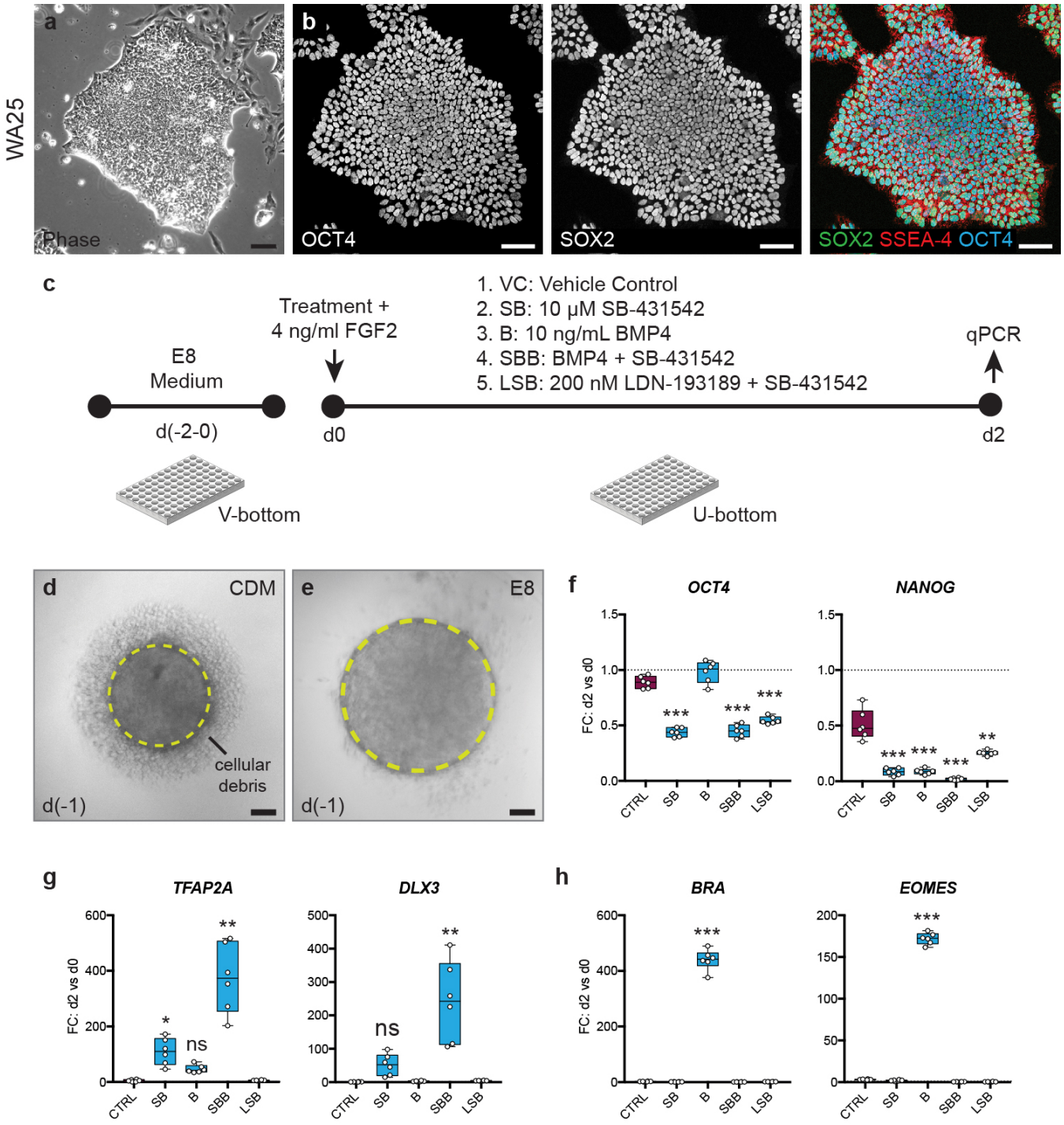
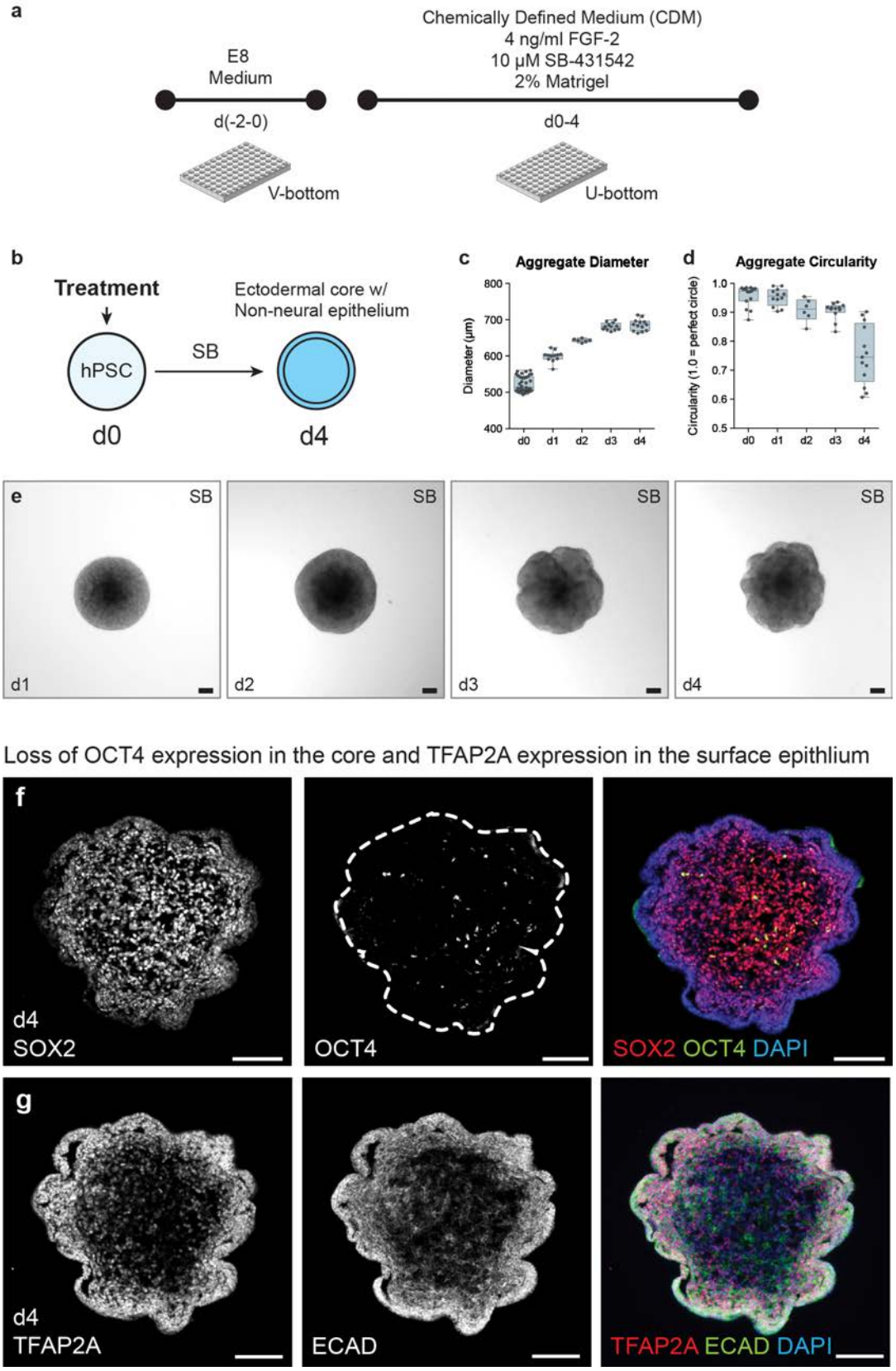
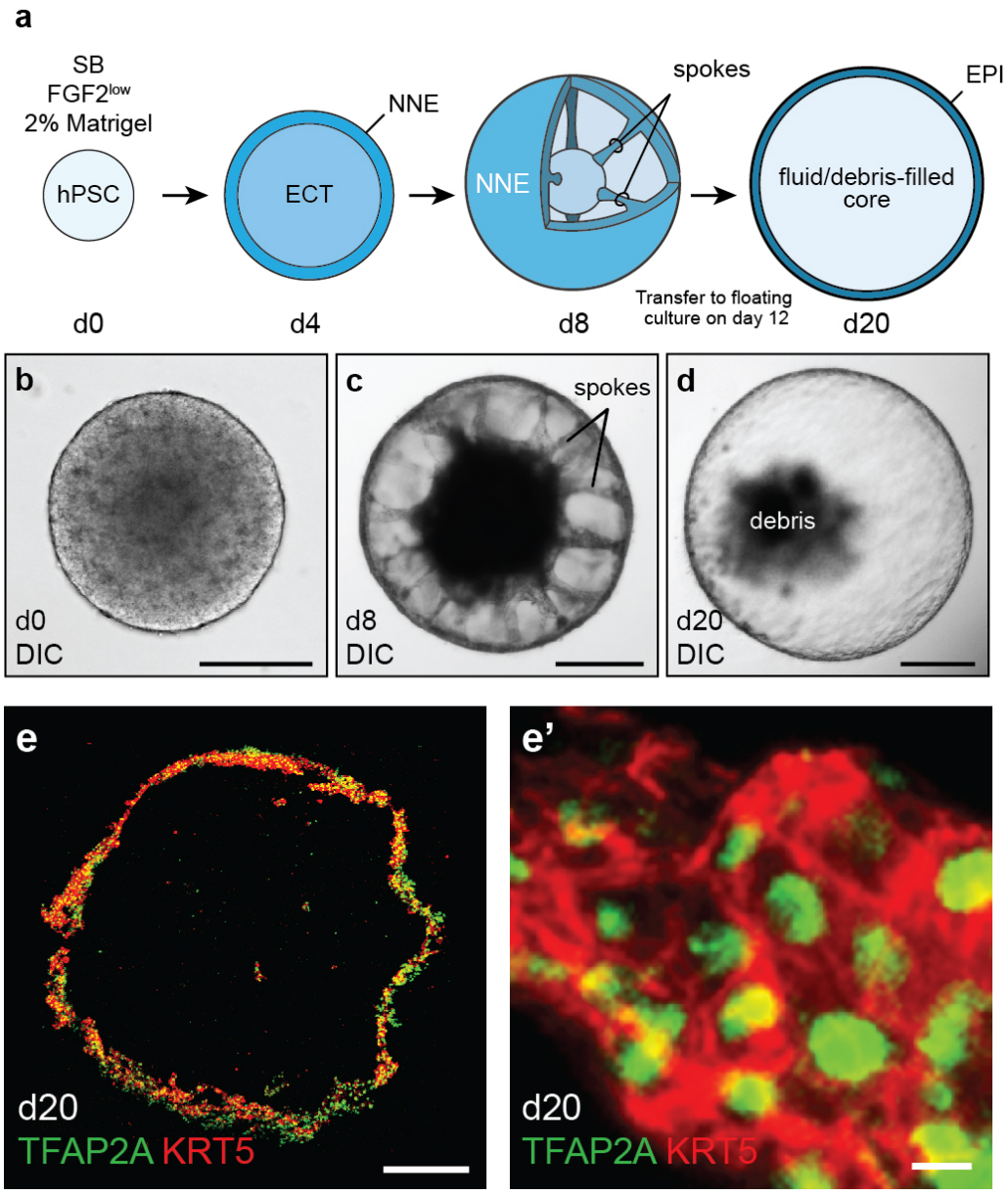
SUPPLEMENTARY FIGURES AND LEGENDS



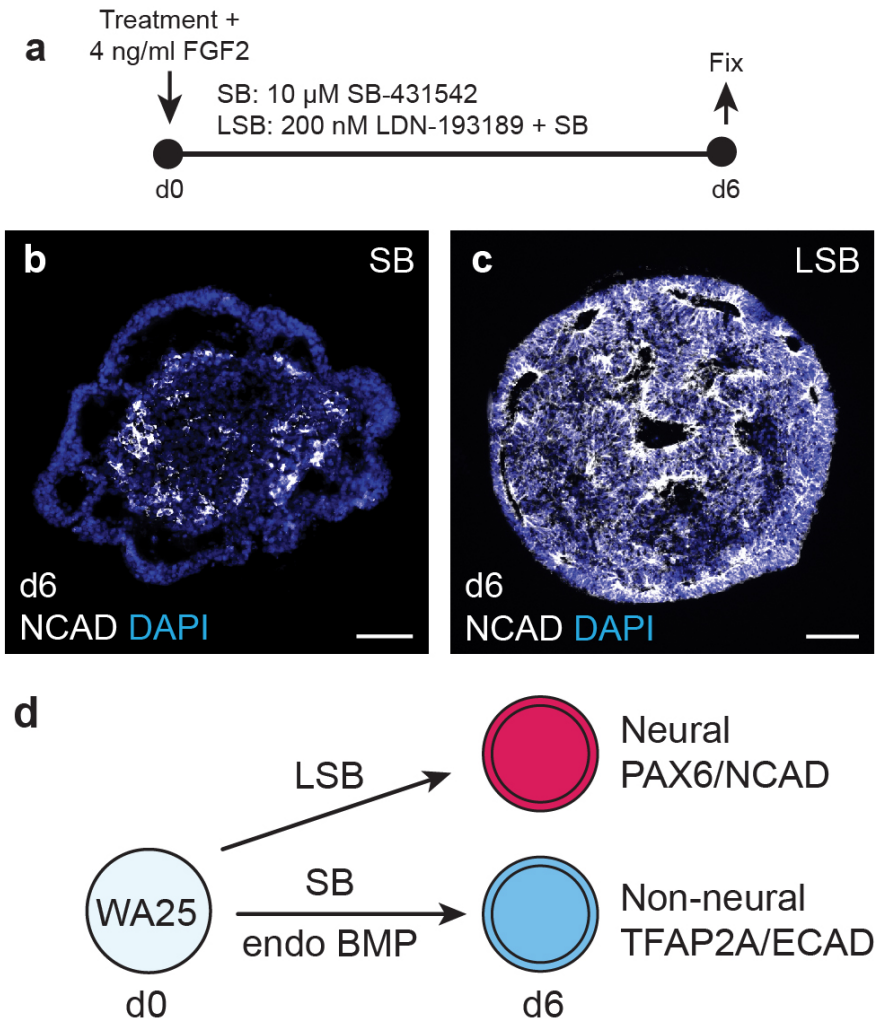
Supplementary Figure 1: Undifferentiated WA25 hESCs, cell aggregation, and initial non-neural ectoderm differentiation analysis. a, **b**, WA25 cells maintained on Vitronectin-N-coated plates in E8 medium express markers of primed pluripotent stem cells. **c**, Overview of differentiation strategy and experimental conditions. **d**, **e**, Aggregation of single-celled hESCs in E8 + 20 µM Y-27632 produced less cellular debris than aggregation in CDM + 20 µM Y-27632. **f**, Relative to undifferentiated cells, pluripotency markers were significantly down-regulated by day 2 in all conditions except vehicle control. **g**, The non-neural markers *TFAP2* and *DLX3* were upregulated in SB and SBB conditions. **h**, The mesendoderm markers *BRA* and *EOMES* were upregulated by BMP4 (B) treatment. Gene expression was normalized to d0 aggregates. For statistical tests treatment values were compared to control (CTRL) values; *n* = 3 biological samples, 2 technical replicates; **P*<0.05, ***P*<0.01, ****P*<0.001, ns = not significant; error bars are max/min. Scale bars, 100 µm (**d, e**), 25 µm (**a, b**).



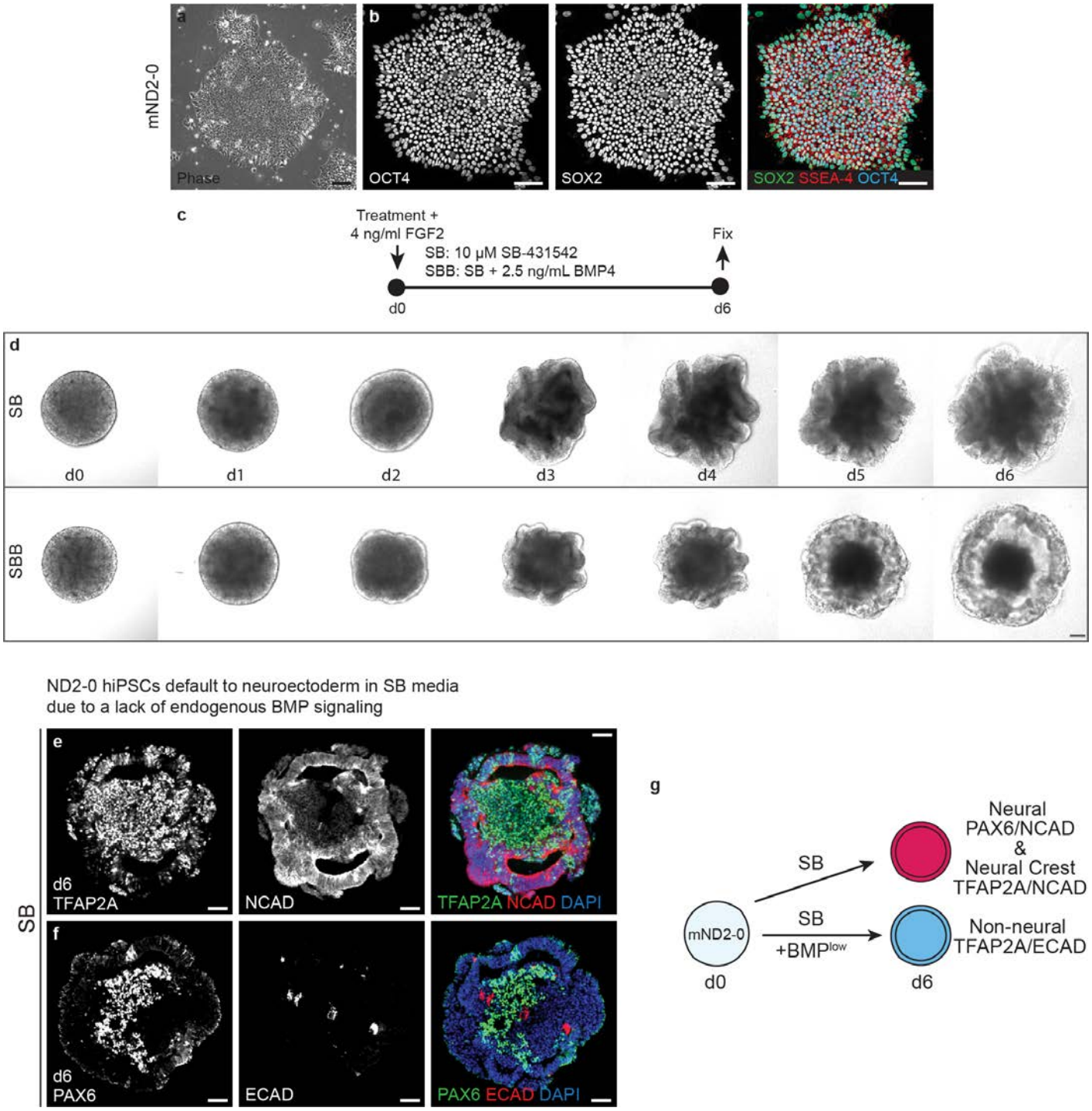
Supplementary Figure 2: Non-neural induction using WA25 hESCs. a, b, Overview of differentiation strategy. **c-e**, Aggregate diameter (**c**) and circularity (**d**) over time in culture. The circularity of the aggregates decreases overtime as the outer epithelium crumples (**e**). **f**, **g**, Representative day 4 aggregate showing a nearly complete lack of OCT4-expressing cells, SOX2-expressing cells in the core, and TFAP2/ECAD-expressing cells in the outer-core and epithelium. Error bars are max/min. Scale bars, 100 µm.



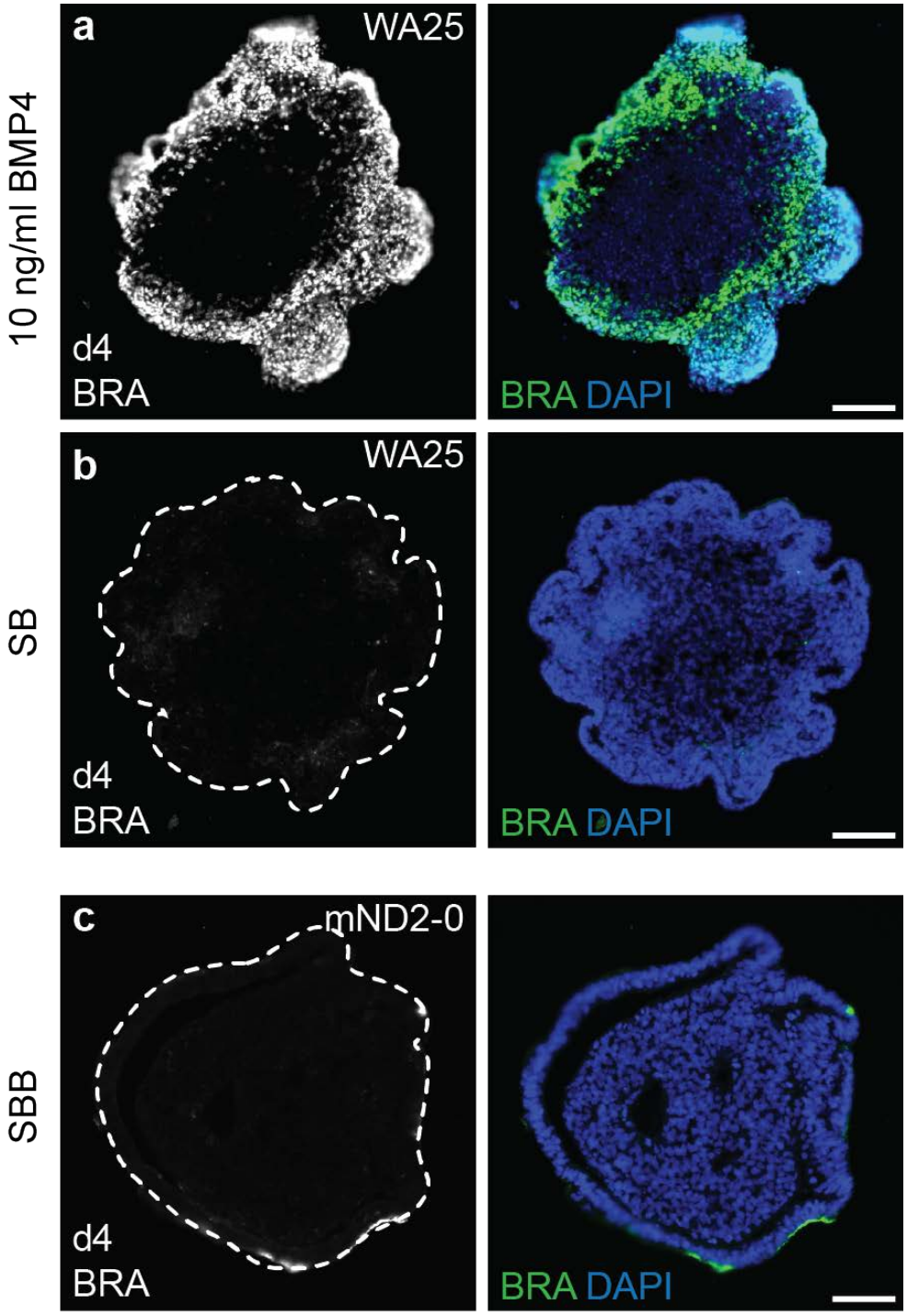
Supplementary Figure 3: SB-treated aggregates generate keratinocytes. a, Overview of non-neural and keratinocyte induction process. **b-d**, In 3D culture, non-neural ectoderm induction is accompanied by characteristic morphological changes. By days 6-8, the epithelium separates from the core, forming a translucent sphere (**c**). The epithelium remains connected to the core via spoke-like structures (**a**, **c**). After 20 days, spokes from the core are absent, and the epithelial sphere is typically filled with cellular debris (**d**). **e**, The day 20 epithelium contains TFAP2A^{+} KRT5^{+} cells, indicative of epidermal keratinocytes. Scale bars, 250 μm (**b-d**), 100 μm (**e**), 5 μm (**e'**).



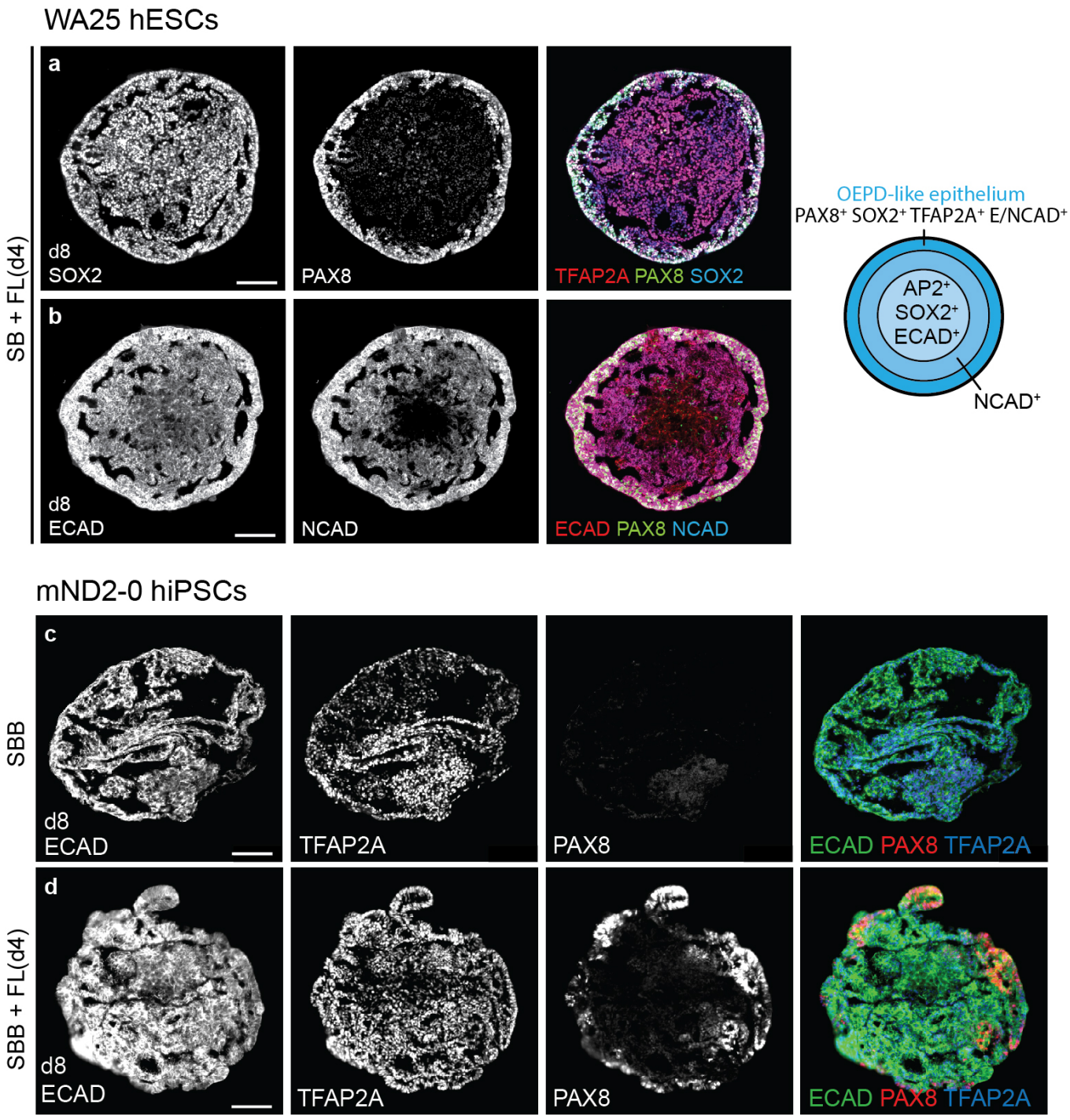
Supplementary Figure 4: Non-neural ectoderm induction in SB-treated WA25 aggregates is due to endogenous BMP signaling. a, Overview of experiment to test whether endogenous BMP signaling influence non-neural induction. **b-d**, LSB treatment leads to NCAD expression throughout the aggregates. Note that a subpopulation of NCAD^{+} PAX6^{+} cells (**Fig. 1e**) do appear in the core of SB treated aggregates. Scale bars, 50 µm.



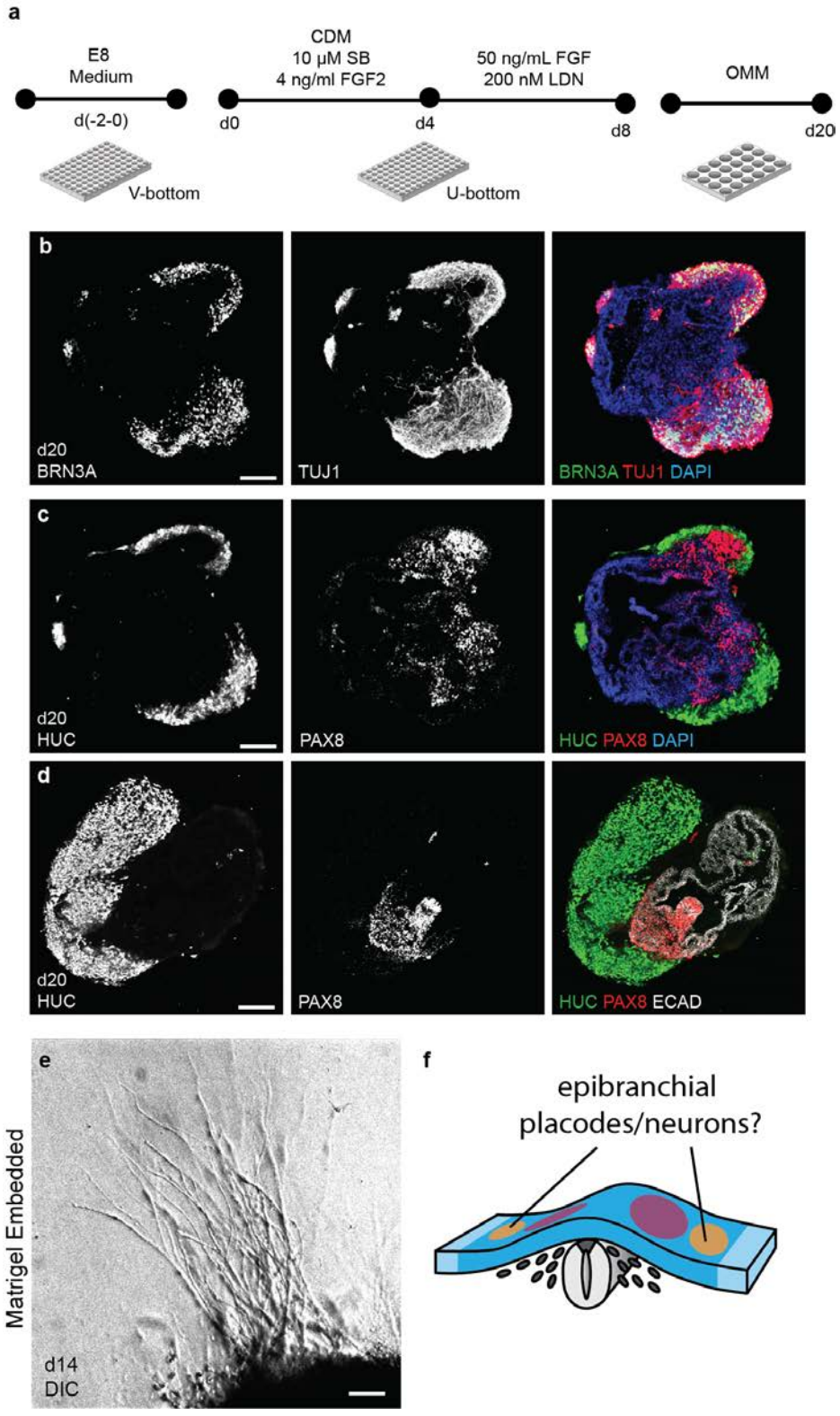
Supplementary Figure 5: Optimization of non-neural ectoderm induction with mND2-0 iPSCs. a, **b**, mND2-0 cells maintained on Vitronectin-N-coated plates in E8 medium express markers of primed pluripotent stem cells. **c**, Overview of differentiation strategy and experimental conditions. Other BMP concentrations were tested in a preliminary experiment (1.25, 2.5, 5, 10, 20, 40 ng/ml), and 2.5 ng/ml was selected as the minimum concentration that produced the morphological changes (i.e. translucent sphere) seen in SB-treated mND2-0 cells (see **Supplementary Fig. 3c**). **d**, Representative images of SB- or SBB-treated aggregates between days 0-6. **e**, **f**, SB-treated aggregates generate PAX6^{+} NCAD^{+} epithelia, TFAP2+ migratory cells, and few ECAD^{+} cells by day 6, suggesting a heterogeneous mix of neuroectoderm and neural crest cells. **g**, Endogenous BMP signaling is insufficient for non-neural conversion in mND2-0 iPSCs; thus, additional BMP4 is necessary. Scale bars, 100 µm (**d**), 25 µm (**a, b, e, f**).



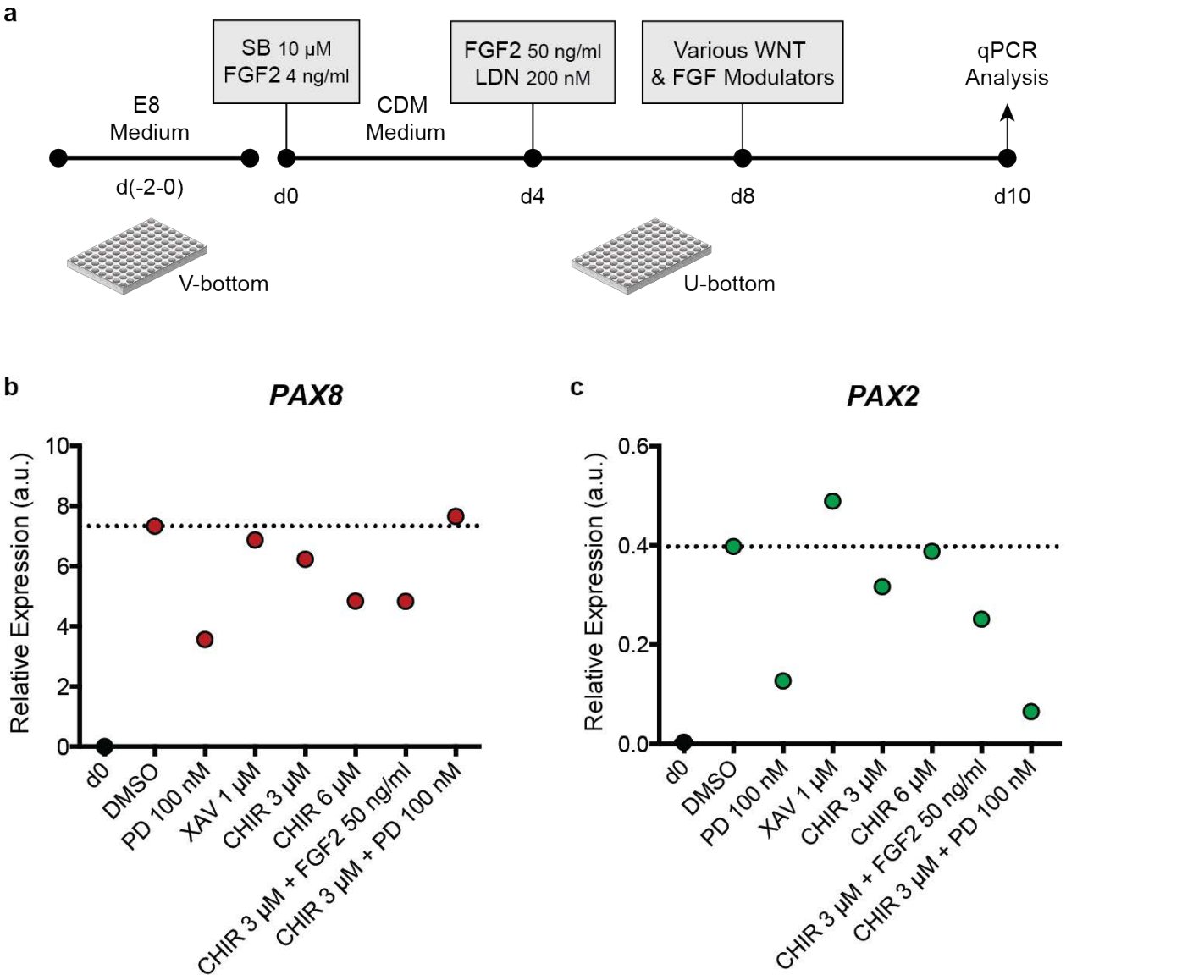
Supplementary Figure 6: Non-neural ectoderm induction occurs without off-target induction of mesendodermal cells. Representative Brachyury (BRA) IHC in day 4 aggregates treated with 10 ng/ml BMP4 (**a**), 10 μM SB (**b**), and 10 μM SB + 2.5 ng/ml BMP4 (**c**) on day 0. Scale bars, 50 μm.



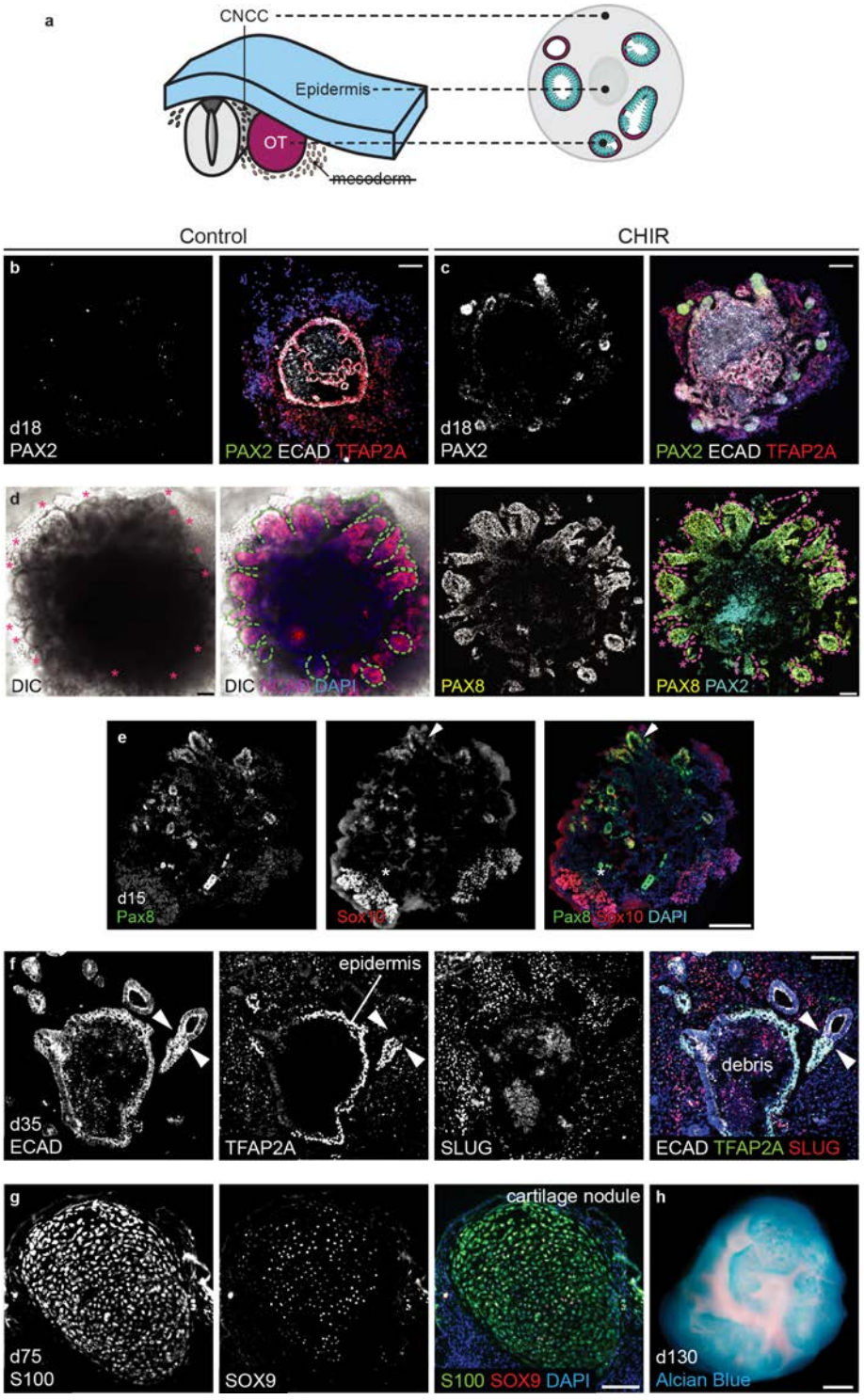
Supplementary Figure 7: Induction of OEPD-like epithelium in WA25 hESC and mND2-0 iPSC aggregates by FGF-2 and LDN treatment. a, **b**, SOX2, TFAP2, and ECAD are expressed throughout SB-treated WA25 aggregates on day 8 following FL treatment on day 4. PAX8 expression is restricted to the outer-epithelium. A unique characteristic of the epibranchial and otic placodes is the co-expression of ECAD and NCAD. Here, NCAD expression was observed throughout the aggregate, except for the interior-most core. **c**, **d**, iPSCs treated with SBB never express PAX8. FL treatment on day 4 induces a thicker outer-epithelium morphology and expression of PAX8. ECAD and TFAP2 are expressed throughout the SBB+FL(d4)-treated iPSC aggregates. Scale bars, 100 μm.



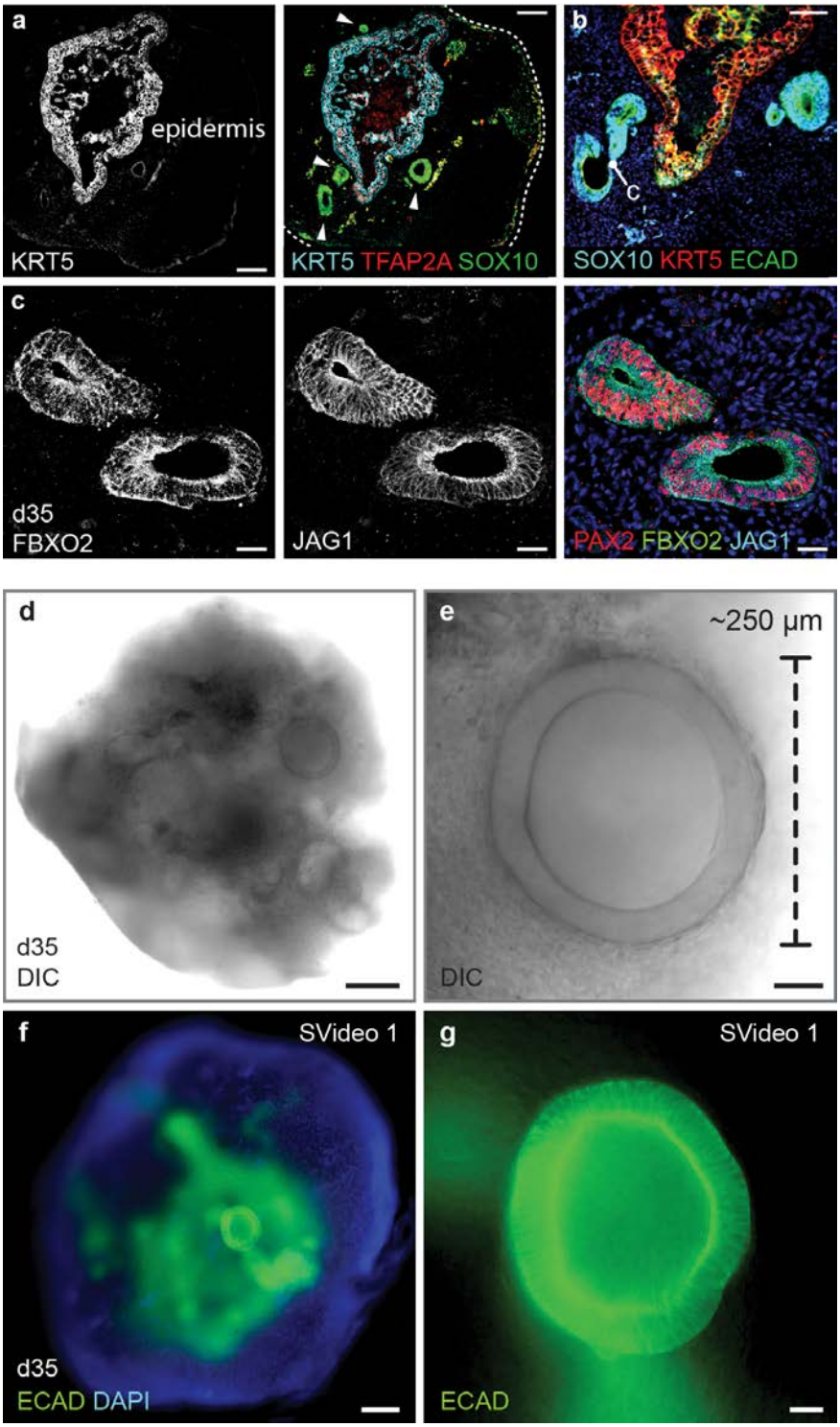
Supplementary Figure 8: OEPD induced aggregates spontaneously generate sensory-like neurons in a minimal medium floating culture. a, Overview of the experiment. SBFL-treated aggregates were transferred to OMM on day 8 of differentiation. **b-d**, On day 20, the aggregates are composed of patches of BRN3A^{+} TUJ1^{+} HUC^{+} neurons surrounding a ECAD+ epithelium. Neuronal patches were typically associated with PAX8^{+} epithelium. When aggregates were plated in Matrigel droplets they produced neurite outgrowths (**e**). **f**, BRN3A^{+} neurons emerging from a PAX8^{+} ECAD^{+} epithelium is consistent with epibranchial placode neurogenesis; however, these data *do not* directly establish the PAX8^{+} ECAD^{+} epithelium as the origin of the sensory neurons. Scale bars, 100 um (**b, c, d**), 50 µm (**e**).



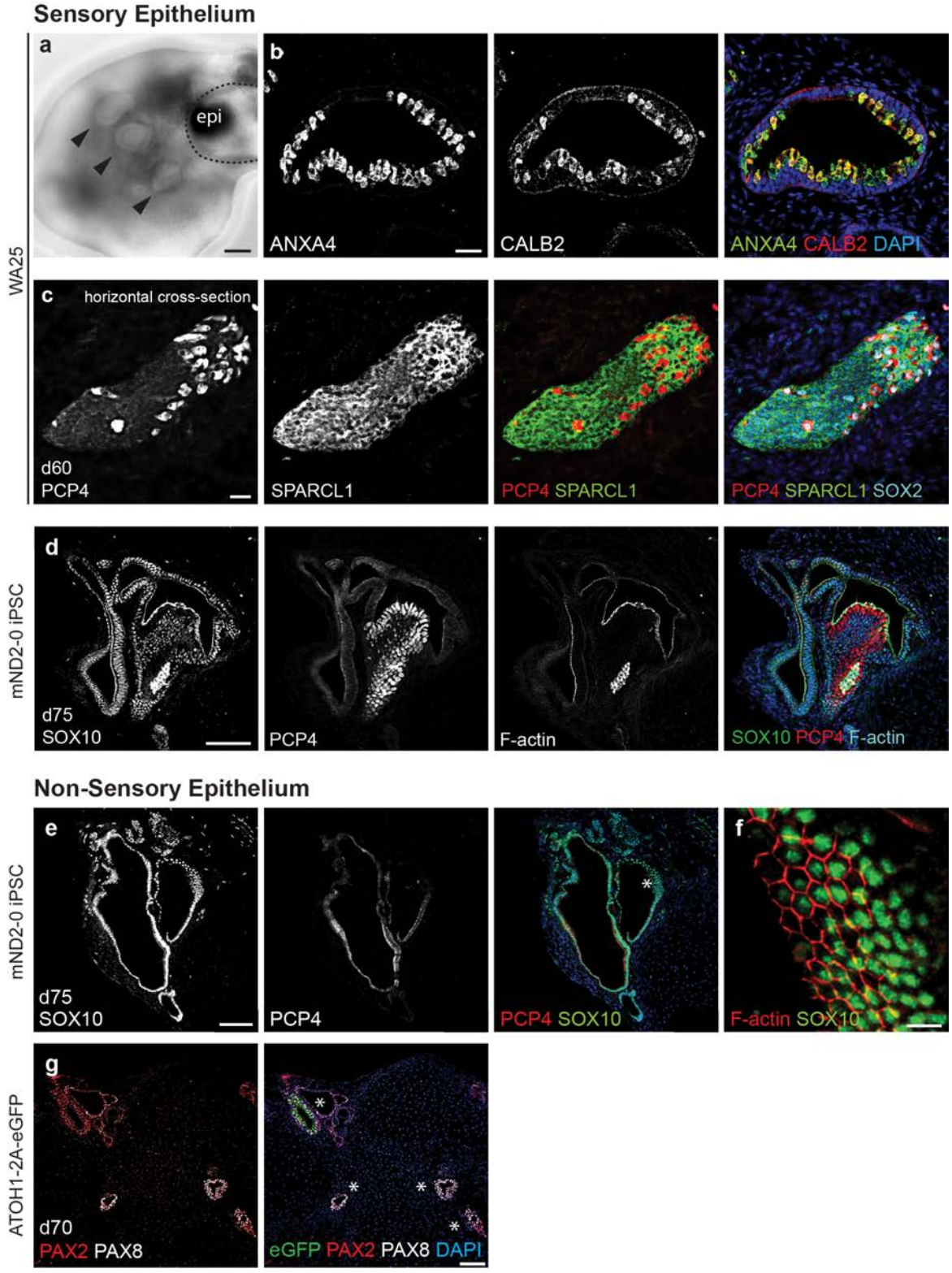
Supplementary Figure 9: WNT and FGF signaling modulation and PAX8/PAX2 expression during days 8-10. a, These qPCR data are representative of one exploratory experiment focused on identifying signaling modulators that could increase PAX2 expression following OEPD induction. **b**, **c**, FGF inhibition using, PD-173074 likely inhibits PAX2 expression, as would be expected based on developmental studies[1]. By contrast, the WNT inhibitor, XAV939, and WNT agonist, CHIR99021, only had a modest positive or negative impact on PAX2 expression compared to a DMSO controls. Based on these results and extensive immunostaining for PAX2 expression, we reasoned that the OEPD epithelium may require more time before it will be responsive to otic inductive cues. Thus, we changed strategies by lengthening the initial culture phase to 12 days (with addition of fresh media on day 8) and testing the effect of transitioning the aggregates to Matrigel droplets.



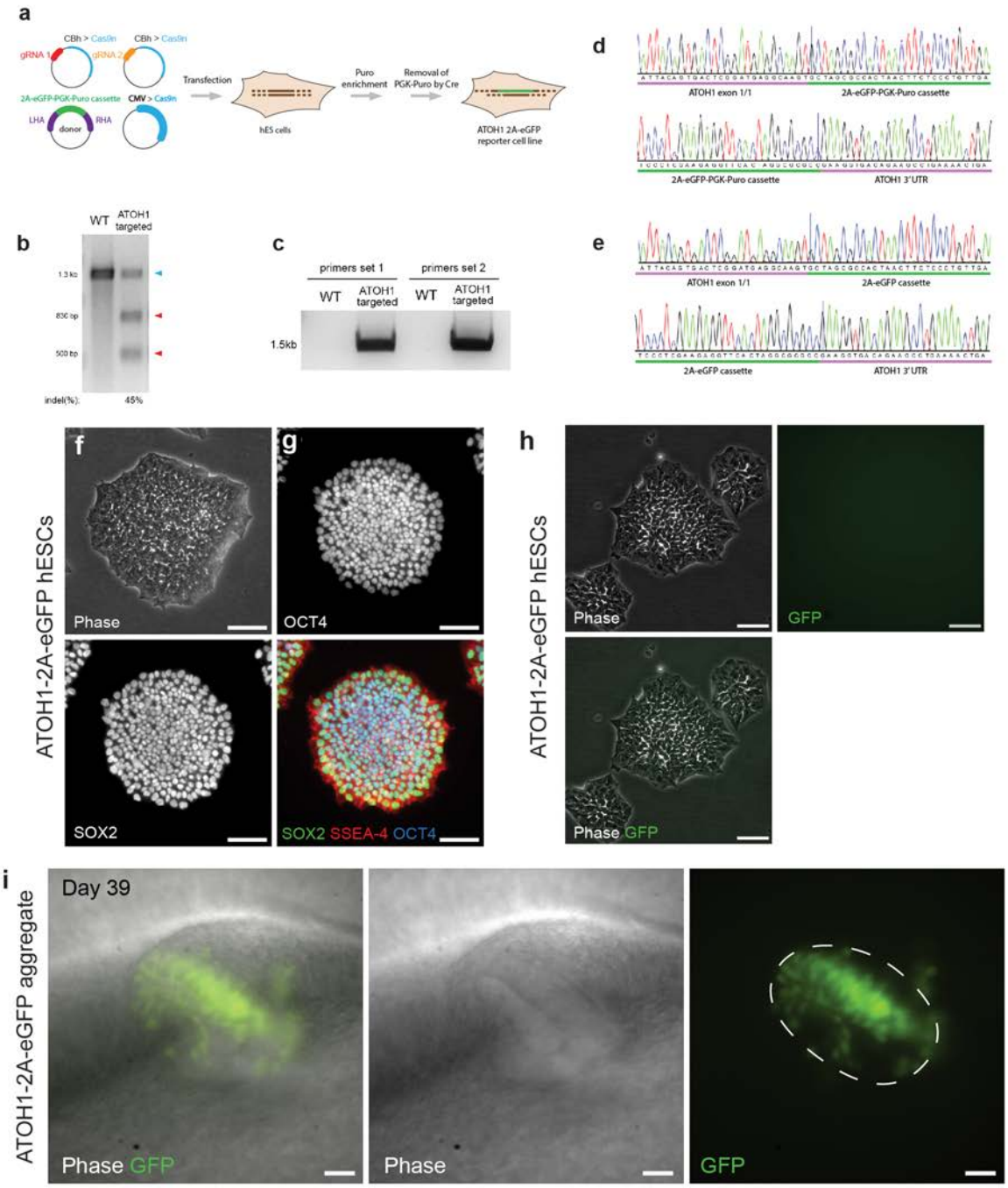
Supplementary Figure 10: Otic vesicles evaginate into a mesenchyme of cranial neural crest (CNC)-like cells. a, During development, the otic vesicle is surrounded by mesenchymal cells—of both CNC and mesodermal origin—with an overlying epidermis. **b, c**, On day 18, CHIR treated aggregates display PAX2+ ECAD+ pit/vesicle-like structures (**c**) that do not appear in control (DMSO) treated samples (**b**). TFAP2A+ migrate radially away from the aggregate in both conditions. **d**, Correlation of otic pit-like structures (asterisks) observed under DIC imaging with cryosections immunostained for NCAD, PAX8, and PAX2. **e**, In CHIR-treated samples, SOX10 is expressed in the otic pit epithelia (arrowhead) as well as in a subset of the migratory cells (asterisks), suggesting the presence of CNC-like cells. **f**, At day 35, the mesenchymal cell layer has thickened relative to day 18 and contains cells expressing TFAP2A and SLUG (**c**) and some otic vesicles have detached from the core epidermis, while others remain attached (arrowheads). **g**, **h**, At days 75-130, the mesenchyme contains multiple cartilage nodules that stain positive for Alcian blue, S100, and S0X9. Scale bars, 250 μm (**h**), 100 μm (**b-g**).



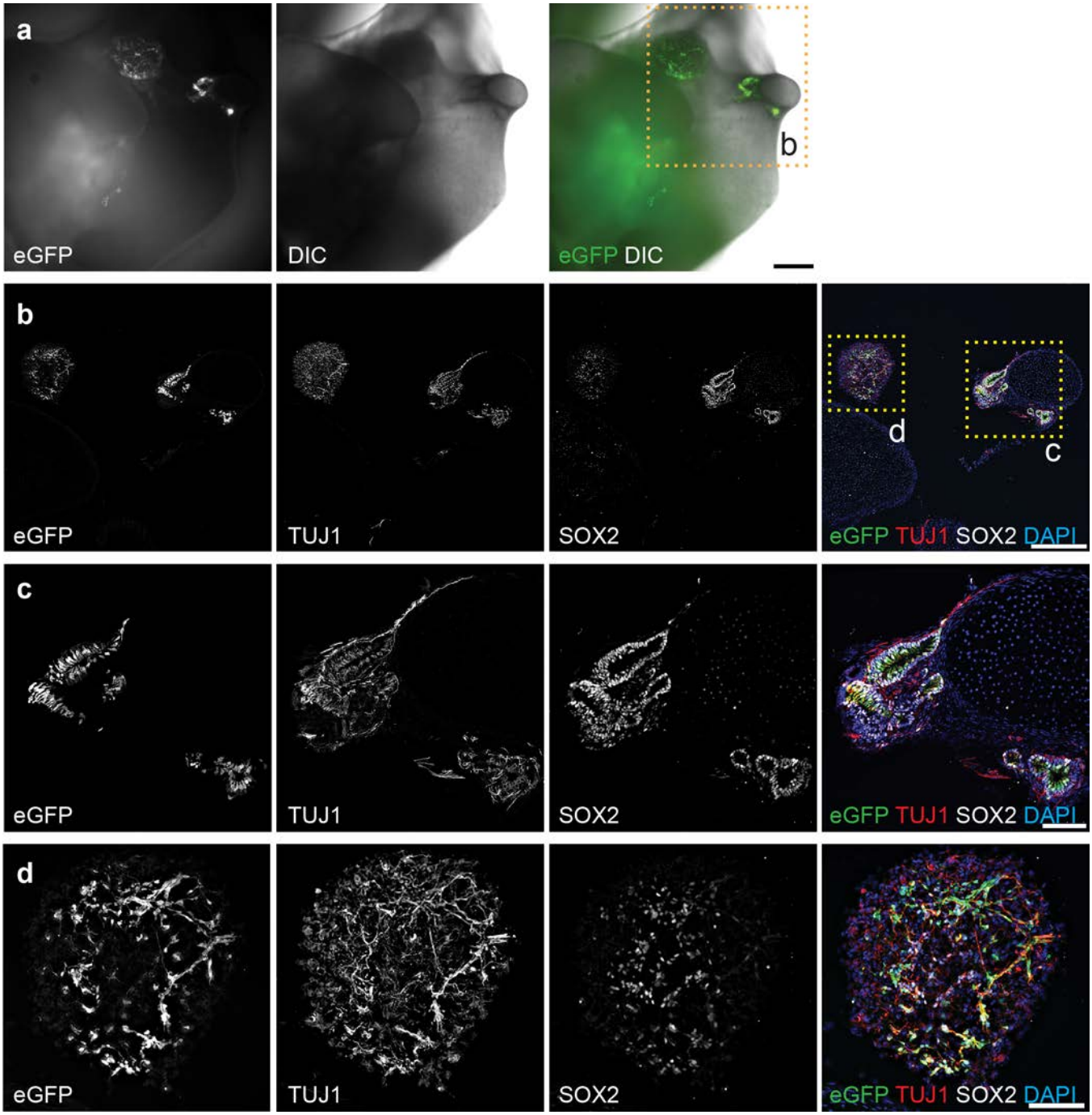
Supplementary Figure 11: Otic vesicles evaginate radially around a core epithelium of epidermal keratinocytes. a-c, Serial sections through a day 35 aggregate showing the internal organization of epidermal and otic vesicle epithelia. KRT5 expression is restricted to the epidermis, whereas ECAD is expressed in both epidermis and otic vesicle epithelial cells. Arrowheads label SOX10^{+} otic vesicles in (**a**). Note the CNC-like SOX10^{+} TFAP2^{-} and SOX10^{+} TFAP2^{+} cells in the mesenchymal layer of the aggregate. The pair of vesicles highlighted in panel (**c**) are labeled, at lower magnification and different orientation, in panel (**b**). The vesicles seen in (**c**) were shown to co-express the otic vesicle markers SOX10, PAX2, FBXO2, JAG1, and ECAD. **d-g**, A day 35 aggregate that was wholemount immunostained for ECAD to reveal the epidermal core and surrounding otic vesicles. See **Supplementary Video 1** for additional analysis of this specimen. Scale bars, 250 µm (**d, f**), 100 µm (**a, b**), 25 µm (**c, e, g**).



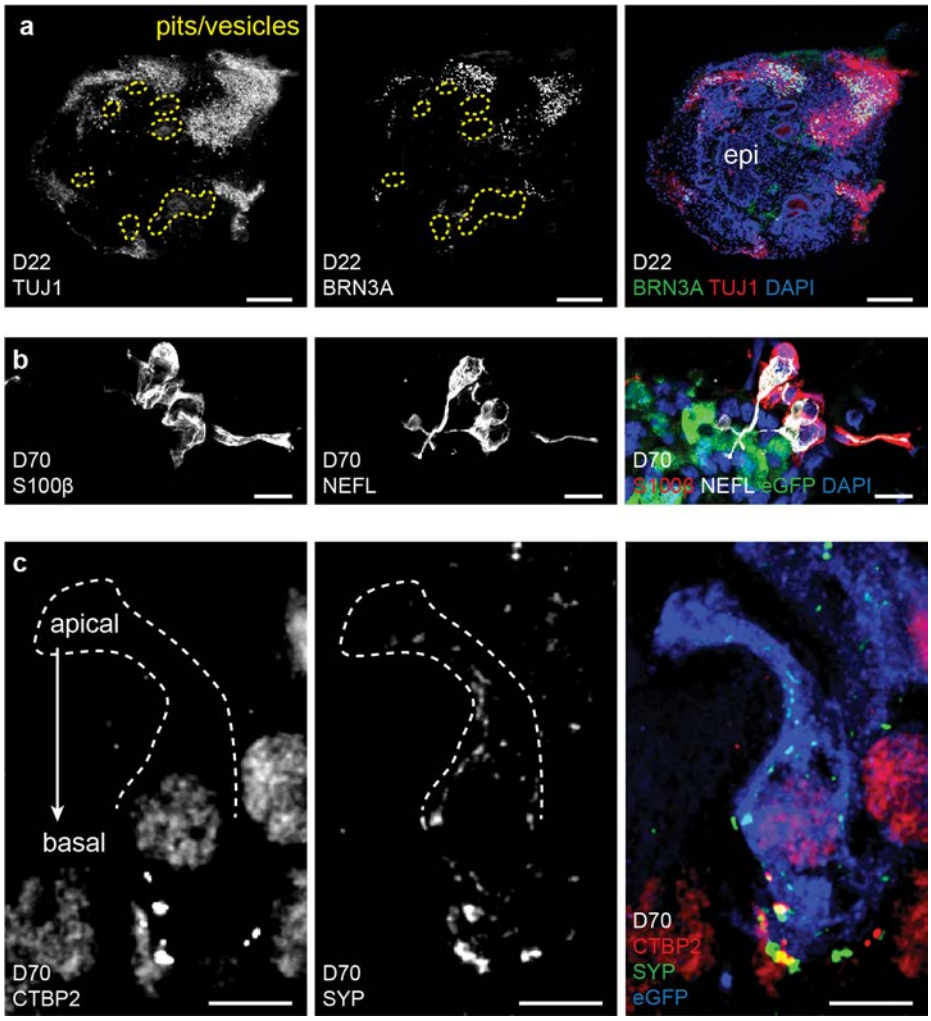
Supplementary Figure 12: Inner ear organoids generate vestibular-like sensory epithelia. a, Day 48 aggregate with three visible inner ear organoids (arrowheads). Note that this specimen was derived from a separate experiment than the specimen seen in **Fig. 2j**. **b**, Organoid hair cells express the type II vestibular and inner cochlear hair cell marker CALB2. **c**, Cross-section through a sensory epithelium showing expression of SPARCL1 throughout the supporting cells. SOX2 is expressing in both supporting cells and PCP4+ hair cells. **d-f**, SOX10 is expressed throughout the supporting and non-sensory epithelial cells. F-actin-rich circumferential belts were observed in both sensory and non-sensory epithelial. **g**, In day 70 aggregates, PAX2 is expressed in all inner ear organoid epithelia, whereas PAX8 is only expressed in non-sensory epithelia. Scale bars, 100 μm (**a, d, e, g**), 25 μm (**b, c**), 10 μm (**f**).



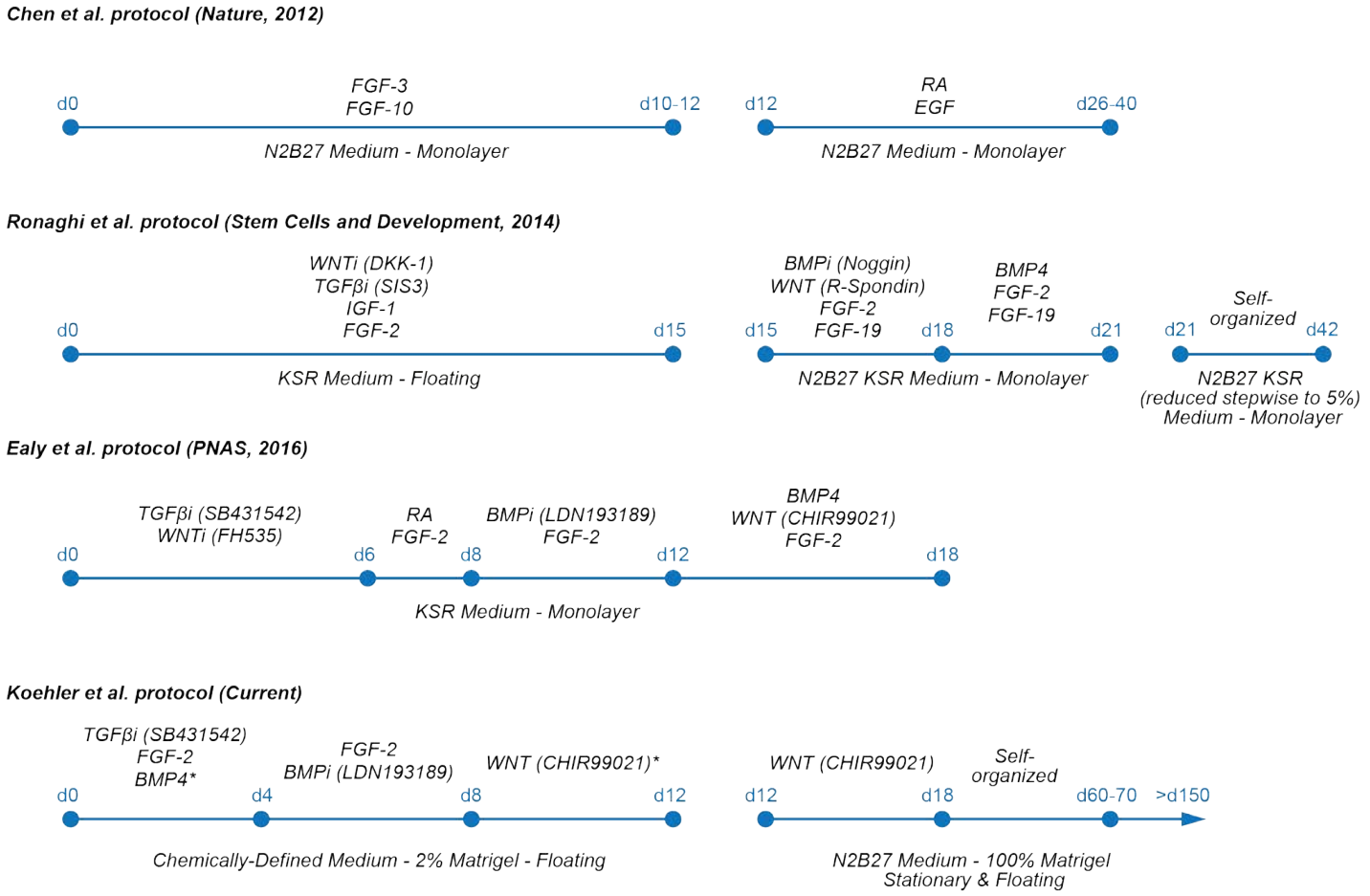
Supplementary Figure 13: Generation of ATOH1-2A-eGFP cell line using CRISPR/Cas9. a, Workflow of ATOH1-2A-eGFP CRISPR. The vector expressing Cas9n under the control of CMV promoter is used for improved expression level of Cas9n. **b**, Testing of the gRNA pairs with T7 Endonuclease I assay suggests high cleavage activities (indel frequency = 45%) of the gRNA pairs. **c**, **d**, PCR amplification from targeted population of cells using primer sets shown in (**Fig. 3a**) demonstrates successful 2A-eGFP-PGK-Puro cassette integration (**c**), which is confirmed by Sanger sequencing at the left and right junctions (**d**). **e**, Sanger sequencing of the left and right junctions of a clonal cell line derived after Cre recombination demonstrates correct integration of the 2A-eGFP cassette at the ATOH1 locus. The 3' end of the 2A-eGFP cassette shown in lower panel of (**e**) is a linker region 3' of the LoxP site(s) not affected by Cre recombination. It is therefore identical to the region shown in (**d**). **f, g**, ATOH1-2A-eGFP cells maintained on Vitronectin-N-coated plates in E8 flex medium express markers of primed pluripotent stem cells. **h**, GFP expression is undetectable in undifferentiated ATOH1-2A-eGFP ES cells. **i**, Inner ear organoid derived from ATOH1-2A-eGFP hESCs containing eGFP^{+} hair cells on day 39. This is the earliest we observed hair cell induction. Scale bars, 50 μm.



Supplementary Figure 14: **eGFP-positive cells in day 70 cell aggregates. a**, Low magnification view of eGFP$^+$ hair cells and neuron-like cells situated in close proximity within a cell aggregate. **b-d**, High magnification images of SOX2, TUJ1, and eGFP expressing cells in sensory epithelia (**c**) and a neuronal cell cluster (**d**). eGFP$^+$ hair cells could be distinguished from eGFP$^+$ neuronal progenitors based on morphology. Note that the TUJ1 antibody labels hESC-derived hair cells. In previous studies, mouse inner ear organoid hair cells were not labeled using the same TUJ1 antibody[2]. Scale bars, 100 μm.



Supplementary Figure 15: **Evidence of neurogenesis and synaptogenesis in inner ear organoid culture.** **a**, On day 22, TUJ1^{+} BRNR3A^{+} neuroblasts were observed in the mesenchyme surrounding otic pits and vesicles. **b**, High magnification view of S100β^{+} Schwann-like cells surrounding neurons and neuronal processes. Lower magnification image is in **Figure 4k**. **c**, High magnification view of CTBP2^{+} and SYP^{+} puncta localized in the basal region of a eGFP^{+} hair cell. Lower magnification image is in **Figure 4m**. Scale bars, 100 μm (**a**), 10 μm (**b**), and 5 μm (**c**).



Supplementary Figure 16: Inner ear induction methods. Comparison of the current inner ear organoid induction protocol to other recent protocols aimed at inner ear induction from Chen *et al.* (2012), Ronaghi *et al.* (2014), and Ealy *et al.* (2016)[3-5]. Treatments are listed above the timeline, whereas the basal medium and format are listed below. The letter (i) denotes inhibitor. Treatments labeled with an asterisks (*) are optional and may be cell line dependent. KSR = Knockout Serum Replacement; SIS3 = Selective Inhibitor of Smad3, a partial TGFβ inhibitor; RA = retinoic acid.

SUPPLEMENTARY TABLES

Supplementary Table 1. Chemically-Defined Differentiation Medium (CDM)

Component	Supplier	Cat. No.	Stock Concentration	Final Concentration	Volume used
Ham's F12 GlutaMax	Gibco	31765-035	-	49% (v/v)	100 ml
IMDM GlutaMax	Gibco	31980-030	-	49% (v/v)	100 ml
Chemically-Defined Lipid	Gibco	11905-031	100x	1x	2 ml
BSA	Sigma	A1470	-	5 mg/ml	1 g
Insulin	Sigma	I9278	10 mg/ml	7 µg/ml	140 µl
Transferrin	Sigma	T8158	20 mg/ml	15 µg/ml	150 µl
1-thioglycerol	Sigma	M6145	11.5 M	450 µM	8 µl
Normocin	Invivogen	Ant-nr-1	50 mg/ml	100 µg/ml	400 µl

Note: This formulation is for 200 mL of medium which should be used for <2 weeks. We sterile filtered the medium before adding Insulin and other factors. A variation of this medium was previously used to generate cerebellar and anterior pituitary organoids[6,7]*.*

Supplementary Table 2. Organoid Maturation Medium (OMM)

Component	Supplier	Cat. No.	Stock Concentration	Final Concentration	Volume (50 ml)
Adv DMEM/F12	Gibco	12491-015	-	49% (v/v)	24.5 ml
Neurobasal	Gibco	21103-049	-	49% (v/v)	24.5 ml
N2 supplement	Gibco	17502-048	100x	0.5x	250 µl
B27 -Vitamin A	Gibco	12587-010	50x	0.5x	500 µl
GlutaMAX	Gibco	35050-079	100x	1x	500 µl
Mercaptoethanol	Gibco	21985-015	55 mM	0.1 mM	91 µl
Normocin	Invivogen	Ant-nr-1	50 mg/ml	100 µg/ml	100 µl

Note: This formulation is for 50 mL of medium, which should be used for <2 weeks. This medium is a custom-made hybrid of two media previously used to generate cerebral and gastric organoids[8,9]*. B27 without Vitamin A was used to limit the influence of endogenously produced retinoic acid.*

Supplementary Table 3. qPCR primers

Gene	Sequence	Rationale
L27		
Forward	CGTGAAGAACATTGATGATGGC	Housekeeping
Reverse	GCGATCTTCTTCTTGCCCAT	
DLX3		
Forward	TACTCGCCCAAGTCGGAATA	Non-neural Ectoderm
Reverse	TTCTTGGGCTTCCCATTCAC	
TFAP2		
Forward	TTTCAGCCATGGACCGTCA	Non-neural Ectoderm
Reverse	GGGAGATTGACCTACAGTGC	
CDX2		
Forward	GGGCTCTCTGAGAGGCAGGT	Extraembryonic
Reverse	CCTTTGCTCTGCGGTTCTG	
BRA		
Forward	TGCTTCCCTGAGACCCAGTT	Mesendoderm
Reverse	GATCACTTCTTTCCTTTGCATCAAG	
OCT4		
Forward	AGTGAGAGGCAACCTGGAGA	Pluripotency
Reverse	ACACTCGGACCACATCCTTC	
NANOG		
Forward	CATGAGTGTGGATCCAGCTTG	Pluripotency
Reverse	CCTGAATAAGCAGATCCATGG	
EOMES		
Forward	CAACATAAACGGACTCAATCCCA	Mesendoderm
Reverse	ACCACCTCTACGAACACATTGT	

Supplementary Table 4. Antibodies

Antibody	Host	Supplier	Catalog No.	Dilution
N-Cadherin	Mouse	BD Biosciences	610920	1:100
SOX10	Mouse	eBiosciences	14-5923-82	1:50
E-Cadherin	Mouse	BD Biosciences	610181	1:250
TFAP2	Mouse	DSHB	3B5	1:5
SOX2	Mouse	BD Biosciences	561469	1:100
PAX8	Rabbit	Abcam	AB97477	1:100
PAX2	Rabbit	Invitrogen	716000	1:100
Jagged-1 (JAG1)	Rabbit	LSBio	LSC138530	1:50
BRN3C	Mouse	Santa Cruz	SC81980	1:25
BRN3A	Mouse	Millipore	AB5945	1:50
MYO7A	Rabbit	Proteus	256790	1:100
Acetylated-α-Tubulin (TUBA4A)	Mouse	Sigma	T6793	1:100
βIII-Tubulin (TuJ1)	Mouse	Covance	MMS-435P	1:500
Calretinin (CALB2)	Mouse	Abcam	AB702	1:100
SPARCL1	Mouse	R&D Systems	AF2728-SP	1:100
PAX6	Mouse	DSHB	PAX6	1:5
ANXA4	Mouse	R&D Systems	AF4146	1:50
PCP4	Rabbit	Sana Cruz	SC74816	1:50
S100β	Rabbit	Abcam	AB52642	1:100
espin (ESPN)	Rabbit	Gift from James Bartles		1:50

Note: All antibodies used for IHC were previously validated in IHC experiments. Citations can be found on the manufacture's website. Previous validation of the custom-made ESPN antibody in IHC on frozen sections of mouse tissue was performed in Koehler et al 2013[2].

Supplementary Table 5. Comparison of human inner ear induction studies.

Study Name	Chen et al. protocol (Nature, 2012)[3]	Ronaghi et al. protocol (Stem Cells Dev., 2014)[4]	Ealy et al. protocol (PNAS, 2016)[5]	Koehler et al. protocol (Current)
Culture format	2D	3D to 2D	2D	3D
Demonstration of non-neural induction	(Not shown)	Yes	Yes	Yes
Analysis of "off-target" lineages included (e.g. mesoderm, neuroectoderm, etc.) to confirm proper lineage commitment	(Not shown)	Yes	Yes	Yes
Demonstration of pre-placodal or otic-epibranchial progenitor domain induction	(Not shown)	Yes	Yes	Yes
Expression of hair cell marker genes	Yes	Yes	Yes	Yes
Multiple hair cell marker genes co-expressed in the same cells	Yes	Yes	(Not shown)	Yes
Hair bundle morphology	Disorganized	Mostly disorganized. Organized morphology "in rare instances"	(Not shown)	Organized
Electrophysiological responses	Yes	(Not shown)	(Not shown)	Yes
Presence of supporting cells	(Not shown)	Yes	(Not shown)	Yes
Correct spatial relationship between supporting cells and hair cells	(Not shown)	No	(Not shown)	Yes
Presence of sensory neurons	Yes	(Not shown)	(Not shown)	Yes
Hair cell or hair cell-like cell generation efficiency	"A small subset" of cells differentiated from the OEPs population	1.79% – 5.89%	(Not shown)	Hair cells present in ~20% of aggregates

2D, two dimensional. 3D, three dimensional. OEPs: otic epithelial progenitors. Note: In Chen et al. (2012) protocol, the efficiency of OEPs generation is dependent on the cell line, plating density and the degree of cell separation. In Ronaghi et al. (2014) protocol, the 1.79% hair cell-like cell generation efficiency was calculated from cells with mid-level *Atoh1*-nGFP expression (19.8% × 9.03% = 1.79%), which is believed to be "an indicator of a potential hair cell phenotype". If also considering cells with high *Atoh1*-nGFP expression and cells negative for *Atoh1*-nGFP expression, the hair cell-like cell generation efficiency is 5.89% (77.1% × 5.24% + 19.8% × 9.03% + 3.1% × 2.08% = 5.89%).

SUPPLEMENTARY DATA

Annotated plasmid sequence and annotated genomic sequence of ATOH1-2A-eGFP cell lines

1. ATOH1-2A-eGFP-PGK-Puro donor plasmid

Elements:
ATOH1 left homology arm (LHA)
2A
eGFP
LoxP
PGK promoter
Puromycin
PGK polyA
ATOH1 right homology arm (RHA)
pUC19 vector backbone

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTC
TGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGG
CTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCAC
AGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGG
CGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGT
TGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACC
CGGGGATCCCCAGCCCCAGCCGCATCATCTCCCGCAACCGCCGCCGCCGCCGCAGCCACCTGCAACTT
TGCAGGCGAGAGAGCATCCCGTCTACCCGCCTGAGCTGTCCCTCCTGGACAGCACCGACCCACGCGCCT
GGCTGGCTCCCACTTTGCAGGGCATCTGCACGGCACGCGCCGCCCAGTATTTGCTACATTCCCCGGAGC
TGGGTGCCTCAGAGGCCGCTGCGCCCCGGGACGAGGTGGACGGCCGGGGGGGAGCTGGTAAGGAGGAG
CAGCGGCGGTGCCAGCAGCAGCAAGAGCCCCGGGCCGGTGAAAGTGCGGGAACAGCTGTGCAAGCTGA
AAGGCGGGGTGGTGGTAGACGAGCTGGGCTGCAGCCGCCAACGGGCCCCTTCCAGCAAACAGGTGAAT
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2. Genomic sequence of the homozygous/bi-allelic ATOH1-2A-eGFP cell line at the ATOH1 locus

Elements:
ATOH1 exon 1/1 without the stop codon
2A
eGFP
LoxP

……
CTTTGATTGGCTGCTCGCACGCGCCTGCCCGCGCCCTCCATTGGCTGAGAAGACACGCGACCGGCGCG
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……

SUPPLEMENTARY VIDEO LEGENDS

Supplementary Video 1: Otic vesicles and epidermal core on day 35. Wholemount aggregate immunostained with antibodies for ECAD (green) and KRT5 (red). The sample was cleared using the ScaleS protocol (see **Methods**) and imaged using confocal microscopy. The central ECAD$^+$ structure is also KRT5$^+$, indicating an epidermal keratinocyte fate. Note the diverse size and morphology of the ECAD$^+$ otic vesicles. We also noted the presence of pore-like structures in the epidermal epithelium. Refer to **Fig. 2g-h** and **Supplementary Fig. 11** for additional characterization of similar day 35 specimens.

Supplementary Video 2: Multi-chambered inner ear organoid viewed through the surface of a day 48 aggregate using DIC imaging. The organoid has an epithelium and lumen that is clearly distinguishable from the surrounding mesenchyme. The two chambers of the organoid appear to be connected via a duct-like structure. A nodule of cartilage is visible in the upper right-hand corner of the frame at the beginning of the video. Refer to **Fig. 2j** to see this specimen in the context of the entire aggregate.

Supplementary Video 3: Inner ear organoids with ATOH1-2A-eGFP$^+$ hair cells (day 100 live cell imaging). The specimen represents three separate inner ear organoids or three chambers of a single inner ear organoid. Note the elongated morphology of hair cells as well as the tight Image segmentation was used to generate an estimation of ~716 eGFP$^+$ hair cells in this specimen.

Supplementary Video 4: Multi-chambered inner ear organoid with ATOH-2A-eGFP$^+$ hair cells in flat-mount preparation (day 100). On day 75, an inner ear organoid containing eGFP$^+$ cells was dissected from an aggregate and placed in a Matrigel droplet in a 24-well plate. After 25 days, the organoid retained eGFP$^+$ cells and had adhered to the culture plate bottom. Part 1: Phalloidin staining revealed F-actin-rich cell junctions on the luminal surface of multiple chambers in the flattened organoid. Part 2: High magnification imaging of chamber 2 reveals eGFP$^+$ cells with hair cell-like morphology and espin (ESPN)$^+$ stereocilia bundle-like structures protruding into the lumen.

Supplementary Video 5: Inner ear organoid with ESPN$^+$ eGFP$^+$ hair cells with innervation by NEFH$^+$ sensory-like neurons. The neuronal morphologies observed are highlighted to demonstrate the mix of unipolar and bipolar neurons found in inner ear organoid culture.

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