SUPPLEMENTARY INFORMATION

Modelling rostro-caudal neural tube regionalisation from human embryonic stem cells with a microfluidic morphogenic gradient

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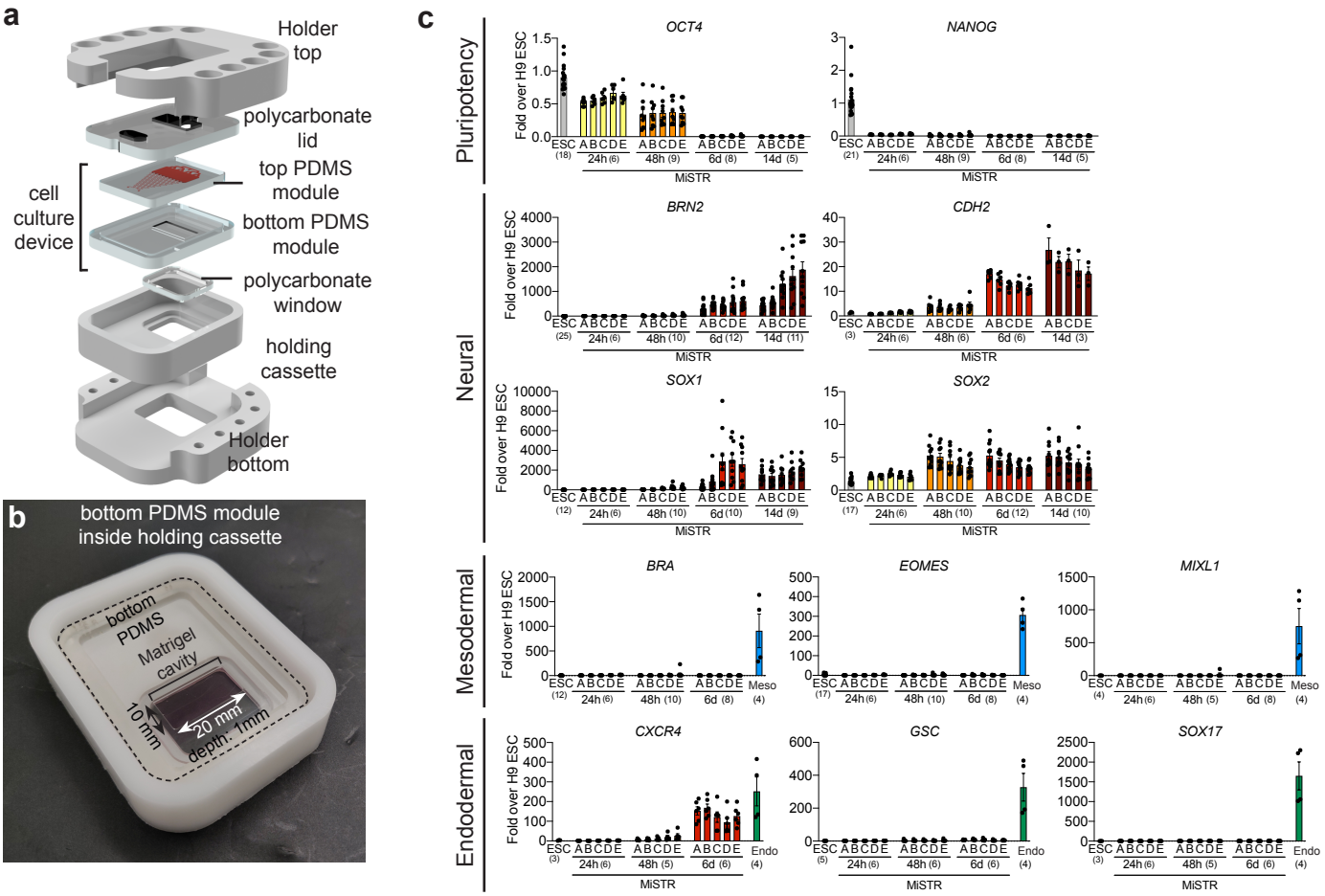
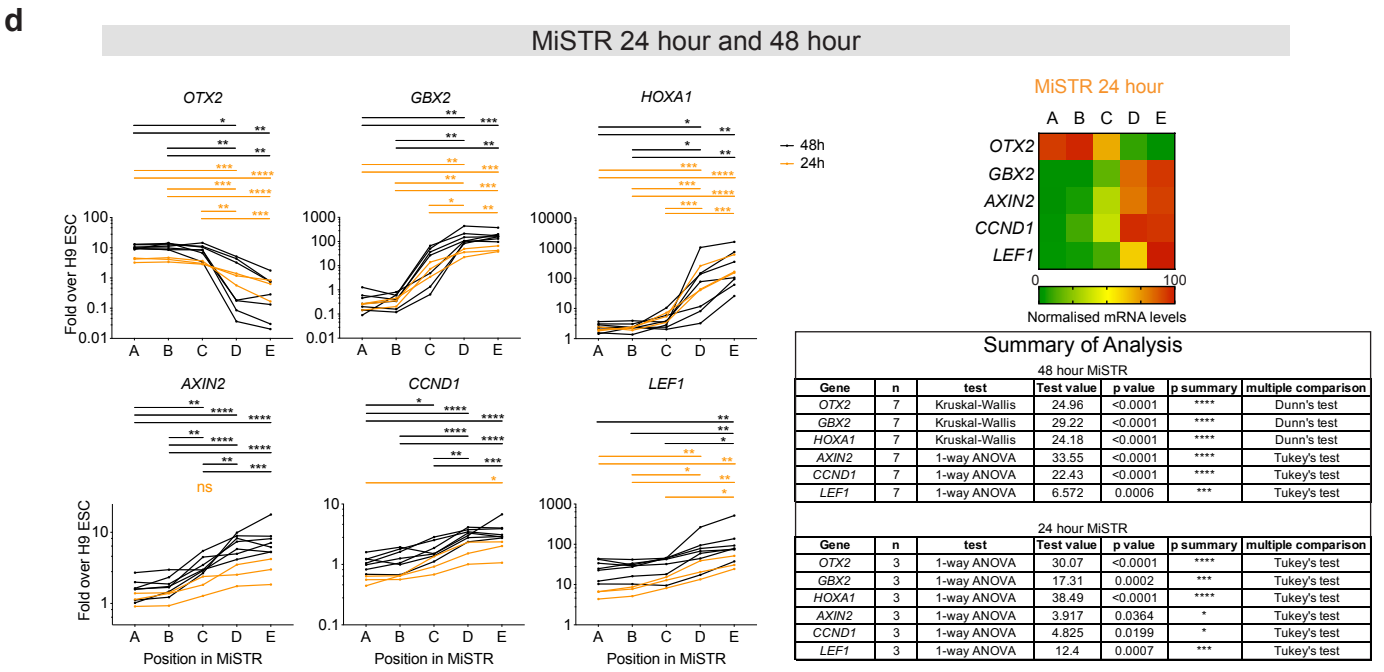
Supplementary Figure 1





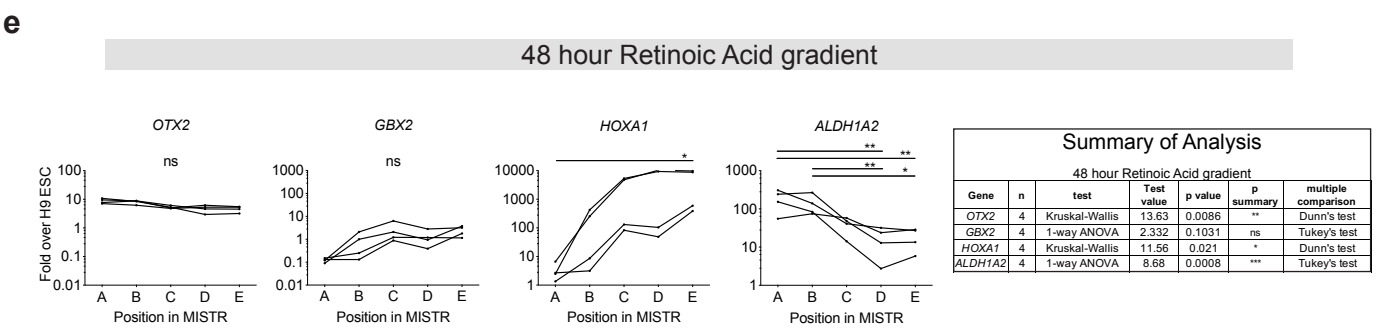
Summary of Analysis

48 hour MiSTR

Gene	n	test	Test value	p value	p summary	multiple comparison
OTX2	7	Kruskal-Wallis	24.96	<0.0001	****	Dunn's test
GBX2	7	Kruskal-Wallis	29.22	<0.0001	****	Dunn's test
HOXA1	7	Kruskal-Wallis	24.18	<0.0001	****	Dunn's test
AXIN2	7	1-way ANOVA	33.55	<0.0001	****	Tukey's test
CCND1	7	1-way ANOVA	22.43	<0.0001	****	Tukey's test
LEF1	7	1-way ANOVA	6.572	0.0006	***	Tukey's test

24 hour MiSTR

Gene	n	test	Test value	p value	p summary	multiple comparison
OTX2	3	1-way ANOVA	30.07	<0.0001	****	Tukey's test
GBX2	3	1-way ANOVA	17.31	0.0002	***	Tukey's test
HOXA1	3	1-way ANOVA	38.49	<0.0001	****	Tukey's test
AXIN2	3	1-way ANOVA	3.917	0.0364	*	Tukey's test
CCND1	3	1-way ANOVA	4.825	0.0199	*	Tukey's test
LEF1	3	1-way ANOVA	12.4	0.0007	***	Tukey's test



Summary of Analysis

48 hour Retinoic Acid gradient

Gene	n	test	Test value	p value	p summary	multiple comparison
OTX2	4	Kruskal-Wallis	13.63	0.0086	**	Dunn's test
GBX2	4	1-way ANOVA	2.332	0.1031	ns	Tukey's test
HOXA1	4	Kruskal-Wallis	11.56	0.021	*	Dunn's test
ALDH1A2	4	1-way ANOVA	8.68	0.0008	***	Tukey's test

Supplementary Fig. 1, related to Fig. 1

(a) Rendered visualisation of the arrangement of the parts and modules composing the MiSTR cell culture system (tubing and external pump system not shown).

(b) Photographic image of bottom PDMS module with Matrigel in the cavity inside the holding cassette. The PDMS contains a 1 mm deep cavity (Matrigel cavity) at the cell culture area. This well was filled with 200 µl Matrigel, onto which the hESC were later seeded. Note the elevations all around, allowing for the immersed alignment of the top PDMS module (containing the serpentine gradient generator) with the cell culture area.

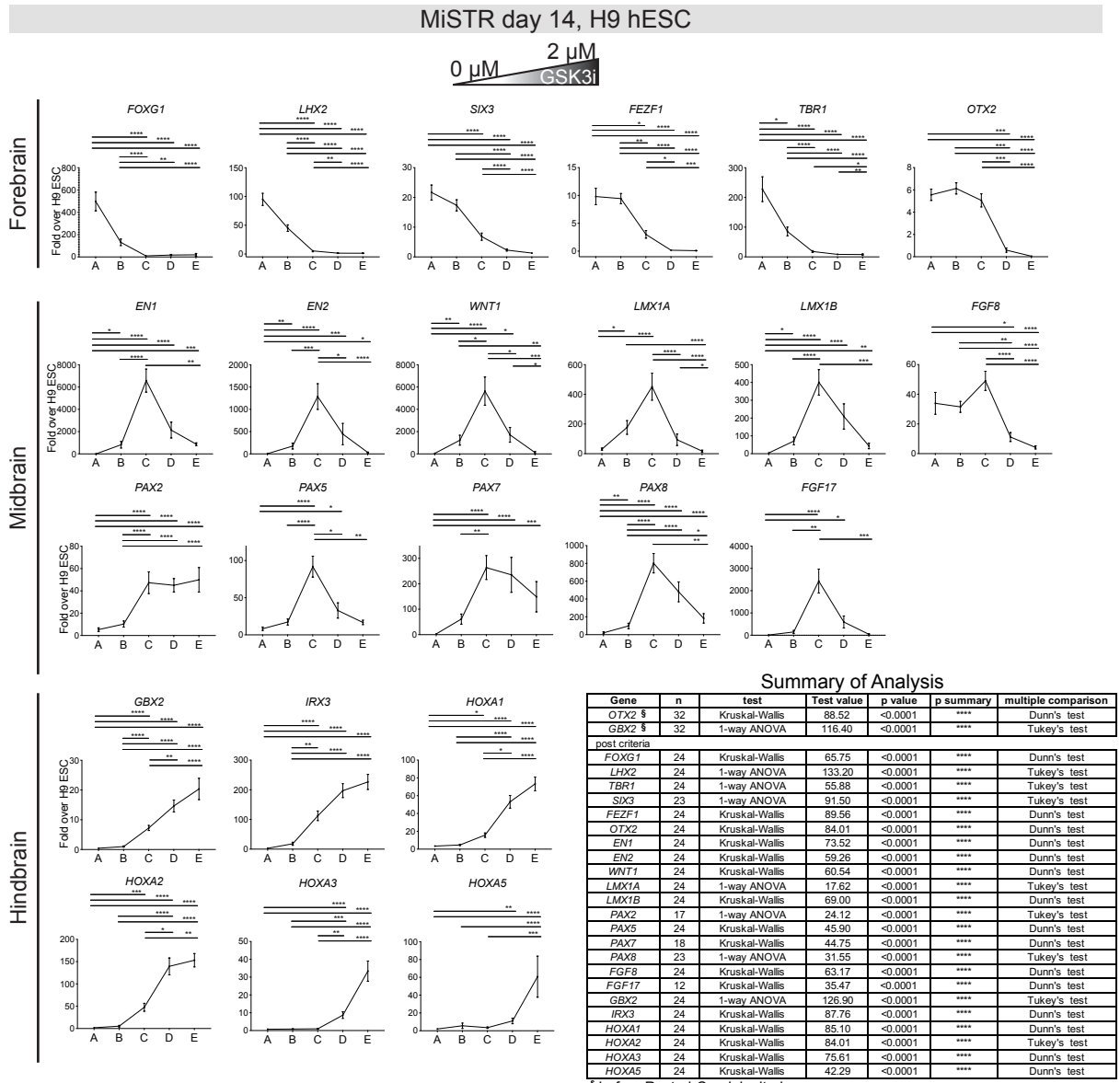
(c) qRT-PCR analysis of hESC and MiSTR tissue for the pluripotency markers *OCT4* (*POU5F1*) and *NANOG*, and neural commitment markers *BRN2* (*POU3F2*), *CDH2* (N-Cadherin), *SOX1* and *SOX2*. Early mesodermal (*BRA*, *EOMES*, *MIXL1*) and endodermal (*CXCR4*, *GSC*, *SOX17*) markers were also analysed for MiSTR tissue as well as a positive sample. Mesodermal and endodermal positive samples were obtained by directed differentiation of hESC as described elsewhere (see Methods). Data as mean ± SEM. The respective n for MiSTR tissues, ESCs and positive samples is in between parenthesis next to each tissue, for each marker.

(d) qRT-PCR analysis along the 5 regions A-E, of early neural genes (*OTX2*, rostral; *GBX2* and *HOXA1*, caudal) and canonical WNT signalling targets (*AXIN2*, *CCDN1*, *LEF1*) at 24 hours and 48 hours of differentiation in the MiSTR (0 µM to 2 µM GSK3i gradient). Line graphs represent individual experiments of 24 hours (orange) and 48 hours (black). Heatmap displays normalized data for each gene at 24 hours. The p-summary of the multiple comparison test is displayed above each graph. *p < 0.05; **p < 0.01; ***p < 0.001; **** p<0.0001. Summary table of the multivariate analysis performed is presented on the right. See also Supplementary Table 2 for the p values of multiple comparison.

(e) qRT-PCR analysis along the 5 regions A-E, of early neural genes (*OTX2*, rostral; *GBX2* and *HOXA1*, caudal) and the retinoic acid synthesis enzyme *ALDH1A2* at 48 hours of differentiation under a Retinoic Acid gradient (0 nM to 200 nM) (n=4 tissues). Line graphs represent individual experiments. The p-summary of the multiple comparison test is displayed above each graph. *p < 0.05; **p < 0.01. Summary table of the multivariate analysis performed is presented on the right side. See Supplementary Table 2 for the p values of multiple comparison.

Supplementary Figure 2

a

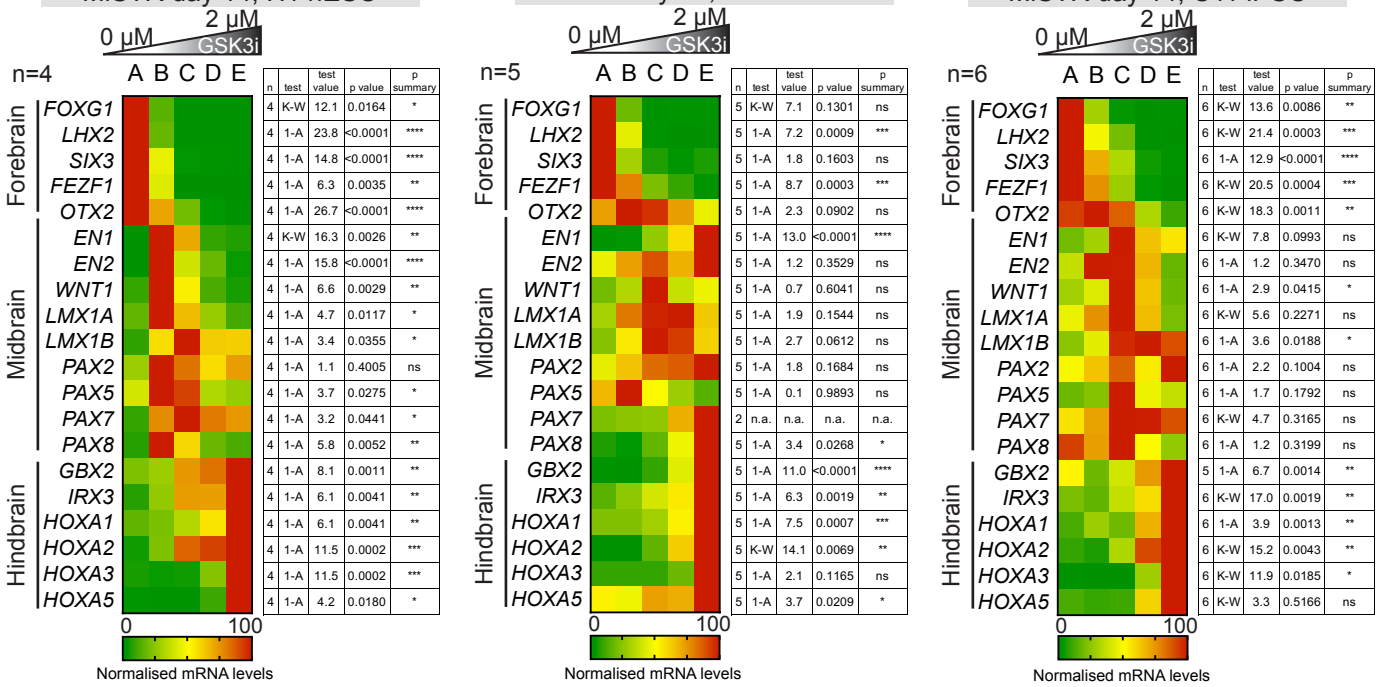


Summary of Analysis

Gene	n	test	Test value	p value	p summary	multiple comparison
OTX2 §	32	Kruskal-Wallis	88.52	<0.0001	****	Dunn's test
GBX2 §	32	1-way ANOVA	116.40	<0.0001	****	Tukey's test
post criteria						
FOXG1	24	Kruskal-Wallis	65.75	<0.0001	****	Dunn's test
LHX2	24	1-way ANOVA	133.20	<0.0001	****	Tukey's test
TBR1	24	1-way ANOVA	55.88	<0.0001	****	Tukey's test
SIX3	23	1-way ANOVA	91.50	<0.0001	****	Tukey's test
FEZF1	24	Kruskal-Wallis	89.56	<0.0001	****	Dunn's test
OTX2	24	Kruskal-Wallis	84.01	<0.0001	****	Dunn's test
EN1	24	Kruskal-Wallis	73.52	<0.0001	****	Dunn's test
EN2	24	Kruskal-Wallis	59.26	<0.0001	****	Dunn's test
WNT1	24	Kruskal-Wallis	60.54	<0.0001	****	Dunn's test
LMX1A	24	1-way ANOVA	17.62	<0.0001	****	Tukey's test
LMX1B	24	Kruskal-Wallis	69.00	<0.0001	****	Dunn's test
PAX2	17	1-way ANOVA	24.12	<0.0001	****	Tukey's test
PAX5	24	Kruskal-Wallis	45.90	<0.0001	****	Dunn's test
PAX7	18	Kruskal-Wallis	44.75	<0.0001	****	Dunn's test
PAX8	23	1-way ANOVA	31.55	<0.0001	****	Tukey's test
FGF8	24	Kruskal-Wallis	63.17	<0.0001	****	Dunn's test
FGF17	12	Kruskal-Wallis	35.47	<0.0001	****	Dunn's test
GBX2	24	1-way ANOVA	126.90	<0.0001	****	Tukey's test
IRX3	24	Kruskal-Wallis	87.76	<0.0001	****	Dunn's test
HOXA1	24	Kruskal-Wallis	85.10	<0.0001	****	Dunn's test
HOXA2	24	Kruskal-Wallis	84.01	<0.0001	****	Tukey's test
HOXA3	24	Kruskal-Wallis	75.61	<0.0001	****	Dunn's test
HOXA5	24	Kruskal-Wallis	42.29	<0.0001	****	Dunn's test

§ before Rostral-Caudal criteria

b



n	test	test value	p value	p summary
4	K-W	12.1	0.0164	*
4	1-A	23.8	<0.0001	****
4	1-A	14.8	<0.0001	****
4	1-A	6.3	0.0035	**
4	1-A	26.7	<0.0001	****
4	K-W	16.3	0.0026	**
4	1-A	15.8	<0.0001	****
4	1-A	6.6	0.0029	**
4	1-A	4.7	0.0117	*
4	1-A	3.4	0.0355	*
4	1-A	1.1	0.4005	ns
4	1-A	3.7	0.0275	*
4	1-A	3.2	0.0441	*
4	1-A	5.8	0.0052	**
4	1-A	8.1	0.0011	**
4	1-A	6.1	0.0041	**
4	1-A	6.1	0.0041	**
4	1-A	11.5	0.0002	***
4	1-A	11.5	0.0002	***
4	1-A	4.2	0.0180	*

n	test	test value	p value	p summary
5	K-W	7.1	0.1301	ns
5	1-A	7.2	0.0009	***
5	1-A	1.8	0.1603	ns
5	1-A	8.7	0.0003	***
5	1-A	2.3	0.0902	ns
5	1-A	13.0	<0.0001	****
5	1-A	1.2	0.3529	ns
5	1-A	0.7	0.6041	ns
5	1-A	1.9	0.1544	ns
5	1-A	2.7	0.0612	ns
5	1-A	1.8	0.1684	ns
5	1-A	0.1	0.9893	ns
2	n.a.	n.a.	n.a.	n.a.
5	1-A	3.4	0.0268	*
5	1-A	11.0	<0.0001	****
5	1-A	6.3	0.0019	**
5	1-A	7.5	0.0007	***
5	K-W	14.1	0.0069	**
5	1-A	2.1	0.1165	ns
5	1-A	3.7	0.0209	*

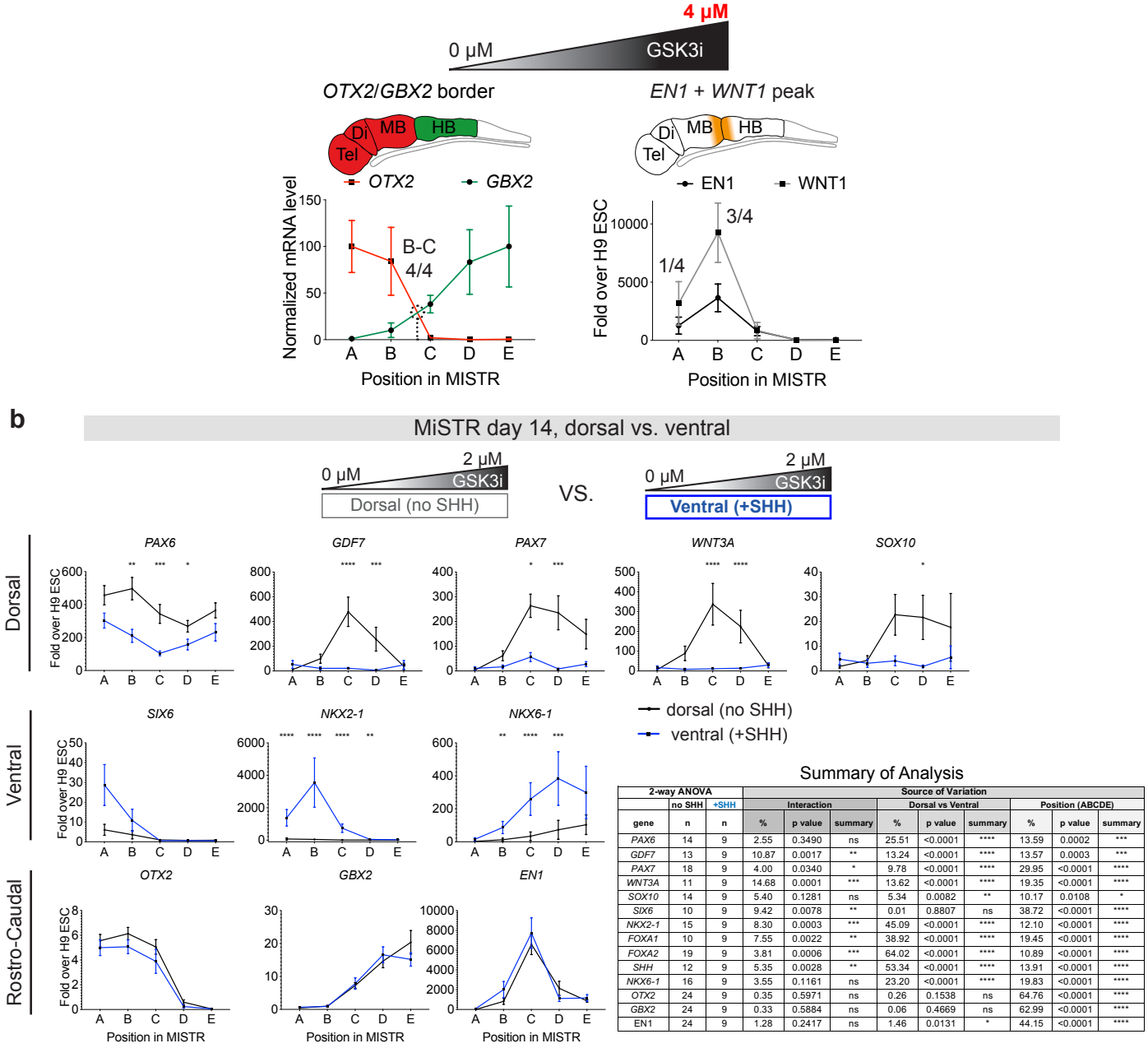
n	test	test value	p value	p summary
6	K-W	13.6	0.0086	**
6	K-W	21.4	0.0003	***
6	1-A	12.9	<0.0001	****
6	K-W	20.5	0.0004	***
6	K-W	18.3	0.0011	**
6	K-W	7.8	0.0993	ns
6	1-A	1.2	0.3470	ns
6	1-A	2.9	0.0415	*
6	K-W	5.6	0.2271	ns
6	1-A	3.6	0.0188	*
6	1-A	2.2	0.1004	ns
6	1-A	1.7	0.1792	ns
6	K-W	4.7	0.3165	ns
6	1-A	1.2	0.3199	ns
5	1-A	6.7	0.0014	**
6	K-W	17.0	0.0019	**
6	1-A	3.9	0.0013	**
6	K-W	15.2	0.0043	**
6	K-W	11.9	0.0185	*
6	K-W	3.3	0.5166	ns

Supplementary Fig. 2, related to Fig. 2

(**a**) qRT-PCR analysis along the 5 regions A-E, of forebrain, midbrain and hindbrain markers in 14 day old MiSTR tissues (0 µM to 2 µM GSK3i gradient). The p-summary of the multiple comparison test is displayed above each graph. *p < 0.05; **p < 0.01; ***p < 0.001; **** p<0.0001. Data as mean ± SEM. Summary of analysis is presented below, containing the respective n of MiSTR tissues. See also Supplementary Table 2 for p-values of multiple comparisons.

(**b**) Normalized mRNA expression of forebrain, midbrain and hindbrain markers along the 5 regions A-E of 14 day old MiSTR tissues (0 to 2 µM GSK3i gradient) derived from two hESC lines (H1, n = 4 MiSTR tissues, and RC17, n = 5 MiSTR tissues) and one hiPSC line (C11, n = 6 MiSTR tissues). Summary of statistical analysis tests are shown on the right side of each heatmap. See also Supplementary Table 2.

Supplementary Figure 3



2-way ANOVA			Source of Variation								
	no SHH	+SHH	Interaction			Dorsal vs Ventral			Position (ABCDE)		
gene	n	n	%	p value	summary	%	p value	summary	%	p value	summary
PAX6	14	9	2.55	0.3490	ns	25.51	<0.0001	****	13.59	0.0002	***
GDF7	13	9	10.87	0.0017	**	13.24	<0.0001	****	13.57	0.0003	***
PAX7	18	9	4.00	0.0340	*	9.78	<0.0001	****	29.95	<0.0001	****
WNT3A	11	9	14.68	0.0001	***	13.62	<0.0001	****	19.35	<0.0001	****
SOX10	14	9	5.40	0.1281	ns	5.34	0.0082	**	10.17	0.0108	*
SIX6	10	9	9.42	0.0078	**	0.01	0.8807	ns	38.72	<0.0001	****
NKX2-1	15	9	8.30	0.0003	***	45.09	<0.0001	****	12.10	<0.0001	****
FOXA1	10	9	7.55	0.0022	**	38.92	<0.0001	****	19.45	<0.0001	****
FOXA2	19	9	3.81	0.0006	***	64.02	<0.0001	****	10.89	<0.0001	****
SHH	12	9	5.35	0.0028	**	53.34	<0.0001	****	13.91	<0.0001	****
NKX6-1	16	9	3.55	0.1161	ns	23.20	<0.0001	****	19.83	<0.0001	****
OTX2	24	9	0.35	0.5971	ns	0.26	0.1538	ns	64.76	<0.0001	****
GBX2	24	9	0.33	0.5884	ns	0.06	0.4669	ns	62.99	<0.0001	****
EN1	24	9	1.28	0.2417	ns	1.46	0.0131	*	44.15	<0.0001	****

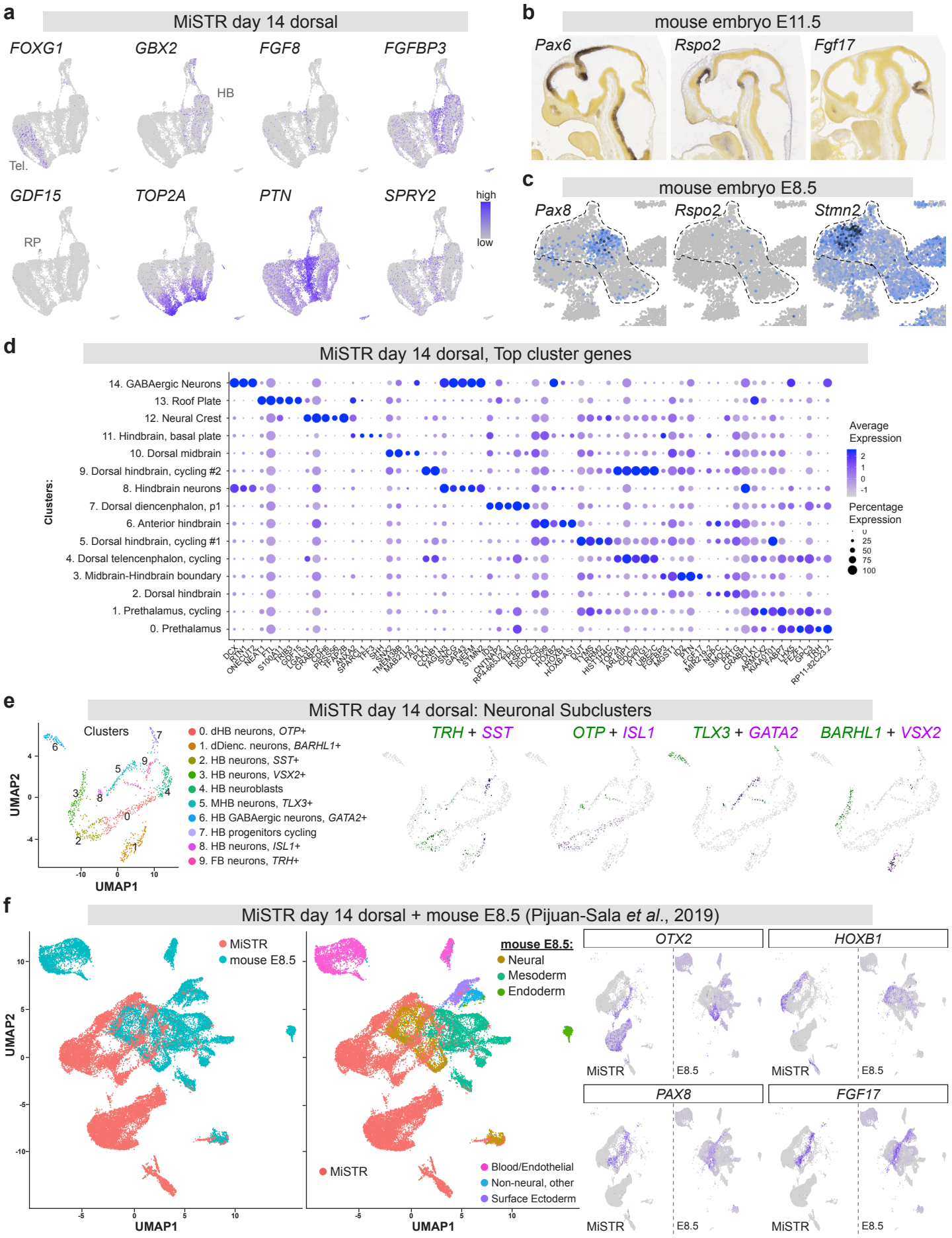
Supplementary Fig. 3, related to Fig. 2

(**a**) Normalized expression of *OTX2* and *GBX2*, and relative expression of *EN1 and WNT1* along the 5 regions A-E of 14-day MiSTR tissues (n=4) formed under the steeper 0 µM to 4 µM GSK3i gradient. The graphical depiction of the *OTX2*/*GBX2* border is displayed together with the quantification (above). Quantification of the location of EN1/WNT1 peaks is displayed above respective region. Data as mean ± SEM.

(**b**) qRT-PCR analysis of dorsal and ventral markers, along the 5 regions A-E of 14 day old dorsal (no SHH) and ventral (+SHH) MiSTR tissues under a 0 µM to 2 µM gradient. P-summary of Sidak's multiple comparison test after 2-way ANOVA between conditions at each region is displayed above each graph. *p < 0.05; **p < 0.01; ***p < 0.001; **** p<0.0001. Summary table of analysis performed for each gene and respective n of MiSTR tissues is presented. Note that *OTX2* and *GBX2* expression are almost indistinguishable between dorsal and ventral samples and only the variation along the A-E regions is revealed in the analysis. Data as mean ± SEM. See Supplementary Table 2 for the p values of multiple comparison.

(**c**) qRT-PCR analysis (normalized heatmap and line average graphs) of various RA target genes and hindbrain markers along the 5 regions A-E of 14 day old MiSTR tissues (n=4) differentiated under a RA gradient. The p-summary of the Tukey's multiple comparison test is displayed above each line graph. *p < 0.05; **p < 0.01; ***p < 0.001; **** p<0.0001. Line data as mean ± SEM. Schematic on the right shows overview of RA gradient differentiation protocol. See Supplementary Table 2 for p-values of multiple comparison.

Supplementary Figure 4



Supplementary Fig. 4, related to Fig. 3

(**a**) UMAP plot of day 14 MiSTR (n = 3, 17683 cells) scRNAseq dataset showing expression patterns of *FOXG1* (= telencephalon, Tel.), *GBX2* (= hindbrain, HB), *GDF15* (= roof plate, RP), *TOP2A* (= cycling cells) as well as *FGF8*, the FGF enhancer *FGFBP3* and FGF target genes *PTN* and *SPRY2* enriched in the MHB cluster.

(**b**) *In situ* hybridization images from sections of E11.5 mouse embryos, showing the expression of *Pax6* (neuroepithelial marker, excluded from the dorsal midbrain region), *Rspo2* (diencephalon) and *Fgf17* (midbrain-hindbrain boundary). Images are from the Allen Institute for Brain Science, available at: http://developingmouse.brain-map.org/.

(**c**) scRNAseq data of E8.5 mouse embryonic stage plotted in UMAP dimensions showing the expression of *Pax8* (midbrain), *Rspo2* (diencephalon) and *Stmn2* (neurons), within the selected neural tube region (FB/MB/HB - highlighted). Note that at this embryonic stage *Rspo2* is not yet expressed. Plots were obtained from the scRNAseq map of mouse early development[17], available at:

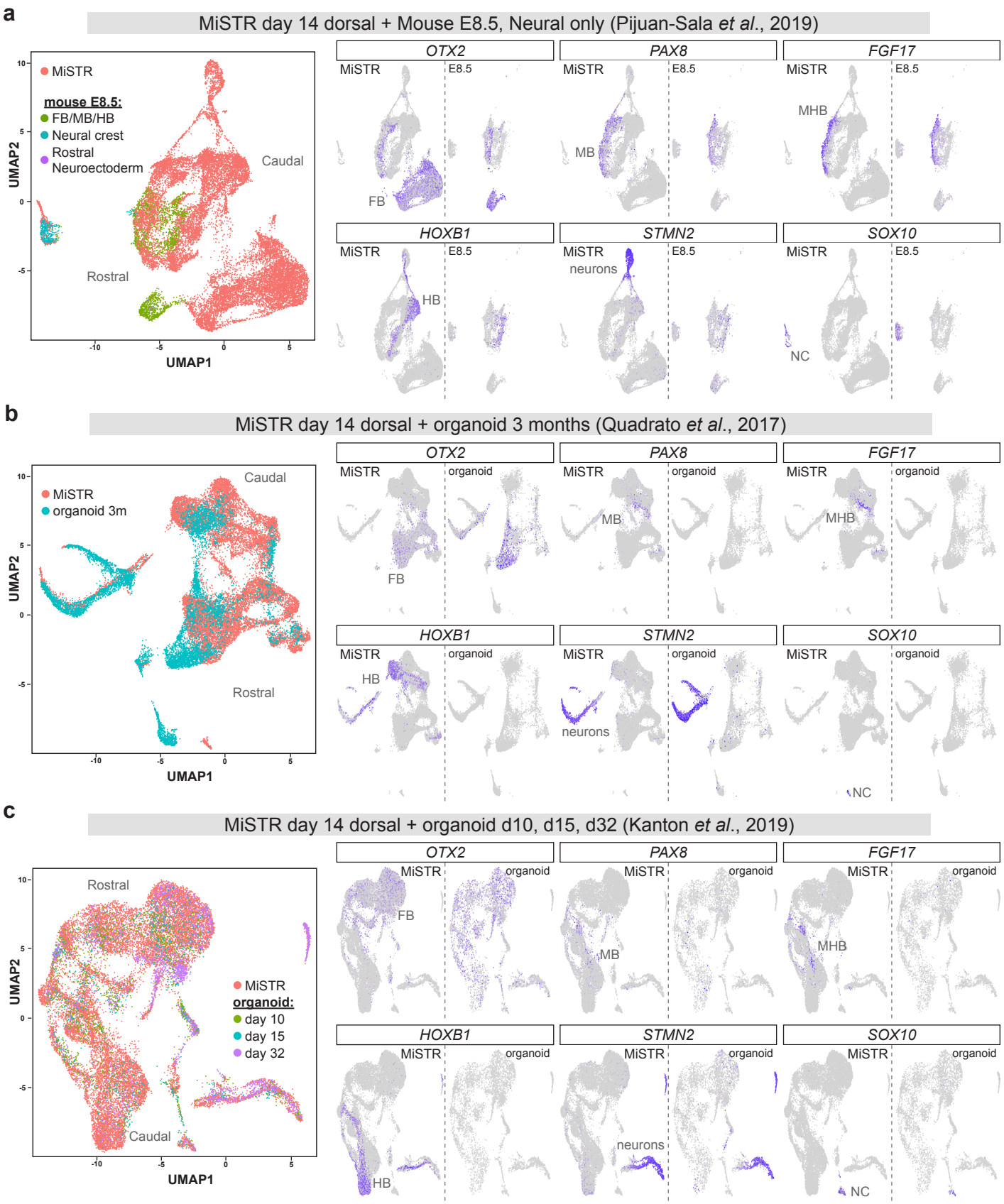
https://marionilab.cruk.cam.ac.uk/MouseGastrulation2018.

(**d**) Dot plot showing top cluster genes found from SNN-based clustering in Seurat on day 14 dorsal MiSTR scRNAseq data. Corresponding cluster map is shown in Fig 3b.

(**e**) Sub-clustering of day 14 dorsal MiSTR neuronal cells (clusters 8+14 from Fig. 3b, 1047 cells combined). for identification of neuronal subtypes and key cluster genes (see also Supplementary Table 1). dHB: Dorsal Hindbrain, dDienc.: Dorsal Diencephalic, MHB: Midbrain-Hindbrain Boundary, FB: Forebrain

(**f**) Integration of day 14 dorsal MiSTR scRNAseq data (17683 cells) with mouse embryo data (16909 cells) from E8.5[17], showing co-localisation of MiSTR cells with neural cells from the mouse. The annotations from the original paper[17] have been applied here.

Supplementary Figure 5



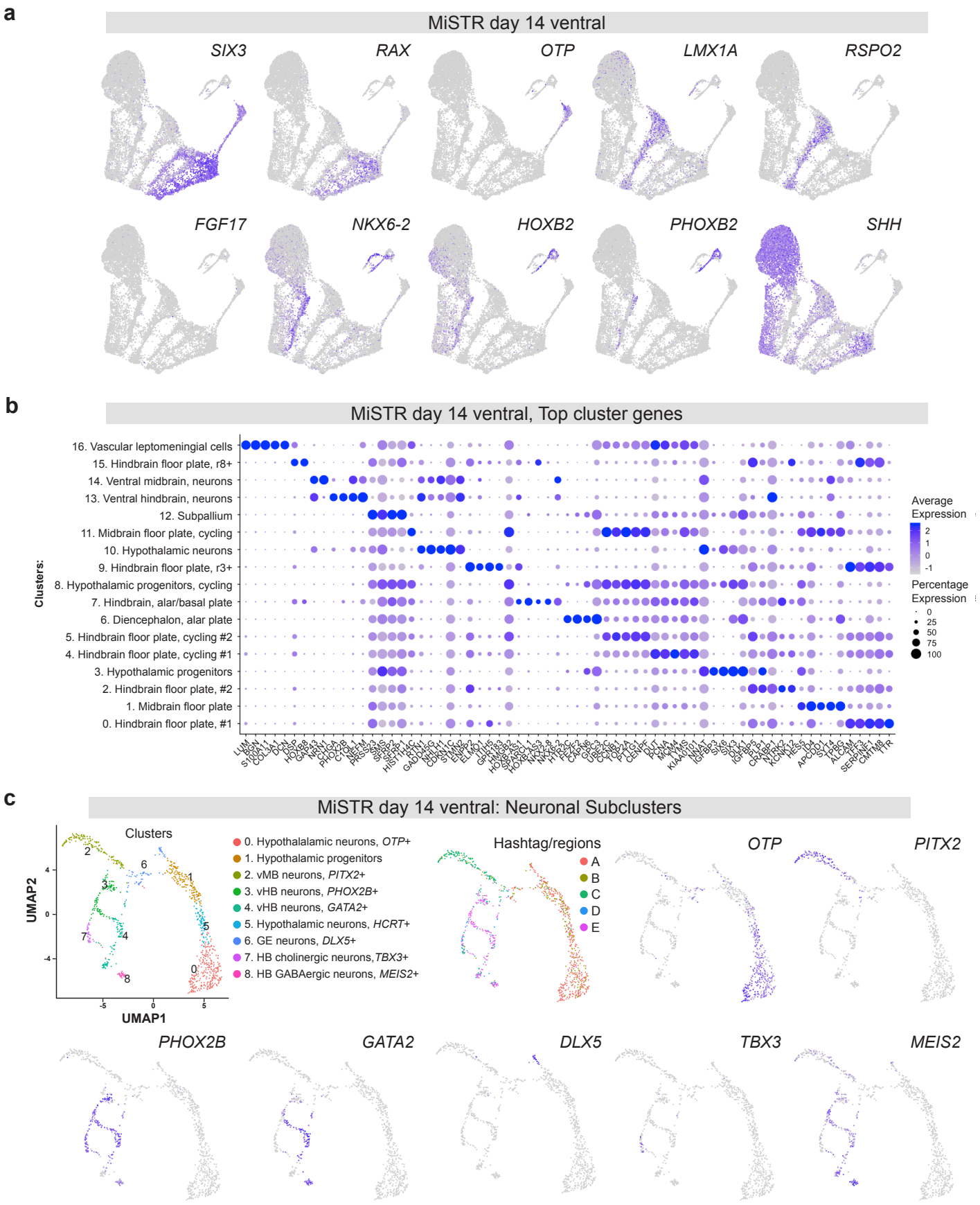
Supplementary Fig. 5, related to Fig. 3

(**a**) Integration of day 14 dorsal MiSTR scRNAseq data (17683 cells) with only CNS neural cell clusters from mouse embryo data E8.5[17], excluding spinal cord and NMPs (i.e. clusters "rostral neuroectoderm", "forebrain/midbrain/hindbrain" and "neural crest", 3089 cells). The integration shows segregation of mouse neural cells to colocalise with either forebrain, midbrain, MHB or hindbrain cells.

(**b**) Integration of day 14 dorsal MiSTR scRNAseq data (17351 cells) with human stem cell-derived organoid data (3-month old, 8426 cells)[18], showing an absence of midbrain, MHB and hindbrain markers from the organoid dataset.

(**c**) Integration of day 14 dorsal MiSTR scRNAseq data (17351 cells) with human stem cell-derived organoid data (10-day, 15-day and 32-day old, 5445 cells combined)[19], showing an absence of midbrain, MHB and hindbrain markers from the organoid dataset. FB: Forebrain, MB: Midbrain, MHB: Midbrain-Hindbrain Boundary, HB: Hindbrain, NC: Neural Crest.

Supplementary Figure 6



Supplementary Fig. 6, related to Fig. 3

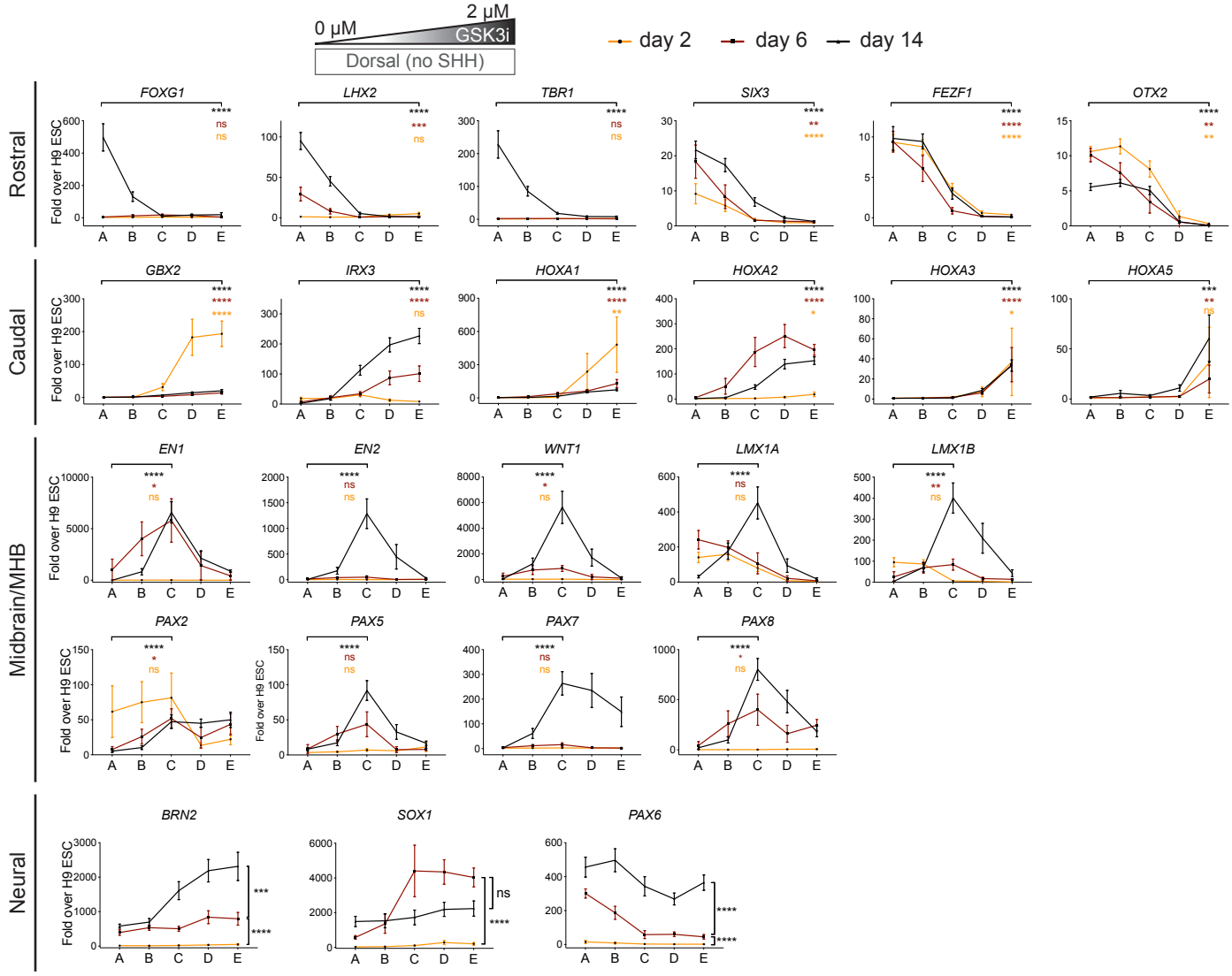
(**a**) scRNAseq of 14 day ventral MiSTR tissue (n = 3 tissues, 12669 cells) plotted in UMAP showing selected marker genes of neural patterning. Note for instance the appearance of *OTP*$^{+}$ hypothalamic neurons emanating from the *RAX*$^{+}$ cluster of hypothalamic progenitors.

(**b**) Dot plot showing top cluster genes found from SNN-based clustering in Seurat on day 14 ventral MiSTR scRNAseq data. Corresponding cluster map is shown in Fig 3f.

(**c**) Sub-clustering of day 14 ventral MiSTR neuronal cells (clusters 10+13+14 from Fig. 3f, 1016 cells combined). for identification of neuronal subtypes and key cluster genes (see also Supplementary Table 1). vMB: Ventral Midbrain, vHB: Ventral Hindbrain, GE: Ganglionic Eminence.

Supplementary Figure 7

a



b

WNT/beta catenin targets at 24h (PC2)

Rank	Gene	References	ChIP-Atlas §
1	*FST*	[24, 25]	
2	*SP5*	[19, 24, 26]	x
3	*TUBB2A*	[19, 24]	x
4	*TUBB2B*	[24]	x
5	*CCND1*	[19, 24, 27]	x
6	*RBP1*	[19, 24, 28]	x
7	*GBX2*	[19, 29]	x
8	*CXXC5*	[19, 26]	x
9	*NQO2*		x
12	*NKD1*	[19, 24]	x
13	*PLA2G2A*	[30]	x
14	*VCAN*	[19, 31]	x

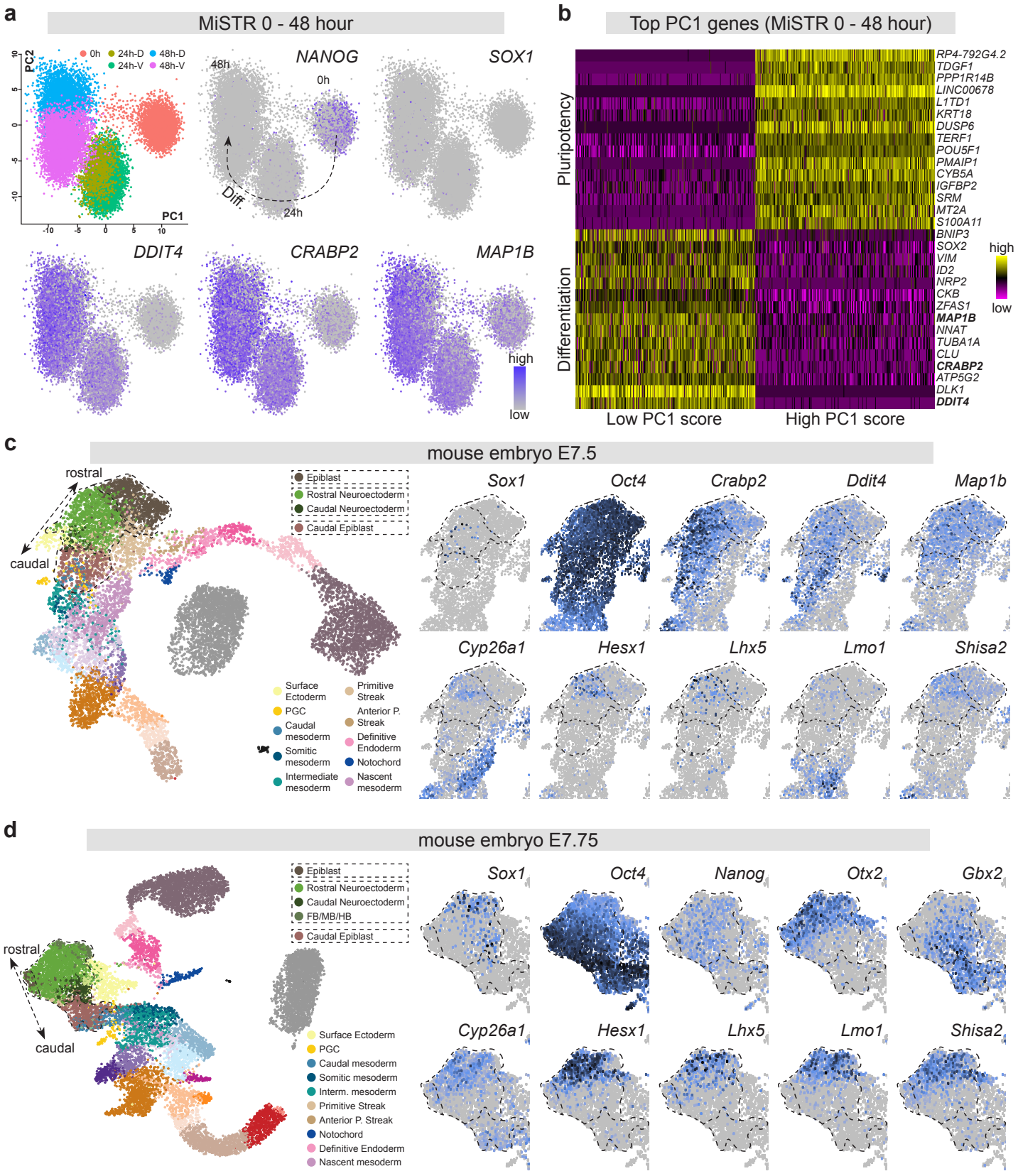
§ Identified on chip-atlas.org

Supplementary Fig. 7, related to Fig. 4

(**a**) qRT-PCR analysis of rostral, caudal, midbrain/MHB and neural markers, along the A-E regions at various time points (day 2, day 6 and day 14), in dorsal (no SHH) MiSTR tissues under a 0 μM to 2 μM gradient. For rostral, caudal and Midbrain/MHB markers: Tukey's or Dunn's multiple comparison test between all regions (A-E) within each timepoint. Comparisons A – E (for Forebrain and Hindbrain markers) and A-C (for Midbrain markers) are shown. For neural markers: 2-way ANOVA (timepoints x regions A-E) followed by Tukey's multiple comparison test between timepoints only. Data as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p<0.0001. See Supplementary Table 2 for summary of analysis and respective n of MiSTR tissues for each marker.

(**b**) List of WNT/β-catenin target genes identified within the 15 top loading negative score genes of PC2 (caudal associated) of 24 hour caudal MiSTR tissue (from **Fig. 4c**). Genes identified as predicted targets on the public Chromatin Immuno-Precipitation database ChIP-Atlas (available at https://chip-atlas.org, entering "*H. sapiens"* as species and "CTNN1B" as antigen for query) and/or through literature search are displayed.

Supplementary Figure 8



Supplementary Fig. 8, related to Fig. 4

(**a**) PCA of scRNAseq data from MiSTR differentiation at 0, 24 and 48 hours, of dorsal (D) and ventral (V) MiSTR tissue (n =3 MiSTR tissues for each of the five conditions, 28555 cells combined). *NANOG* expression indicates the differentiation path of MiSTR tissue from left to right on the PC1 axis. Plots of selected top loading negative genes on PC2 (*DDIT4*, *CRABP2* and *MAP1B*, i.e. genes associated with differentiation) reveal increasing expression of these genes from 0 to 48 hours. In contrast, the pan-neuroectodermal marker *SOX1* is not yet expressed at 48 hours of MiSTR differentiation.

(**b**) Heatmap (yellow, high expression; purple, low expression) of the top 15 positively- and negatively loading genes on principal component 1 (PC1) in 0 - 48 hour MiSTR tissues, i.e. low PC1 score = differentiation and high PC1 score = pluripotency. The top 500 cells with lowest and highest PC1 scores, respectively, are shown in columns. Top genes displayed in **a)** are highlighted in bold.

(**c**) scRNAseq of the E7.5 mouse embryo plotted in UMAP dimensions showing the expression of the top loading PC1 genes identified during the 0 to 48-hour MiSTR differentiation. Differentiation-associated genes identified in MiSTR (i.e. *Ddit4*, *Crabp2* and *Map1b*), are not specific to neural fates but encompass both the epiblast and neuroectodermal compartments in the mouse. The pan-neuroectodermal marker *Sox1* is only expressed in few cells at this time point. In contrast, early rostral neuroectoderm markers identified in Fig. 4f (*Cyp26a1, Hesx1, Lhx5, Lmo1* and *Shisa2*) are exclusively expressed in mouse *OTX2*$^{+}$ presumptive neuroectodermal cells. Note that the mouse cluster annotated as "rostral neuroectoderm" contains both *OTX2*$^{+}$ and *GBX2*$^{+}$ cells, i.e. covering both presumptive forebrain, midbrain and hindbrain cells. Plots were obtained from the scRNAseq map of mouse early development[17], available at: https://marionilab.cruk.cam.ac.uk/MouseGastrulation2018.

(**d**) scRNAseq of the E7.75 mouse embryo plotted in UMAP dimensions showing the expression early rostral neuroectoderm markers obtained from analysis of 0 – 48 hour MiSTR scRNAseq dataset (Fig. 4f), within the selected regions, highlighted in the overall map (left). Early rostral neuroectodermal markers identified in MiSTR data (i.e. *Cyp26a1, Hesx1, Lhx5, Lmo1* and *Shisa2*) are strongly upregulated in only the OTX2+ compartment of the mouse neuroectodermal cells at E7.75. Plots were obtained from the scRNAseq map of mouse early development[17], available at: https://marionilab.cruk.cam.ac.uk/MouseGastrulation2018.

Supplementary Table 1

Supplementary Table 1 is provided as a separate .xlsx File:

“Supplementary Table 1 scRNAseq markers.xlsx”

This table contains the marker genes for the cell clusters obtained for the following datasets: whole day 14 dorsal MiSTR, whole day 14 ventral MiSTR, neuronal sub-clustering of day 14 dorsal MiSTR, neuronal sub-clustering of day 14 ventral MiSTR, 0 – 48 hours MiSTR (data in Figures 3b, 3f 4b, 4e and 4f and Supplementary Figures 4d, 4e, 6b, 6c, 8a and 8b).

Supplementary Table 2

Supplementary Table 2 is provided as a separate .xlsx File:

“Supplementary Table 2 Summary Statistics.xlsx”

This table contains the extended summary of the statistical analyses graphically displayed in the main text and supplementary info: Figure 1e, 1i, Figure 2d, 2i, 2k, Figure 4a, and Supplementary Figures 1d, 1e, 2a, 2b, 3b, 3c, 7a. It also contains descriptive statistics for violin plots on Figure 4f.

Supplementary Table 3. Primary Antibodies used for immunostaining

Epitope	Species	Source	Cat #	RRID	Dilution
NKX2-1	Mouse	AbCam	ab133737	N/A	1:200
OTX2	Goat	R&D Systems	AF1979	AB_2157172	1:500
PAX8	Rabbit	ProteinTech	10336-1-AP	AB_2236705	1:100

Supplementary Table 4. Human primer sequence for quantitative real time PCR

Primer	Gene (full name)	Forward Primer	Reverse Primer
ACTB	Actin beta	CCTTGCACATGCCGGAG	GCACAGAGCCTCGCCTT
ALDH1A2	aldehyde dehydrogenase 1 family member A2	GTTGAGCCACGGTCCTTACTTA	GCTTTAGTTGTGCAGTGACCTG
AXIN2	axin 2	GAAACCATGCCCAGCGAGCAGT	CTCCGTGCCTTTCCCATTGCGT
BRA	T brachyury transcription factor	AAAAATGTTTGCCAGGGTCCAG	TGCCAAAGTTGCCAATACACTG
BRN2	*POU3F2*, POU class 3 homeobox 2	ATGCGCGGCTCCTTTAACCGG	TTAGACGCTGCGGTCGCCATG
CCND1	cyclin D1	GATGGGGCAAGGGCACAAGTCC	CCTCAGACTTGCGCGTCACAGG
CDH2	Cadherin 2	ACAATTGCTGTTTTGGACCGAG	AGCGTTCCTGTTCCACTCATAG
CXCR4	C-X-C motif chemokine receptor 4	CACCGCATCTGGAGAACCA	GCCCATTTCCTCGGTGTAGTT
CYP26A1	cytochrome P450 family 26 subfamily A member 1	CCTTAGGAGCTGTGTAGGCAAA	CTGGCCAGCTCCACTGTAAATA
EN1	engrailed homeobox 1	CGTGGCTTACTCCCCATTTA	TCTCGCTGTCTCTCCCTCTC
EN2	engrailed homeobox 2	CCTCCTGCTCCTCCTTTCTT	GACGCAGACGATGTATGCAC
EOMES	eomesodermin	GCGAGAGAACCGTGCCACAGAC	GCCACCTCTTCGCTCTGTTGGG
FEZF1	FEZ family zinc finger 1	GGTACATTCCACATTCGTGAGC	TCACGTGCAATAATCAAAACCA
FGF17	Fibroblast growth factor 17	AAATCTGCTTCTCGGATCTCCC	CTACAGTCTAGCCAGGAGGAGT
FGF8	fibroblast growth factor 8	ACAGCGCTGCAGAATGCCAAGT	GAAGTGGACCTCACGCTGGTGC
FOXA1	forkhead box A1	GGGCAGGGTGGCTCCAGGAT	TGCTGACCGGGACGGAGGAG
FOXA2	forkhead box A2	CCGTTCTCCATCAACAACCT	GGGGTAGTGCATCACCTGTT
FOXG1	forkhead box G1	TGGCCCATGTCGCCCTTCCT	GCCGACGTGGTGCCGTTGTA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	TTGAGGTCAATGAAGGGGTC	GAAGGTGAAGGTCGGAGTCA
GBX2	gastrulation brain homeobox 2	GTTCCCGCCGTCGCTGATGAT	GCCGGTGTAGACGAAATGGCCG
GDF7	growth differentiation factor 7	GACGCTGCTCAACTCCATGGCA	TTGGCGGCGTCGATGTAGAGGA
GSC	goosecoid homeobox	AGACCAAGTACCCGGACGTGGG	TCTCCTCGCGGAGGTGCACTTT
HOXA1	homeobox A1	GTACGGCTACCTGGGTCAAC	ACTTGGGTCTCGTTGAGCTG
HOXA2	homeobox A2	CGTCGCTCGCTGAGTGCCTG	TGTCGAGTGTGAAAGCGTCGAGG
HOXA3	homeobox A3	GGCCAATCTGCTGAACCTCA	GAGTTCAGATAGCCACCGGC
HOXA4	homeobox A4	ACGCTCTGTTTGTCTGAGCGCC	AGAGGCCGAGGCCGAATTGGA
HOXA5	homeobox A5	TCATAGTTCCGTGAGCGAGC	ATCCATGCCATTGTAGCCGT
HOXB1	homeobox B1	GGCCTTCTCAGTACTACCCTCT	CCGTAGCTCGAGGGATGAAAAT
HOXB3	homeobox B3	ATGAACGCCTTACACTCCATGA	ATTCTGGTGGGCTTTACCGAAG
HOXB4	homeobox B4	TGCTTGAGTCTAGAACCCTTCG	TGCTGGTCACAAGAAACCAAAC
HOXB6	homeobox B6	CACGGTTCAATGGTAGATTCGC	GGTAGTATGTGCTCCTTCCAGT
HOXC4	homeobox C4	TAGGAGGGCTTTATGGAGCAGA	TCGGATCGATGTAGTTAGAGTCCA
IRX3	iroquois homeobox 3	GGCTTGCGCCCCGTAGAAATGT	AGGAGCCAGGTCAGGTCCGAAC
LEF1	lymphoid enhancer binding factor 1	GGTCCTCCTGGTCCCCACACAA	TCATGCTGAGGCTTCACGTGCA
LHX2	LIM homeobox 2	GGGCGACCACTTCGGCATGAA	CGTCGGCATGGTTGAAGTGTGC
LMX1A	LIM homeobox transcription factor 1 alpha	CGCATCGTTTCTTCTCCTCT	CAGACAGACTTGGGGCTCAC
LMX1B	LIM homeobox transcription factor 1 beta	CTTAACCAGCCTCAGCGACT	TCAGGAGGCGAAGTAGGAAC
MIXL1	Mix paired-like homeobox	CCGAGTCCAGGATCCAGGTA	CTCTGACGCCGAGACTTGG
NANOG	Nanog homeobox	TTGGGACTGGTGGAAGAATC	GATTTGTGGGCCTGAAGAAA
NKX2-1	NK2 homeobox 1	AGGGCGGGGCACAGATTGGA	GCTGGCAGAGTGTGCCCAGA
NKX6-1	NK6 homeobox 1	GGATCCCAACTCGGACGACGAGA	AGGATGAGCTCTCCGGCTCGG
OCT4	*POU5F1*, POU class 5 homeobox 1	TCTCCAGGTTGCCTCTCACT	GTGGAGGAAGCTGACAACAA
OTX2	orthodenticle homeobox 2	ACAAGTGGCCAATTCACTCC	GAGGTGGACAAGGGATCTGA
PAX2	paired box 2	CTTGATGCCCCCTCCCCCGA	CCACTTCCACCCCGCAGCAG
PAX5	paired box 5	CCCCATTGTGACAGGCCGTGAC	TCAGCGTCGGTGCTGAGTAGCT
PAX6	paired box 6	TGGTATTCTCTCCCCCTCCT	TAAGGATGTTGAACGGGCAG
PAX7	paired box7	CTTCAGTGGGAGGTCAGGTT	CAAACACAGCATCGACGG
PAX8	paired box 8	ATAGCTGCCGACTAAGCATTGA	ATCCGTGCGAAGGTGCTTT
RARA	retinoic acid receptor alpha	AAGATTACTGACCTGCGAAGCA	GGATCTCCATCTTCAGCGTGAT
SHH	sonic hedgehog	CCAATTACAACCCCGACATC	AGTTTCACTCCTGGCCACTG
SIX3	SIX homeobox 3	ACCGGCCTCACTCCCACACA	CGCTCGGTCCAATGGCCTGG
SIX6	SIX homeobox 6	CTCAACAAGAATGAGTCGGTGC	ACTCCTTGGTGAACTTGTGGTT
SOX1	SRY-box 1	GGGAAAACGGGCAAAATAAT	TTTTGCGTTCACATCGGTTA
SOX10	SRY-box 10	CTTTCTTGTGCTGCATACGG	AGCTCAGCAAGACGCTGG
SOX17	SRY-box 17	AAGGGCGAGTCCCGTATC	TTGTAGTTGGGGTGGTCCTG
SOX2	SRY-box 2	CATGGCAATCAAAATGTCCA	TTTCACGTTTGCAACTGTCC
TBR1	T-box, brain 1	TCGTCCCCGCTCAAGAGCGA	CCTTGGCGCAGTTCTTCTCGCA
WNT1	Wnt family member 1	GAGCCACGAGTTTGGATGTT	TGCAGGGAGAAAGGAGAGAA
WNT3A	Wnt family member 3A	GCGATGGCCCCACTCGGATACT	TAGCTGCCCAGAGCCTGCTTCA

Supplementary Table 5. Summary of Single-Cell RNA-seq

	Day 0	24 hours		48 hours		day14 dorsal			day 14 ventral
		dorsal	ventral	dorsal	ventral	run1	run2	run3	(5x hashtags)
Number of cells captured	8160	1018	8850	4266	6325	7076	5246	5919	16744
Median genes per cell	2889	3196	3200	1694	3517	2644	2722	3183	3903
Mean reads per cell	23933	191636	25782	51531	43360	32035	33131	54781	43378
Median UMIs per cell	8497	12045	11426	4646	14787	9115	9477	11961	13643
Total genes detected	22501	20216	22647	20897	22428	22871	22070	23029	25638

SUPPLEMENTARY Notes

Péclet number calculations; Fluorescein Sodium Salt and GSK3i.

Considering a molecule like fluorescein sodium salt (FSS) flowing inside the cell culture chamber with the following dimensions:

$$\begin{cases} width = 2 * 10^{-2}\, m \\ length = 1 * 10^{-2}\, m \\ height = 5 * 10^{-4} m \end{cases}$$

Then we have a cross sectional area A given by:

$$A = width * height$$

$$= 1 * 10^{-5}\, m^2$$

The average flow speed v inside the cell culture chamber can be calculated from the total flow rate Q.

$$v = \frac{Q}{A} = \frac{160}{1 * 10^{-5}} \frac{\frac{\mu l}{h}}{m^2} = \frac{4.44 * 10^{-11}}{1 * 10^{-5}} \frac{\frac{m^3}{s}}{m^2}$$

$$= 4.44\, \frac{\mu m}{s}$$

The hydraulic diameter d_h of the cell culture chamber is given by:

$$d_h = \frac{4A}{P} = \frac{4(width * height)}{2(width + height)} = \frac{2 * (1 * 10^{-5})\, m^2}{(2 * 10^{-2} + 5 * 10^{-4})\, m}$$

$$= 976\, \mu m$$

where P is the wetted perimeter of the channel.

The Péclet number for FSS is given by:

$$P\acute{e} = \frac{diffusion\ time}{convection\ time} = \frac{\frac{(L)^2}{D}}{\frac{L}{v}} = \frac{v * L}{D} = \frac{4.44 * 10^{-6} * 9.76 * 10^{-4}}{7.3 * 10^{-10}} \frac{\frac{m}{s} * m}{\frac{m^2}{s}}$$

$$= 5.74$$

where L is the distance the molecule can travel by either diffusion or convection and D is the diffusion coefficient for the molecule ($D_{FSS} = 7.55 * 10^{-10} m^2/s$). We set L to be equal to d_h.

For GSK3i with a smaller diffusion coefficient that FSS ($D_{GSK3i} = 6.60 * 10^{-10} m^2/s$), the Péclet number is instead equal to: $P\acute{e} = 6.57$.

For RA with a smaller diffusion coefficient than that of FSS ($D_{RA} = 6.67 * 10^{-10} m^2/s$), the Péclet number was also >1, equal to: $P\acute{e} = 6.50$.

Shear Stress calculations for NPM cell culture medium.

The shear stress reported to affect the differentiation of stem cells and progenitor cells are in the order of $1 - 10\ dynes/cm^2$ [37], and other reported physiological shear stresses are within these orders of magnitude. For instance, the wall shear stresses in a human aorta was estimated to be in the range of $4.7 - 10.4\ dynes/cm^2$ [38], while wall shear stress of urine flow in the proximal renal tubule was reportedly to be of 0.17 $dynes/cm^2$ [39].

The wall shear stress in a parallel plate flow chamber is given by[40]:

$$\tau_{wall} = \frac{6\mu Q}{h^2 w}\ [dynes/cm^2]$$

where μ is the dynamic viscosity of the fluid, Q is the flow rate, h is the height of the culture chamber and w is the width of the culture chamber. The cross-sectional area of the culture chamber has a high aspect ratio (width/height = 40) which could be approximated with the parallel plate model.

The dynamic viscosity of the neural proliferation media (NPM) was estimated at 37℃ by a falling-ball viscometer:

$$\mu_{NPM}^{37\,℃} = 0.77 * 10^{-3}\ Pa * s$$

similar to water ($\mu_{Water}^{37\,℃} = 0.69 * 10^{-3}\ Pa * s$).

Given this value of the dynamic viscosity along with previously stated parameters, it is possible to make an estimation of the wall shear stress,τ_{wall}, on the cells under flow:

$$\tau_{wall} = \frac{6\mu Q}{h^2 w} = \frac{6 * 0.77 * 10^{-3} * 4.44 * 10^{-11}}{(5 * 10^{-4})^2 * 2 * 10^{-2}}\ \frac{Pa * s * \frac{m^3}{s}}{m^3} = 4.1 * 10^{-5}\ Pa$$

$$\approx 4 * 10^{-4}\ dynes/cm^2$$

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