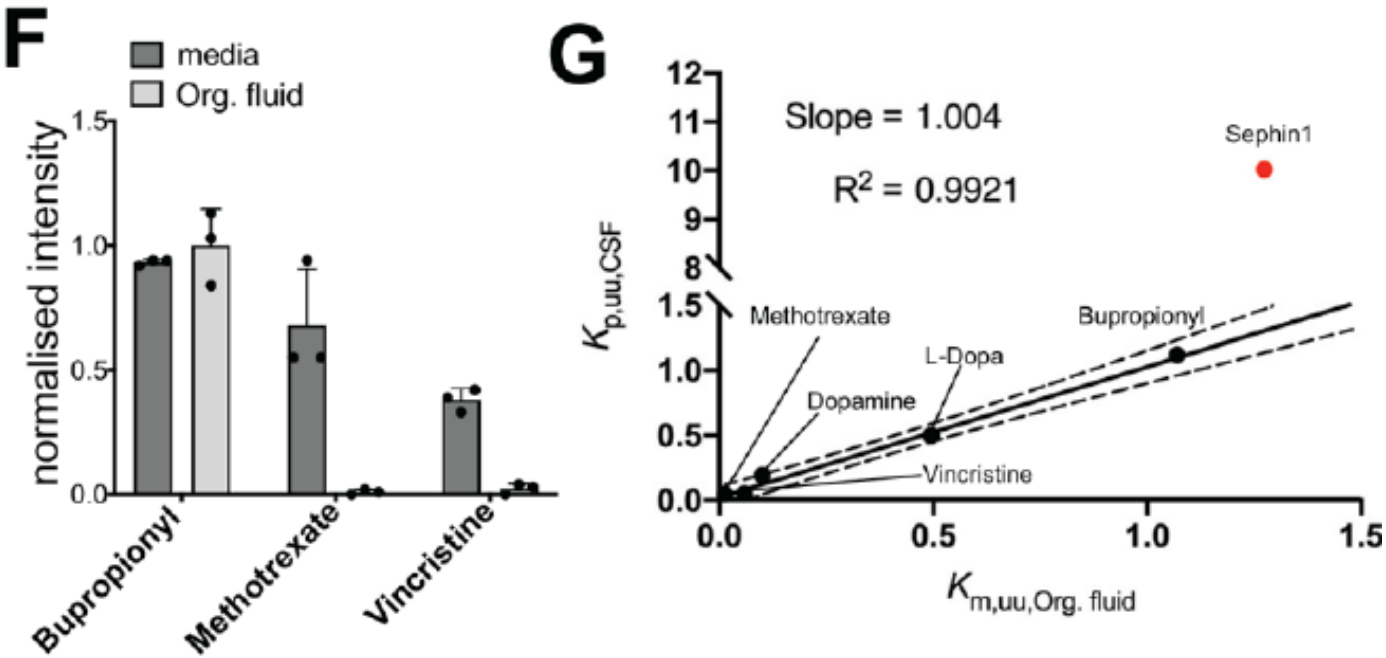






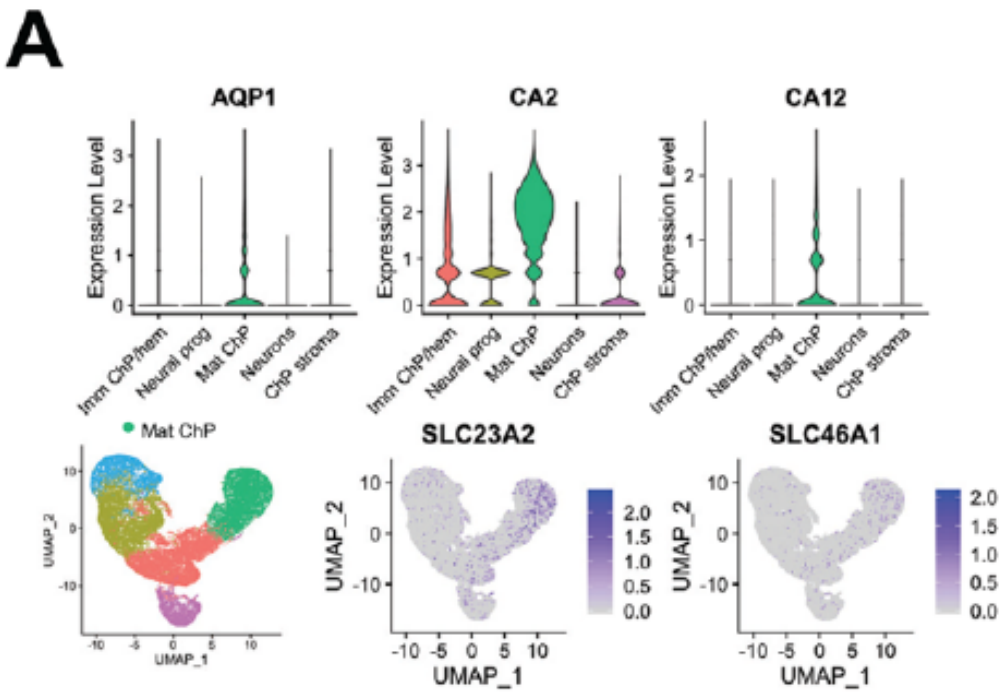
- Dopamine did not enter the organoid when injected into fluid compartment (NMR), whereas L-Dopa is probably transported by LAT1 (amino acid transporter)
- Efflux pumps were also expressed (Pgp, MRP1), probably pump molecules from CSF into blood in vivo

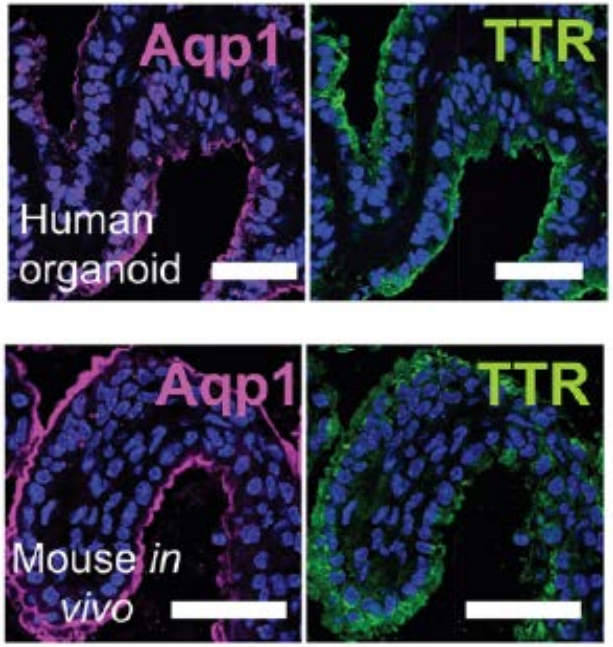
Transport of therapeutic molecules correlates with in vivo observations



- Bupropion (antidepressant, crosses BBB), methotrexate and vincristine (anti-cancer drugs, don’t cross BBB) diffuse as expected
- Drug concentrions in organoid fluid correlate with in vivo CSF concentrations
- Sephin1: experimental drug for Charcot-Marie-Tooth disease, human BBB permeability unknown (mouse data available), diffuses into organoid

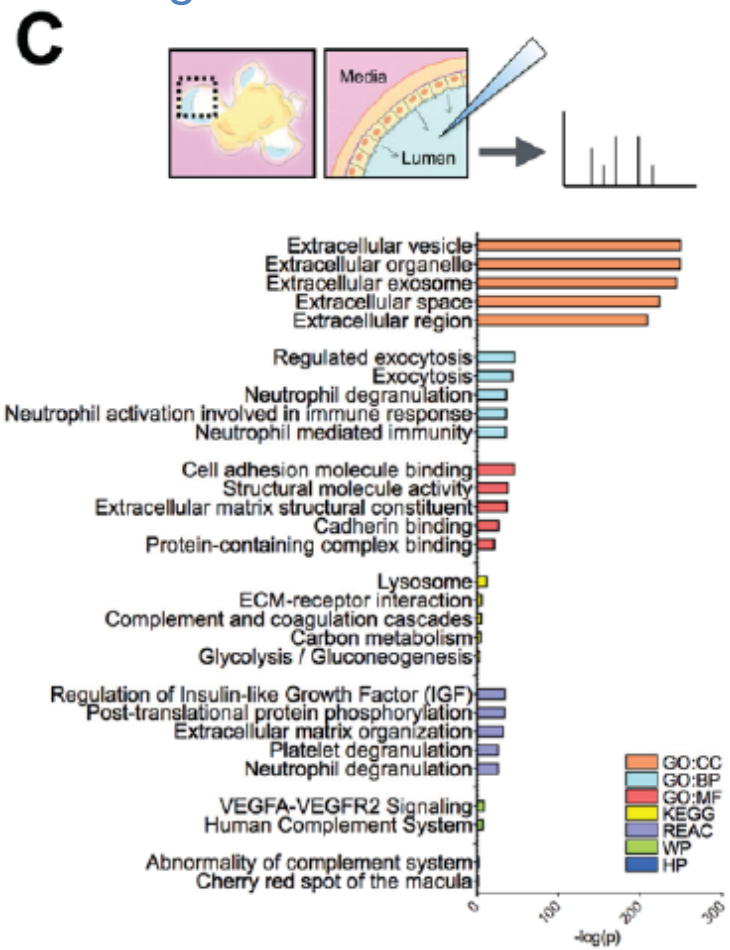
CSF production in organoids: Expression of required proteins

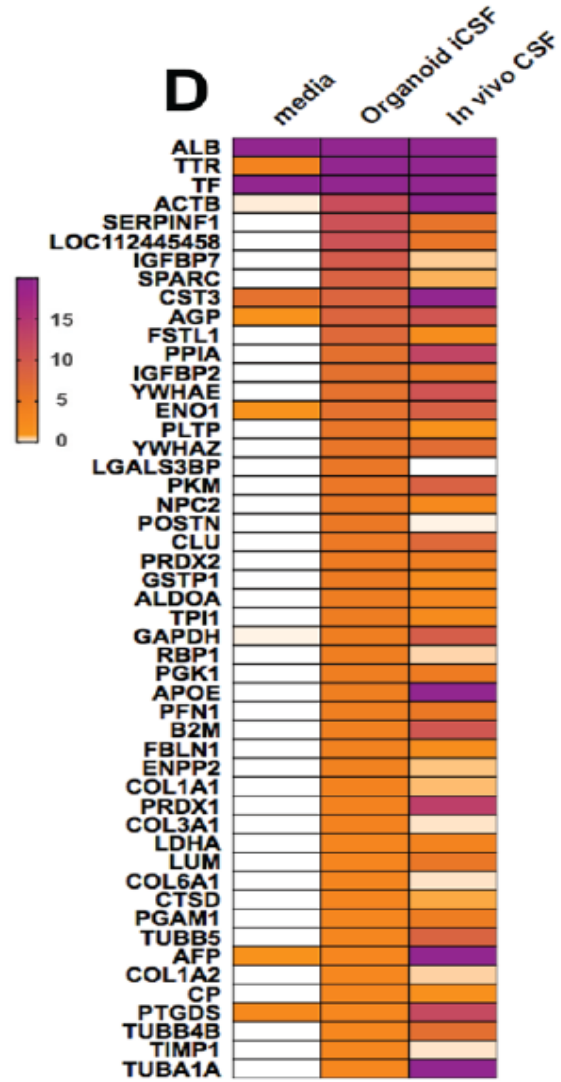




- Expression of Aquaporin 1, enzymes that drive CSF secretion gradient (Carbonic anhydrase 2 and 12) and ion channels

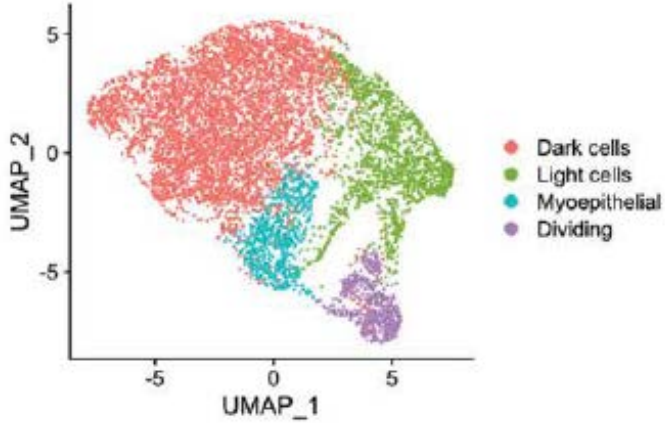
CSF production in organoids: Composition of organoid fluid

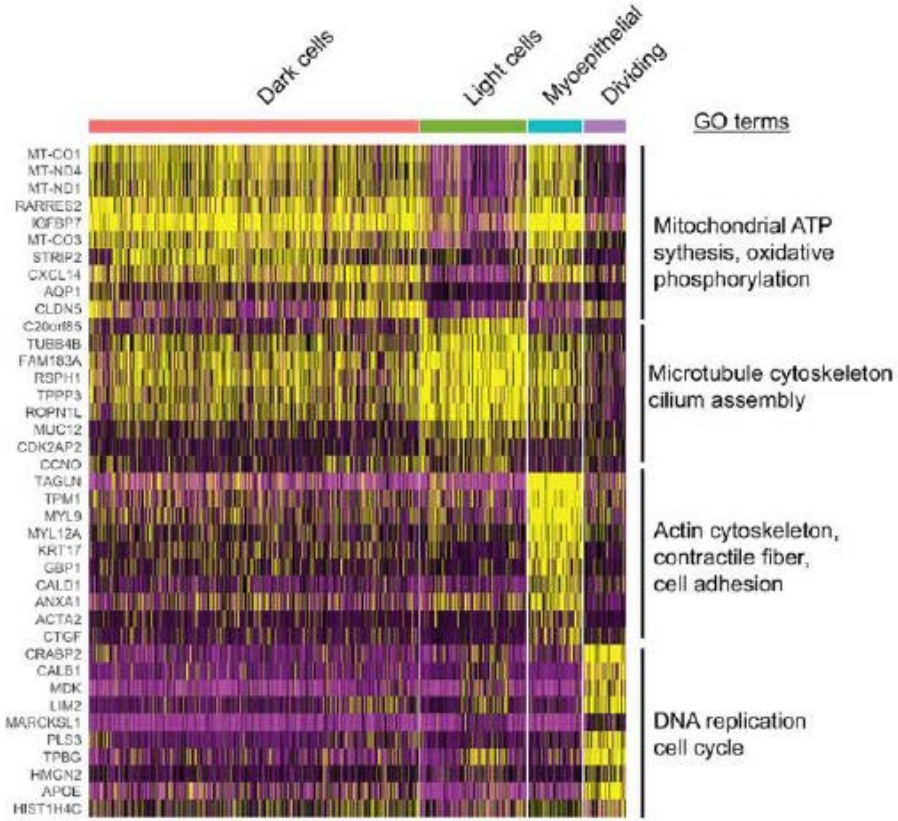




- Mass spectrometry, gene ontology analysis of detected proteins
  → Proteins related to vesicular transport, extracellular vesicles, strong overlap with in vivo CSF proteins
- Mixed ChP-neuronal organoids secreted a limited number of additional proteins, e.g. secretogranin-1 → probably of neuronal origin (whereas most CSF proteins come from ChP)

More ChP cell subtypes





- EM: «Dark» mitochondria-rich and «light» mitochondria-poor cells have been described within the ChP, but their significance is unclear
- Epithelial cells could be subclustered to clusters with high / low mitoch. gene expression, probably corresponding with dark / light cells
- Additionally, myoepithelial (express actin and myosin) and dividing cells (cell cycle markers) were found
- Several CSF proteins identified by mass spec were only expressed in dark / light cells, e.g. IGF2 in dark cells.

Summary

- ChP organoids were created by modifying the cerebral organoid protocol, using factors known to influence ChP differentiation in vivo
- ChP organoids show ChP-like morphology, intact barrier function and secrete CSF-like fluid
- Diffusion properties of certain drugs in organoids match known in vivo pharmacokinetics → might allow modelling
- Origin of CSF proteins was traced to ChP or neurons
- Identification of novel cell subtypes, presumably also found in vivo

nature methods

ARTICLES

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

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Limitations of cortical organoids

- Lack functional blood vessels
- Limited formation of microglia and six distinct cortical layers
- Millimetre-scale organoids under long-term culture exhibit apoptotic cell death at the inner-most regions
- Lack of vasculature impairs differentiation of neuronal progenitors

But organoids can become vascularised: Human brain organoids transplated on to the cortex of living mice induced the outgrowth of murine vessels in to the human tissue (Mansour et al. Nat Biotechnol 2018).

Method

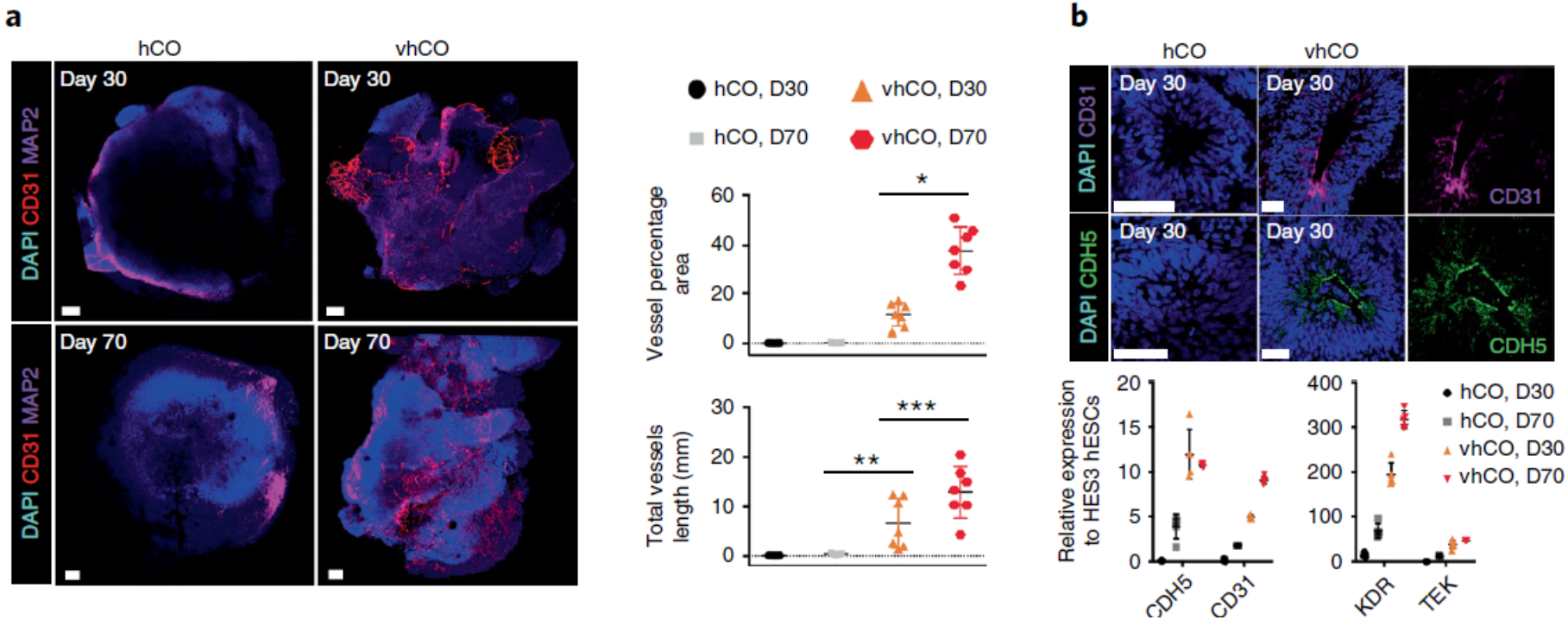
- The expression of the transcription factor *ETV2* (*ETS variant 2*) reprograms human dermal fibroblasts into endothelial cells (ECs)

- hESCs are transduced with *ETV2* lentiviral construct (dox-inducible) → standard cortical organoid protocol
- *ETV2* is activated by adding doxycycline
- (Vitamin A growth medium was supplemented with BDGF and ascorbic acid)

- Overexpression of *ETV2* triggers EC differentiation in hESCs regardless of prior differentiation conditions, as shown in:
  1. Embryoid body differentiation
  2. Neuronal differentiation
  3. EC differentiation

hESC: human embryonic stem cells
BDNF: Brain-derived neurotrophic factor

Organoids are vascularised

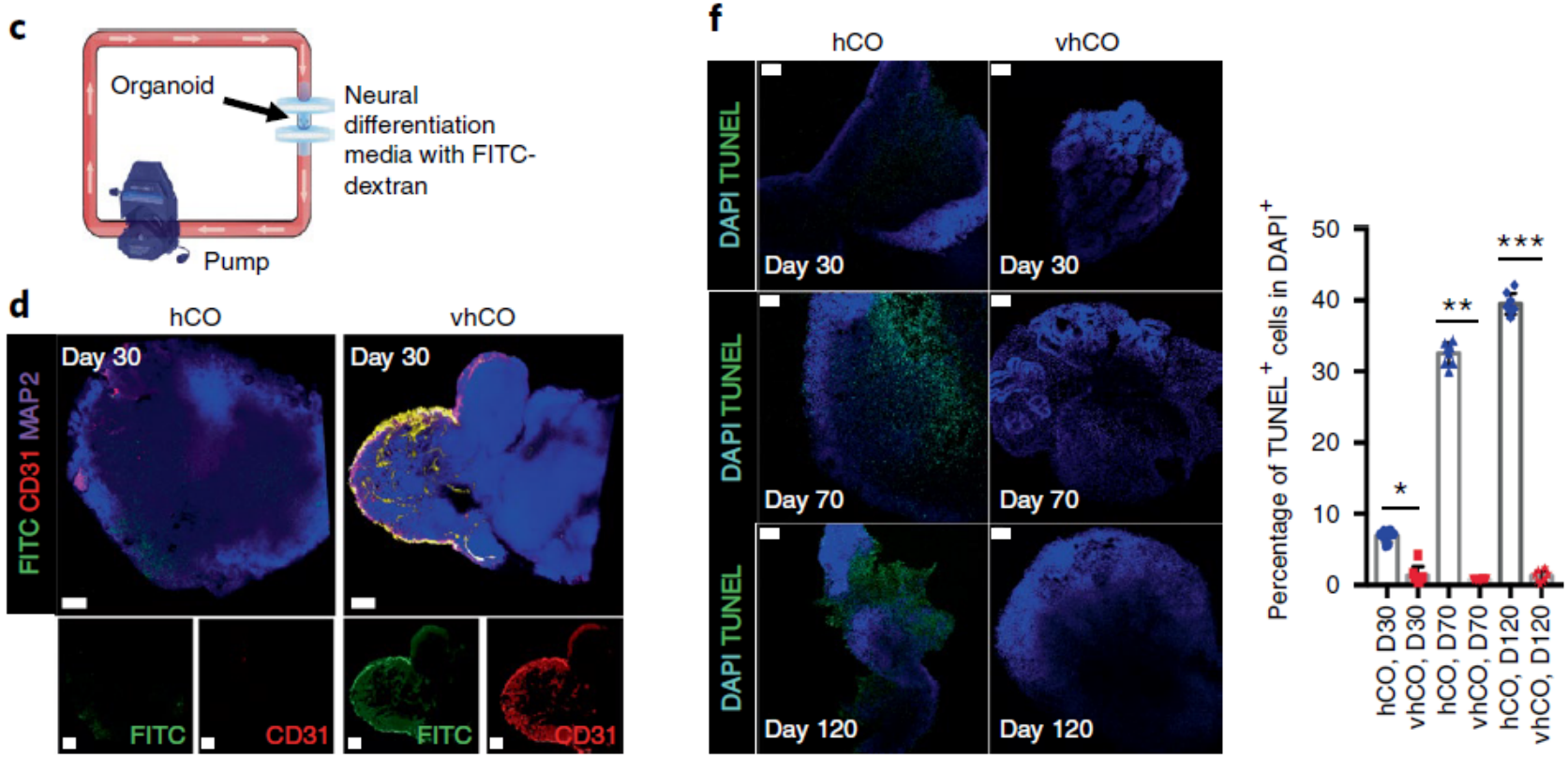


- *ETV2* expression induces formation of CD31+ and CDH5+ blood vessels (markers of endothelium)
- On day 70, vessel network is larger and more complex than on day 30
- Expression of endothelial markers increases over time (qPCR)

hCO: human cortical organoid
vhCO: vascularised human cortical organoid

CDH5: Cadherin 5
KDR: VEGF receptor 2
TEK: Angiopoietin-1 receptor

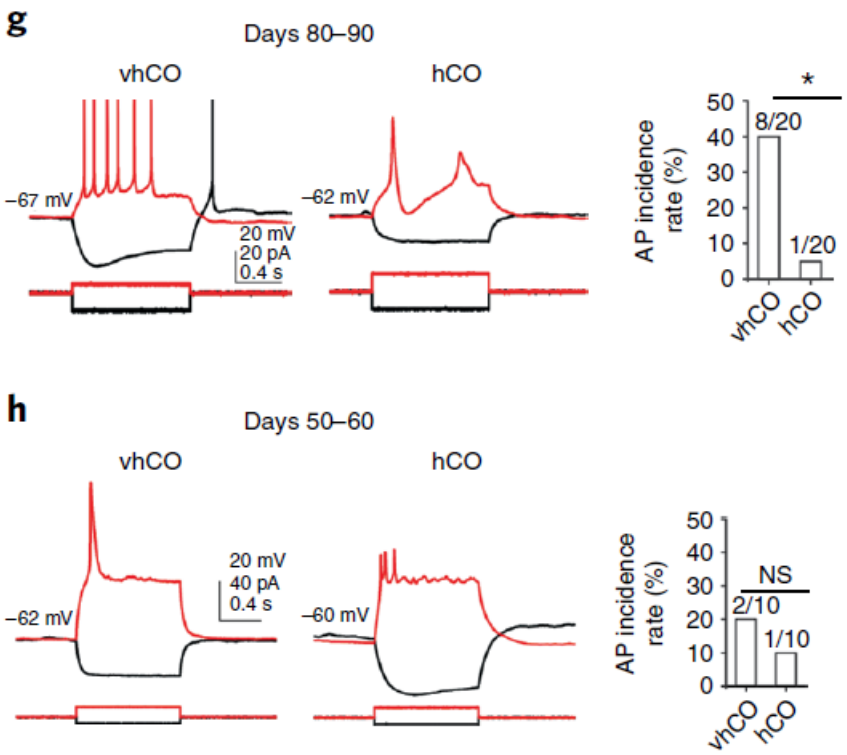
vhCO can be perfused



- Organoids were perfused with FITC-Dextran, followed by whole-mount immunostaining → FITC detected in vasculature

- TUNEL cell-death stain increased over time in control organoids, but not in vhCO
- *HIF1a* expression was also decreased in vhCO (not shown)

vhCO neurons are more likely to produce action potentials



- Depolarising current pulses were more likely to induce APs in vhCO neurons
- In regular organoids (d 80-90) only 1 out of 20 studied cells produced an AP

scRNA-seq (day 70)

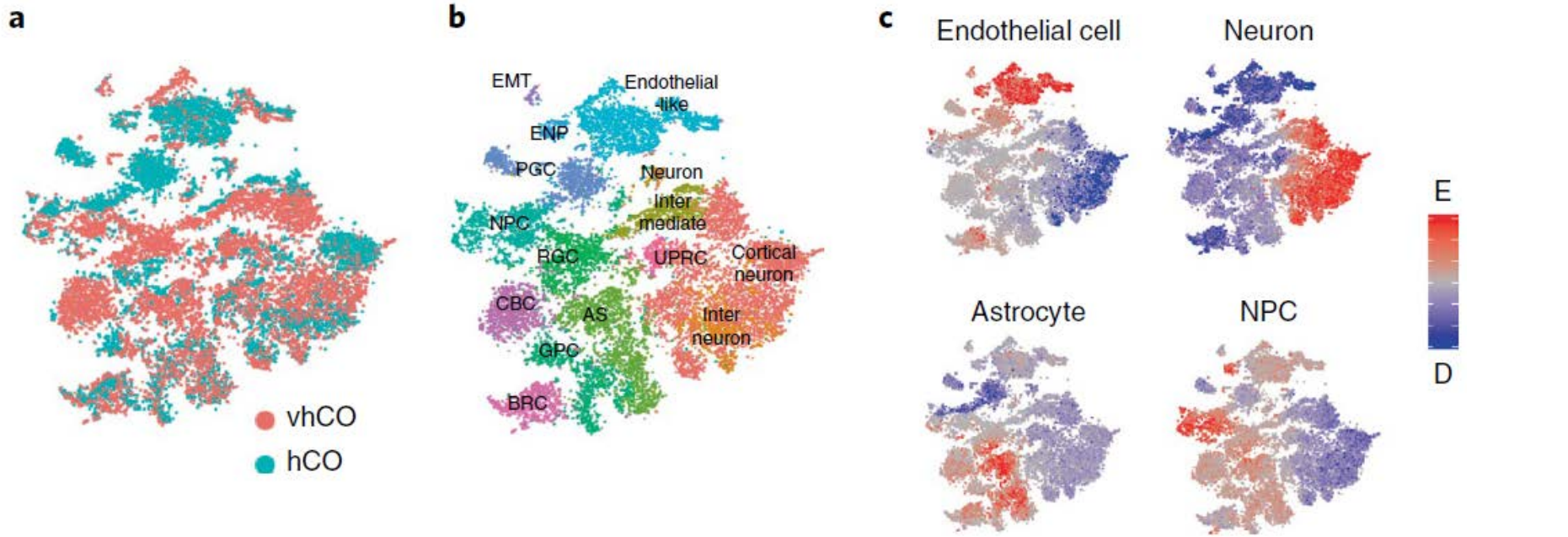
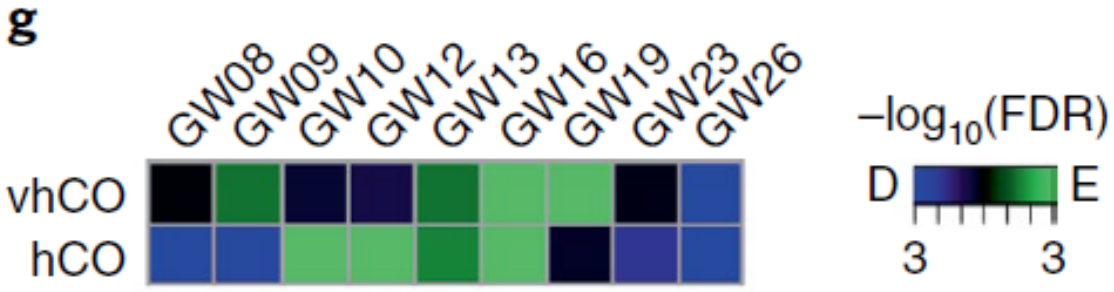


Fig. 2 | Single-cell analysis of vhCOs. a,**b**, tSNE plot of single cells distinguished by organoid type (**a**) and cell annotation (**b**). RGC, radial glia cell; GPC, glia progenitor cell; NPC, neuronal progenitor cell; CBC, Cilium-bearing cell; BRC, BMP signal-related cell; EN, endothelial-like cell; ENP, endothelial-like progenitor; PGC, proteoglycan-expressing cell; EMT, epithelial-mesenchymal transition-related cell and UPRC, unfolded protein response-related cell. Data depicts results from 20,026 cells. **c**, Enrichment of gene signatures for ECs, astrocytes, neurons and NPCs. Data depicts results from 20,026 cells

- Endothelial-like cells were found both in control and vascularised hCOs
- But vhCOs expressed **higher levels of vasculogenesis-related genes** (FLT1, MME) and pericyte genes

FLT1: VEGF receptor 1
MME: Neprilysin (metallo metalloendopeptidase)

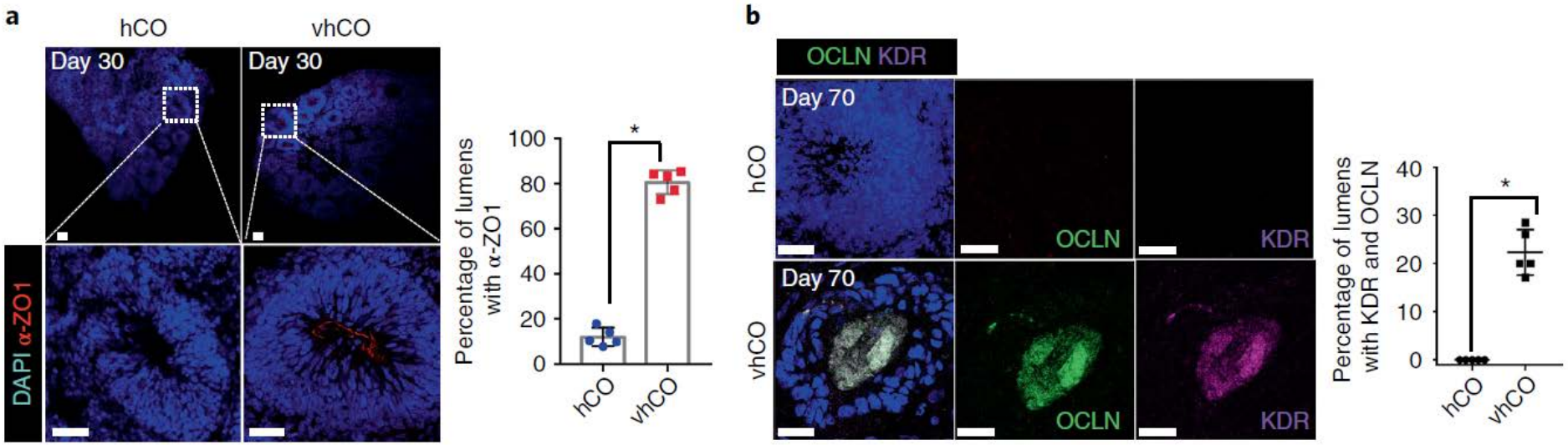
scRNA-seq (day 70)



Comparison with developing human brain time points (GW: gestational week)

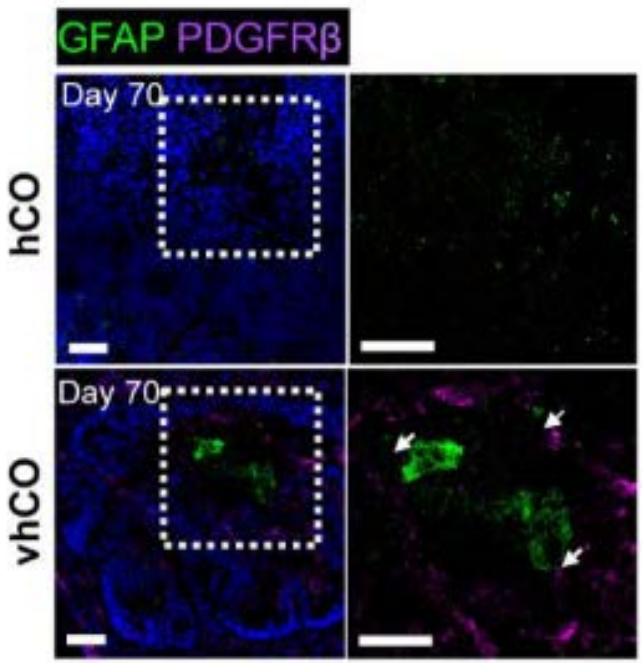
- vhCO cells resemble GW13-19, whereas hCO resemble GW10-16
- Suggests that vascularisation aided maturation

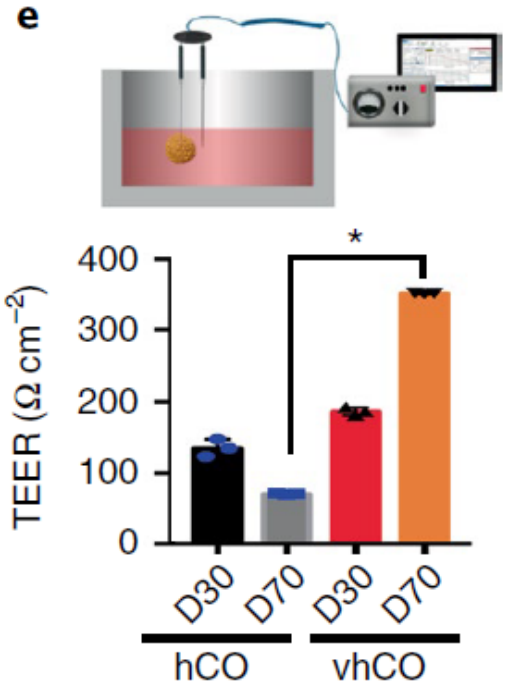
vhCOs exhibit BBB-like characteristics (1)



- In lumina, vhCO cells express tight junction protein ZO1 (IHC, qPCR), Occludin (OCLN) and VEGFR2 (KDR)

vhCOs exhibit BBB-like characteristics (2)





- In lumina, vhCO cells express tight junction protein ZO1 (IHC, qPCR), Occludin (OCLN) and VEGFR2 (KDR)
- vhCO lumina are surrounded by GFAP+ glia and PDGFRβ+ pericytes
- Barrier function is increased, as determined by Transendothelial electrical resistance measurements (tight junctions increase resistance)

Summary

- Vascularised cortical organoids were created by infecting EC cultures with lentivirus containing inducible *ETV2* construct
- *ETV2*-reprogrammed EC cells in organoid culture formed a vasculature-like network
- Vascular network aided organoid differentiation and prevented hypoxia and cell death within inner tissues
- Vasculature contained tight junctions and formed BBB-like structures with astrocytes and pericytes

Thank you for your attention