Molecular characterization and prospective isolation of human fetal cochlear hair cell progenitors

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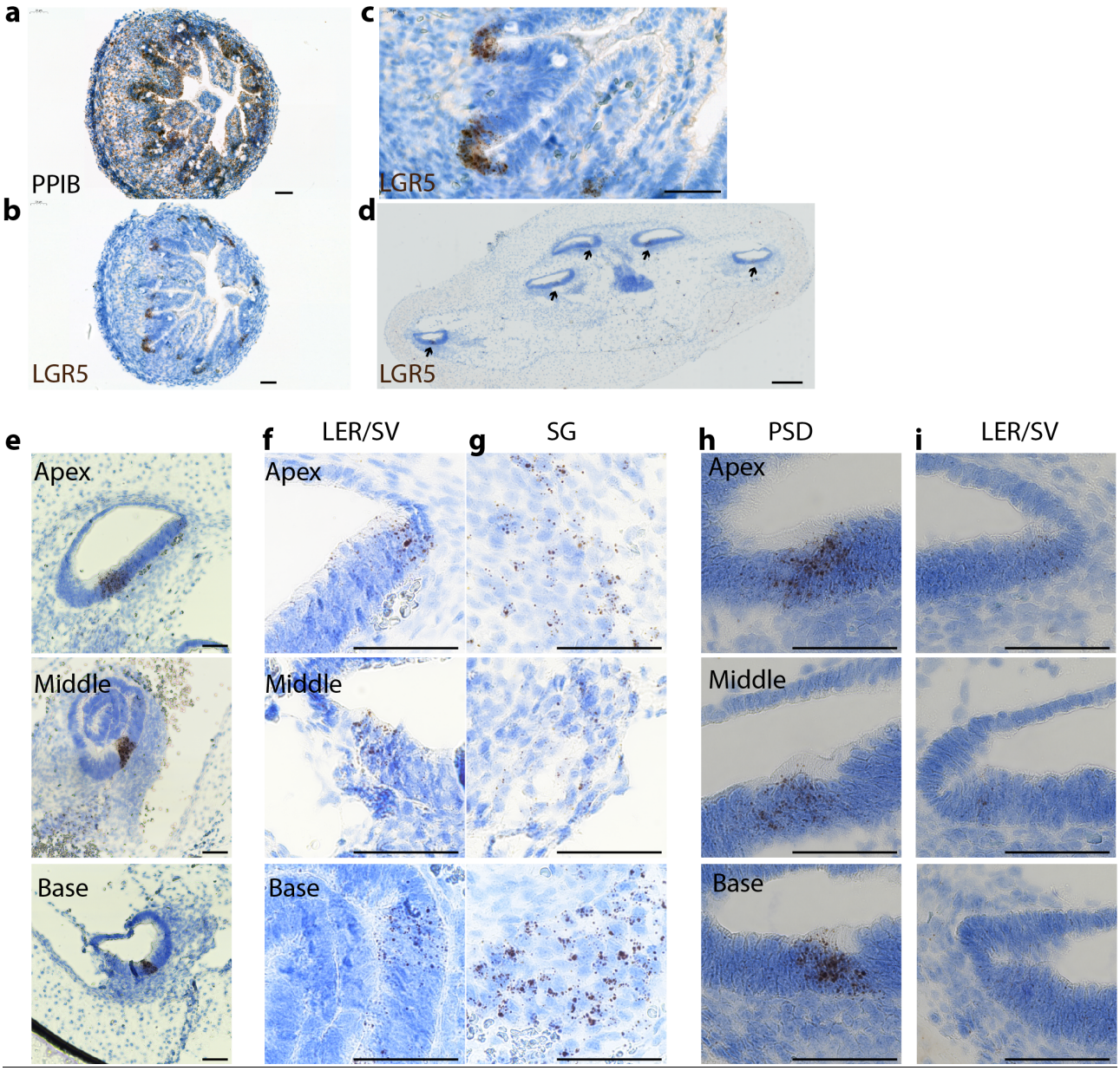
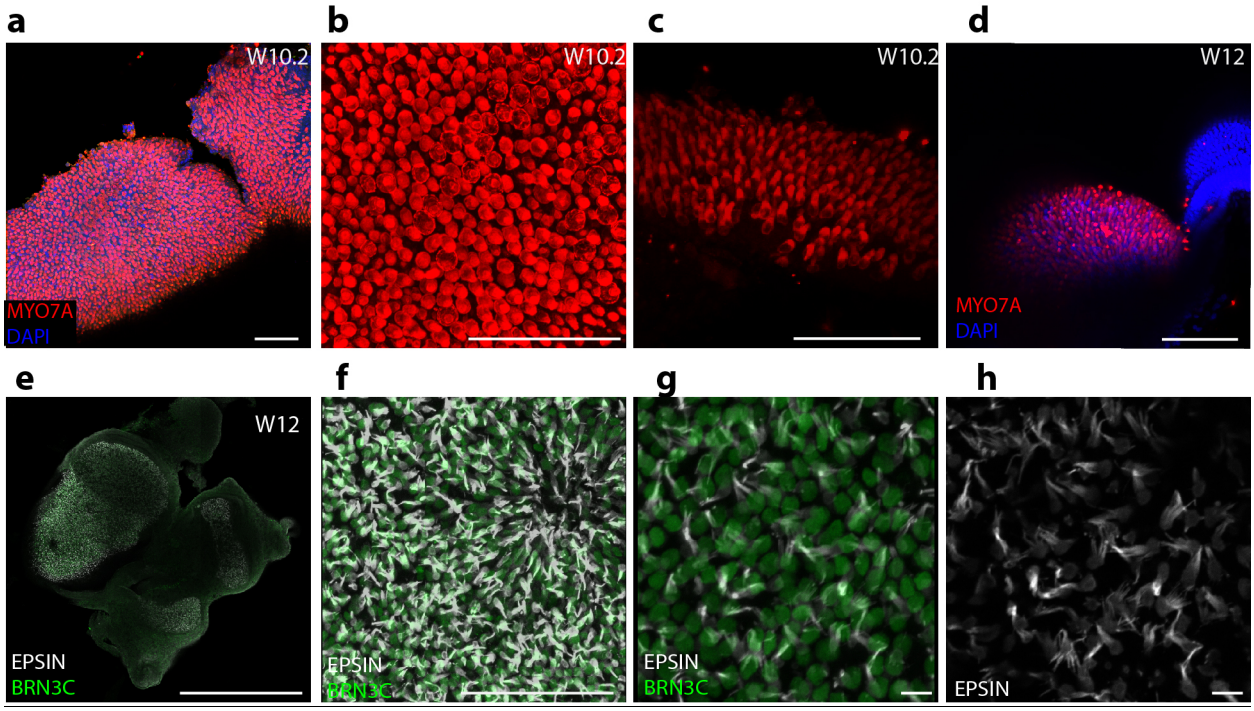
Supplementary figure 1: In Situ Hybridization (RNAscope) for human LGR5



Supplementary figure 1

(**a-c**) Human fetal intestine at W9.5-10 of development (E1273). In Situ Hybridization (ISH) for *PPIB* (housekeeping gene) and *LGR5* is shown as brown dots. Haematoxylin staining (in blue) to illustrate tissue morphology. Scale bar= 50μm c) Higher magnification of the intestinal crypts showing positivity for *LGR5*. Scale bar= 50μm**.**
(d) Fetal cochlea at W9.3 of development (E1278). ISH positive signal for *LGR5*, indicated by arrows. Scale bar= 200μm
(**e-g**) *LGR5* ISH in a human cochlea W9.2 (E1276)**.** (**e**) Shown are apical, middle and basal turns. (**f**) Higher magnification of the cochlear duct in the region of the lesser epithelial ridge/spiral ligament/stria vascularis (LER/SV). Spiral ganglion (SG) is shown in (**g**).
(**h-i**) *LGR5* ISH in a human cochlea W9.3 (E1278)**.** Apical, middle and basal turns are shown. PSD (h) and lesser epithelial ridge/spiral ligament/stria vascularis (LER/SV) (i) are shown. Scale bar= 50μm

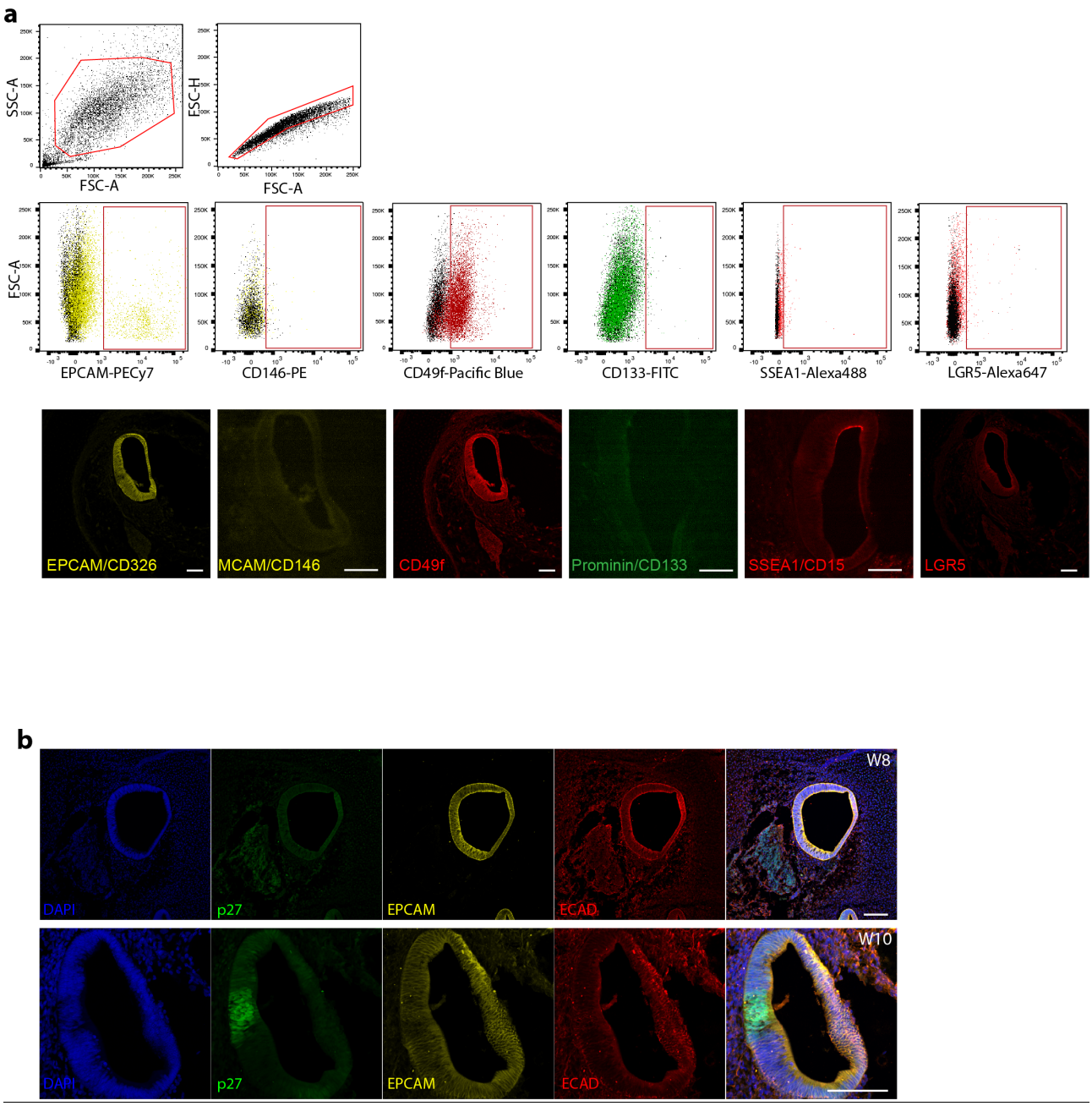
Supplementary Figure 2 : Hair cell staining vestibular sensory epithelia



Supplementary figure 2

(**a**) Whole mount confocal imaging of a human utricle at week 10.2 of development (sample E1213). The sample is immunostained for MYO7A (red). Nuclei are labeled by DAPI (blue). Scale bar =100 μm.
(**b**) Higher magnification of the same sample. Scale bar =100 μm.
(**c**) Whole mount confocal imaging of a human ampulla at week 10.2 of development immunostained for MYO7A (red). Nuclei are labeled by DAPI (blue). Scale bar =100 μm.
(**d**) Whole mount confocal imaging of a human ampulla at week 12.1 of development immunostained for MYO7A (red). Nuclei are labeled by DAPI (blue). (sample E1210). Scale bar =100 μm.
(**e**) Whole mount confocal imaging of a human utricle and ampullae at week 12 of development (sample E1289) immunostained for BRN3C (green) and ESPIN (white). Scale bar =1mm.
f-h) Details of the utricle sensory epithelium. Scale bar =100 μm (f), Scale bar =10 μm (g-h)

Supplementary figure 3: Surface marker characterization

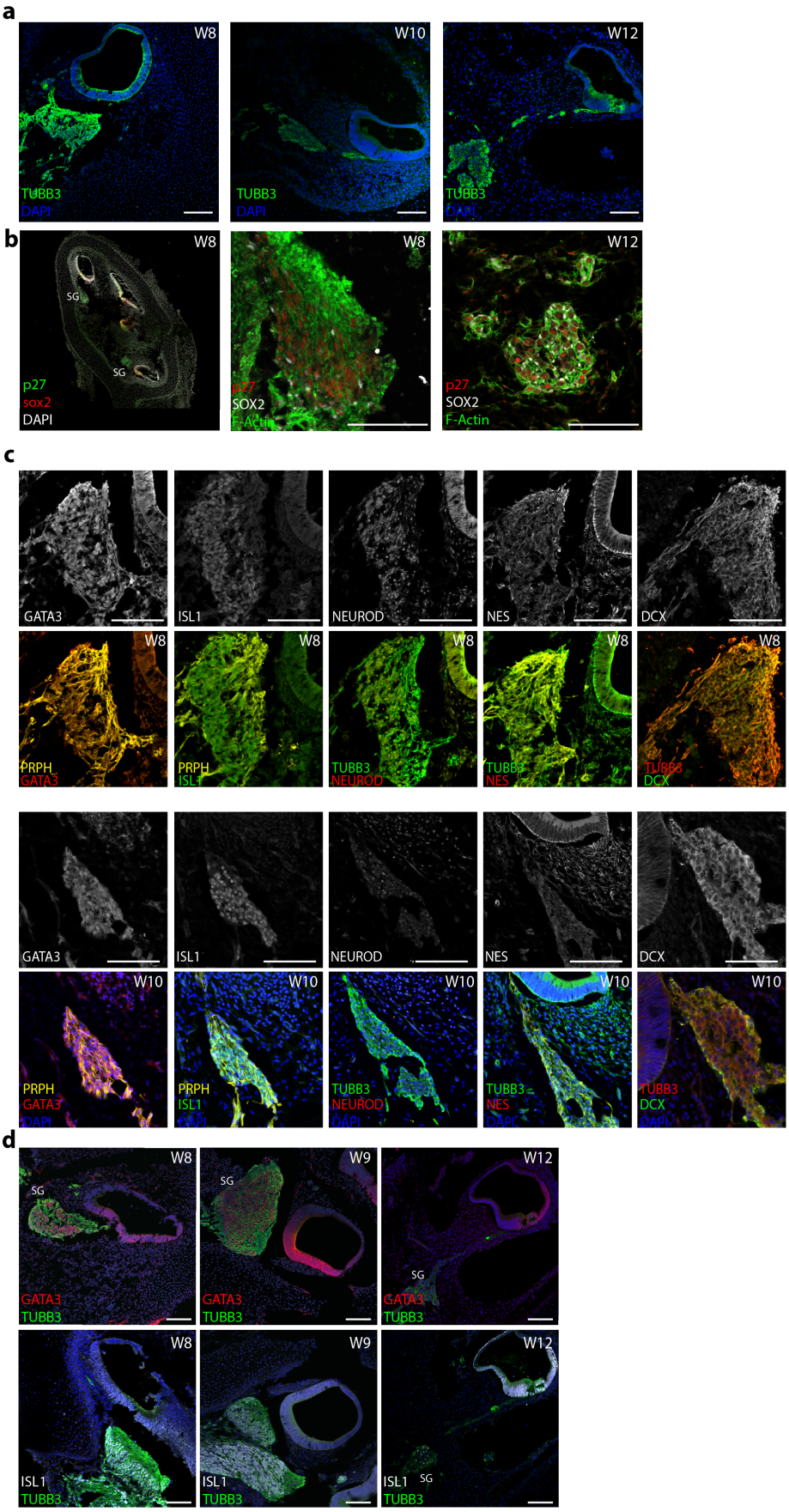


Supplementary figure 3

(**a**) Flow cytometry analysis for the markers EPCAM/CD326, MCAM/CD146, CD49f, Prominin/CD133, SSEA1/CD15 and LGR5. Samples (E1213, E1206 and E1299). Representative example of the gating strategy is shown in upper panel for gates p1(left) and p2(right). Lower panels show cells from p2 gates with unstained samples (black) and stained populations (in color) for each marker. Representative images of basal turn cochlear duct immunostained for EPCAM/CD326, MCAM/CD146, CD49f, Prominin/CD133, SSEA1/CD15 and LGR5 (sample E1201) are shown. Scale bar =100 μm.

(**b**) EPCAM, ECAD and p27Kip1 co-staining of cochlear duct samples at W8 (E1202) and W10 (E1201) of development. Scale bar =100 μm.

Supplementary figure 4: Spiral ganglion characterization



Supplementary figure 4

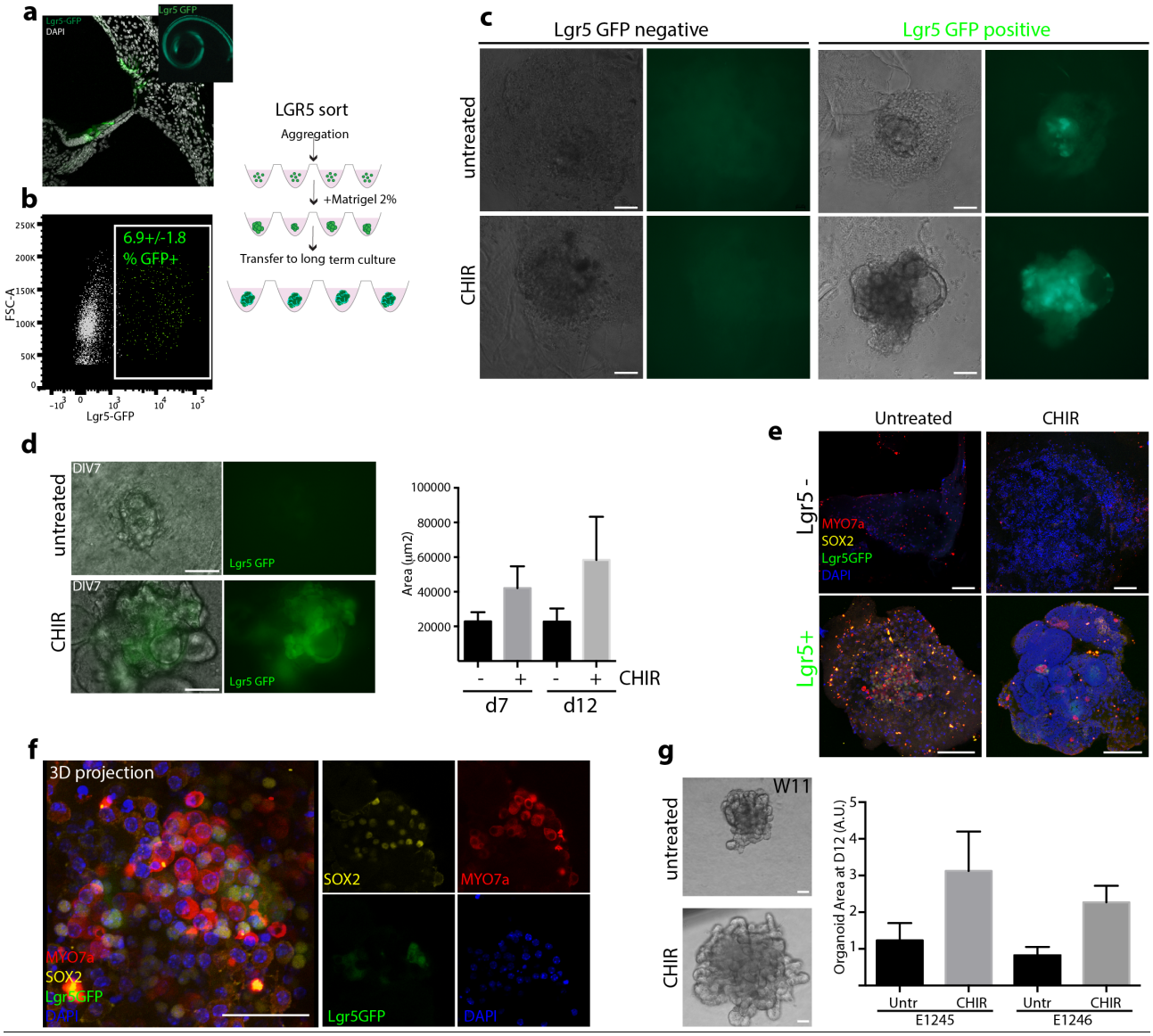
(**a**) Representative images of 3 stages of development (W8, W10 and W12). Cochleas were cyosectioned and immunostained for the neuronal marker βIII Tubulin (TUBB3) (green). The basal turn is shown. Scale bar =100 μm.

(**b**) Immunostaining of W8.4 (sample E1251) cochlea for SOX2 (red) and p27Kip1 (green) shows the lack of SOX2 expression in the p27+ SG cells. Higher magnification of the ganglion at W8.4 (E1251) and W12 (E1203) are shown. Scale bar =100 μm.

(**c**) Cochlear tissue immunostained at W8 (E1202) and W10 (E1201) for GATA3, Islet1 (ISL1), NEUROD, Nestin (NES) and Doublecortin (DCX). βIII tubulin (TUBB3) or peripherin (PRPH) staining is used to visualize the ganglion. Scale bar=100μm

(**d**) Immunostaining of W8 (E1202), W9 (E1228) and W12 (E1203) cochlear tissue for GATA3, TUBB3 or Islet1 and TUBB3. The cochlear duct also expresses these two markers at all time points analyzed. Scale bar =100 μm.

Supplementary figure 5: Organoids culture validation using Lgr5-GFP murine supporting cells.



Supplementary figure 5

(**a**) GFP expression in Lgr5-GFP reporter mice labels supporting cells in the organ of Corti, cross section and a whole mount preparation are shown.

(**b**) Flow cytometry analysis and FACS sorting of the GFP positive cells isolated from the early postnatal murine sensory epithelium and schematic of the re-aggregation protocol and organoid generation.

(**c**) Epithelial organoids expressing GFP can be expanded by CHIR99021 (CHIR) supplementation. Lgr5-GFP negative cells, grow largely as mesenchyme in these culture conditions. Scale bar=100µm.

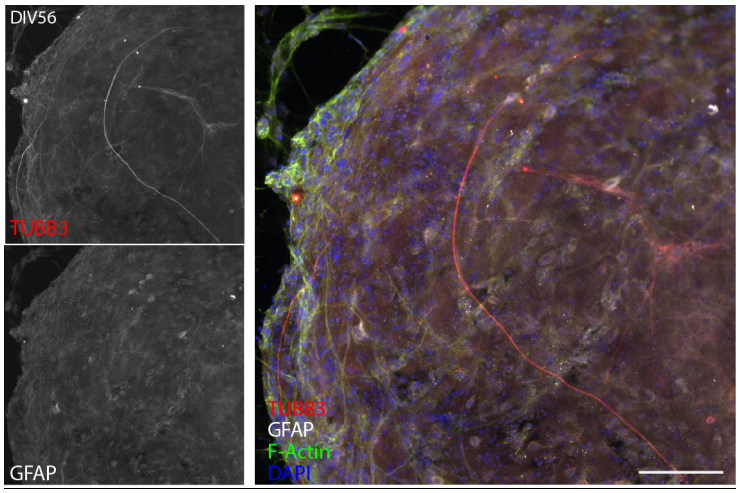
(**d**) Assessment of the organoids area at day 7 and day 12 in culture in presence/absence of CHIR99021. Bar graph shows mean+/- s.d. N=4 organoids per condition/time point are assessed. Scale bar = 100µm.

(**e**) Hair cell differentiation is assessed at day 15-20 *in vitro* by immunostaining the organoids for Myo7a (red) and sox2 (yellow). Hair cell-like cells are obtained only from the GFP+ organoids (n=3 independent experiments). Scale bar=100µm

(**f**) Representative example of hair cells derived from the Lgr5-GFP+ sorted cells. 3D projection (left) and a selected single stack for the separate channels are shown (right). Scale bar=50µm.

(**g**) Assessment of organoid size for two independent W11 human fetal samples (E1245 and E1246) in presence/absence of CHIR99021 added at 3µM twice during two weeks expansion. 8 organoids per sample per treatment were assessed. Bar graph shows Mean+/- s.d. Scale bar=100µm.

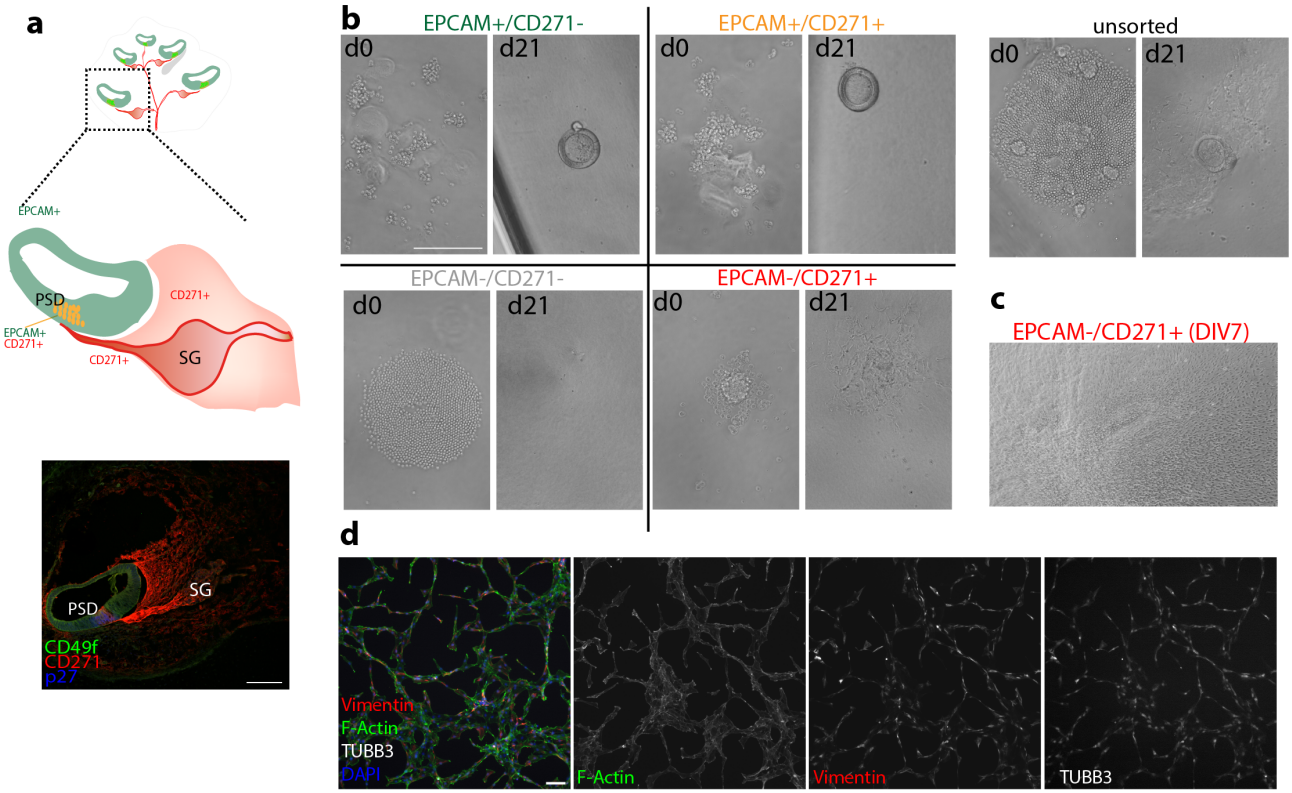
Supplementary figure 6: EPCAM negative cell differentiation



Supplementary figure 6

Immunostaining for βIII Tubulin (TUBB3) (red) and GFAP (green) in organoids derived from sorted EPCAM- cells, cultured for 56 days *in vitro* (sample E1220). Scale bar =100 μm.

Supplementary figure 7: Sorting strategy to isolate PSD resident cells



Supplementary figure 7

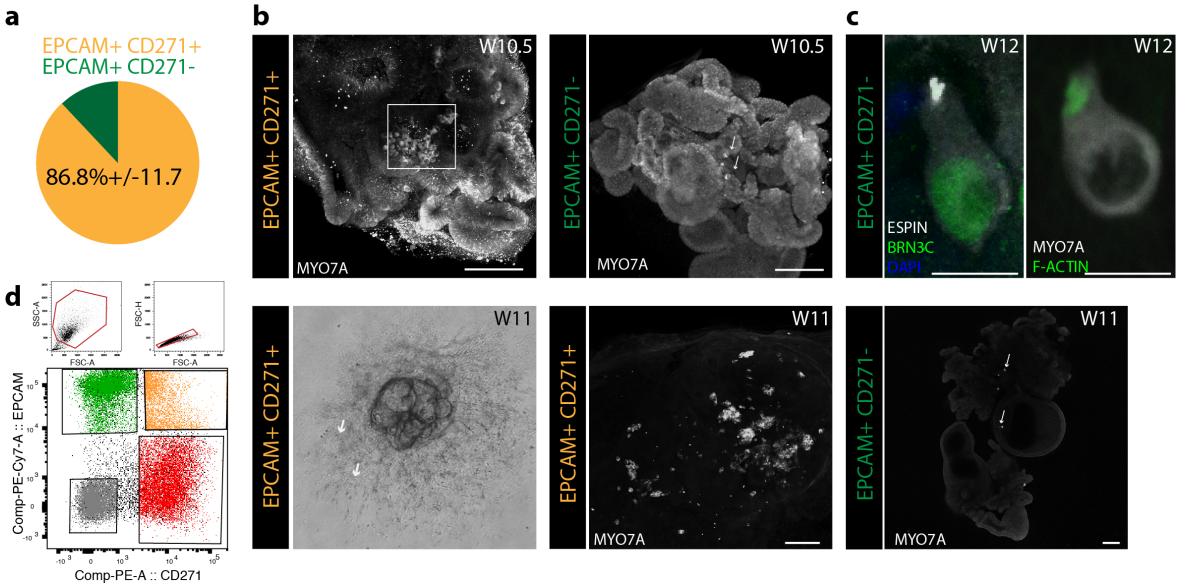
(**a**) Schematic representing the expression pattern of the selected markers EPCAM and CD271 and immunostaining of a W10 cochlea sample. The PSD is visualized by SOX2 staining (blue). CD271 expression is not restricted to the SG, but also expressed by mesenchymal tissue in the region of the developing spiral limbus starting from W10 of development. Scale bar =100 μm

(**b**) Representative images of the cultures derived from the four sorted populations immediately after sort (day 0) and at day 20 *in vitro*. The unsorted cell pool undergoing the same procedure is shown for comparison. (sample E1219). Scale bar =100 μm

(**c**) Representative image of a confluent culture derived from EPCAM-/CD271+ cells after 1 week in culture.

(**d**) Representative images of EPCAM-/CD271+ derived cultures immunostained for Vimentin (red), βIII Tubulin (TUBB3) (gray) and co-labelled with phalloidin and DAPI. Scale bar =100 μm.

Supplementary figure 8: Characterization EPCAM+/CD271+ organoids



Supplementary figure 8

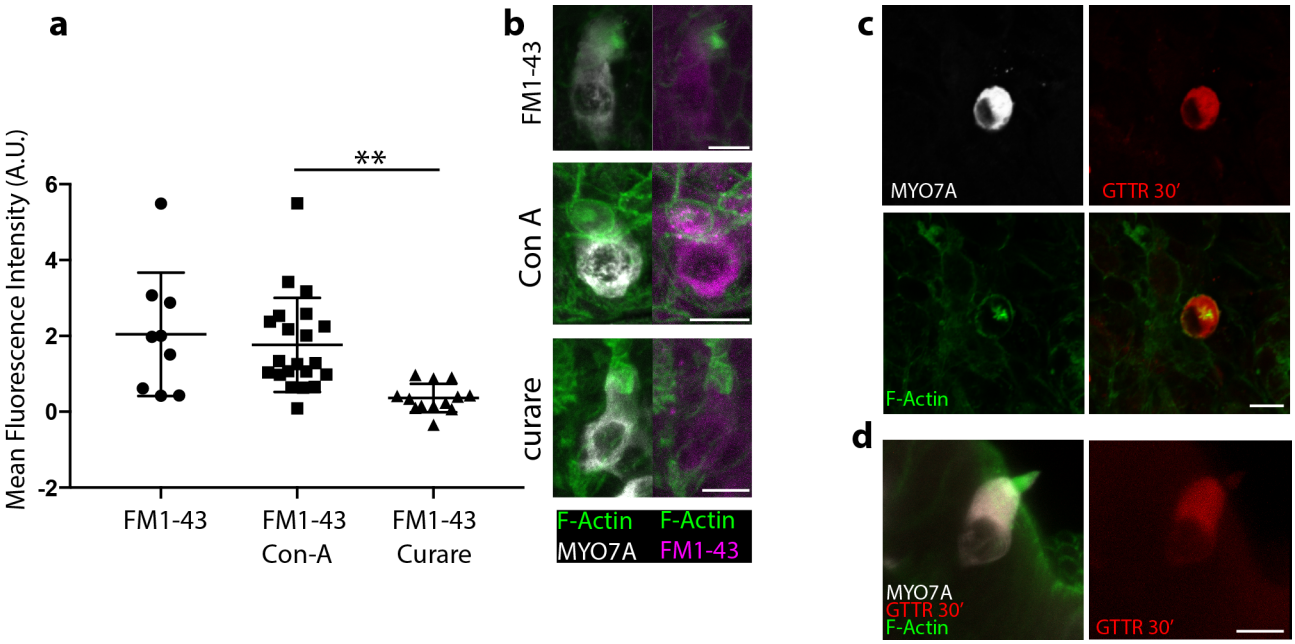
(**a**) Quantification of the contribution to total hair cell-like cell generation from the two sorted populations. N=5 different fetal sample. Values are mean +/- s.d.

(**b**) Immunostaining of EPCAM+/CD271+ (left) and EPCAM+/CD271- (right) organoids for MYO7A (sample E1270). Scale bar=100μm. Arrows point at single MYO7A+ cells obtained in the single positive population.

(**c**) Representative images of ESPIN and BRN3C immunostaining and MYO7A and F-Actin staining in cells derived from EPCAM+/CD271- organoids (sample W12, E1289) Scale bar=10μm.

(**d**) Example of FACS sorting based on EPCAM and CD271 co-staining (W11 sample E1254). Gating strategy indicated on top. Yellow gate (double positive) including cells expressing lower level of EPCAM leads to isolation cells which eventually outgrow with a mesenchymal phenotype as shown in the light microscopy image and indicated by the arrows. Right panels show immunostaining for MYO7A in organoids derived from EPCAM+/CD271+ or EPCAM+/CD271- sorted cells (W11 sample E1254). Maximum intensity projection of the confocal stack is shown. Scale bar=100μm.

Supplementary figure 9: Functional characterization of the in vitro generated hair cell-like cells



Supplementary figure9

(**a**) Quantification of FM1-43 loading in hair cell-like cells derived from a W12 sample (E1289). Cells were pretreated with concanavalin A or curare for 10 minutes or left untreated prior to the application of FM1-43 for 30 seconds. Fluorescence intensity (mean gray value) for the FM1-43 channel was quantified in the volume identified by MYO7A staining using FIJI and background corrected.
Samples were prepared in parallel and imaged by confocal scanning in sequential mode using the same parameters. FM1-43-only: n=9 cells from 2 different organoids, Curare-FM1-43: n=13 cells from 2 different organoids; Concanavalin A-FM1-43: n=21 cells from 3 different organoids. Unpaired t test (** $p<0.01$)
(b) Representative images of the hair cell-like cells quantified in the 3 conditions. Merged channels for F-Actin and MYO7A and F-Actin and FM1-43 are shown. Scale bar=10μm.
(**c-d**) GTTR loading in hair cell-like cells from a W9.6 sample (E1286). Single channels and merged image are shown for two different morphologically distinct hair cells types. Scale bar=10μm.

SUPPLEMENTARY TABLES

Supplementary Table 1: Human fetal samples used in the study

sample ID	Week P.C.	experiment	
Histochemistry			
E1209	7.7	immunostaining/Cryosection	
E1202	7.9	immunostaining/Cryosection	
E1251	8.4	immunostaining/Cryosection	
E1241	9.1	immunostaining/Cryosection	
E1228	9	immunostaining/Cryosection	
E1192	10.3	immunostaining/Cryosection	
E1201	10.3	immunostaining/Cryosection	
E1213	10.2	immunostaining/Cryosection	
E1195	10.9	immunostaining/Cryosection	
E1210	12.1	immunostaining/Cryosection	
E1203	12	immunostaining/Cryosection	
E1274	6	immunostaining/Cryosection	**
E1257	11.6	immunostaining/Cryosection	**
In Situ Hybridization (ISH)			
E1273	9.5-10*	intestine ISH	
E1276	9.2	cochlea ISH Lgr5	
E1278	9.3	cochlea ISH Lgr5	
EPCAM+ sort organoids			
E1253	10.4	differentiation to hair cells with co-culture	
E1248	11.4	differentiation to hair cells with co-culture	
E1246	11.5	differentiation to hair cells with co-culture	
E1245	11.4	differentiation to hair cells with co-culture	
E1229	11	differentiation to hair cells with co-culture	
E1224	8.9	short term culture or diff with other protocols	
E1220	9.3	short term culture or diff with other protocols	
E1237	9.7	short term culture or diff with other protocols	
E1232	9.7	short term culture or diff with other protocols	
EPCAM+/CD271+ sort organoids			
E1254	11	differentiation to hair cells with co-culture	
E1242	12.5	differentiation to hair cells with co-culture	
E1238	9.7	differentiation to hair cells with co-culture	
E1270	10.5	differentiation to hair cells with co-culture	
E1273	9.5-10*	differentiation to hair cells with co-culture	
E1286	9.6	differentiation to hair cells with co-culture	
E1289	12	differentiation to hair cells with co-culture	
E1227	10.4	flow cytometry only	
E1225	9.9	flow cytometry only	
E1221	9.8	flow cytometry only	
E1214	8.5	flow cytometry only	
E1219	9.4	flow cytometry only	
Gene expression Fluidigm			
E1236	9		
E1235	11.8		
E1208	8.3		
E1204	11.1		
Flow cytometry			
E1206	11.1	Flow cytometry	
E1213	10.2	Flow cytometry	
E1199	10.5	Flow cytometry	

notes

* calculated 9.1, p.m. W12, morphology W10

** only for staining controls

Supplementary Table 2: antibodies used in the study

antigen		conjugated	cat#	company	dilution /final conc.
CD49f/α6Integrin	Rat IgG2a, κ	eFluor 450	48-0495	eBioscience	1 : 100
CD271/p75	Mouse IgG1, κ	Alexa fluo 647	560877	BD	1 : 100
CD271/p75	Mouse IgG1k	PE	557196	BD Pharmigen	1 : 100
Lgr5	Rat IgG2b, λ	alexa Fluor 647	562912	BD	1 : 100
CD146/MCAM	Mouse IgG1k	PE	561013	BD	1 : 100
CD326/EPCAM	Mouse IgG1, κ	PE-Cy7	25-9326	eBioscience	1 : 100
CD15/SSEA1	Mouse IgM	Alexa 488	60060AD.1	stem cell technologies	1 : 100
CD133/Prominin	mouse IgG1κ	VioBright FITC	130-105-225	Milteny	1 : 100
p27	Rabbit monoclonal		ab32034	AbCAM	1 : 200
sox2	Rabbit polyclonal		48-1400	Invitrogen	1 : 100
sox2	Mouse IgG1k		561469	BD	1 : 100
sox2	Mouse IgG2b		MAB4343	Millipore	1 : 200
Ki67	Rabbit polyclonal		NCL-ki67p	Novocastra	1 : 100
Myo7a	Rabbit polyclonal		25-6790	Proteus	1 : 200
Jag1	Rabbit polyclonal		AB7771	ABCAM	1 : 100
Jag1	mouse IgG1k (E12)		SC-390177	Santa Cruz	1 : 100
βcatenin	mouse		610154	BD Transduction laboratories	1 : 100
E-cadherin	Rat IgG1		ab11512	abcam	1 : 200
FBXO2	Rabbit monoclonal		ab133717	AbCAM	1 : 100
FBXO2	mouse (A-12)		sc-393873	Santa Cruz	1 : 100
FBXO2	goat (D-19)		sc-69400	Santa Cruz	1 : 100
ZO-1	Rabbit policlonal		AB59720	ABCAM	1 : 100
Sox9	Rabbit monoclonal		ab185966	AbCAM	1 : 100
Nestin	Mouse IgG1, κ		611658	BD Transduction Laboratories	1 : 200
Peripherin	Rabbit polyclonal		AB1530	Millipore	1 : 100
GATA3	Mouse monoclonal IgG1		MA1-028	Thermofisher scientific	1 : 100
Islet1	Mouse IgG2b		39.4D5(sup)	DSHB	1 : 10
NeuroD	Rabbit polyclonal		AB16508	ABCAM	1 : 100
Doublecortin	Goat		SC8066	Santa Cruz	1 : 100
βIII tubulin	Mouse IgG2a		MAB1195	R&D	1 : 200
Vimentin	Mouse IgG1k		SC6260	Santa Cruz	1 : 100
Espin	Rabbit polyclonal			Gift Jim Bartles	2.5mg/ml
BRN3C	Mouse monoclonal IgG1		SC81980	Santa Cruz	1 : 100
Fluorescently labeled compounds					
FM1-43 FX			F35355	Thermofisher scientific	5μM
Gentamycin-Texas Red				Gift Anthony Ricci	0.3mg/ml
Phalloidin		ATTO-488	49409	Sigma	1 : 100