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Supplemental Information

EPHRIN-B1 Mosaicism Drives Cell Segregation in Craniofrontonasal Syndrome hiPSC-Derived Neuroepithelial Cells

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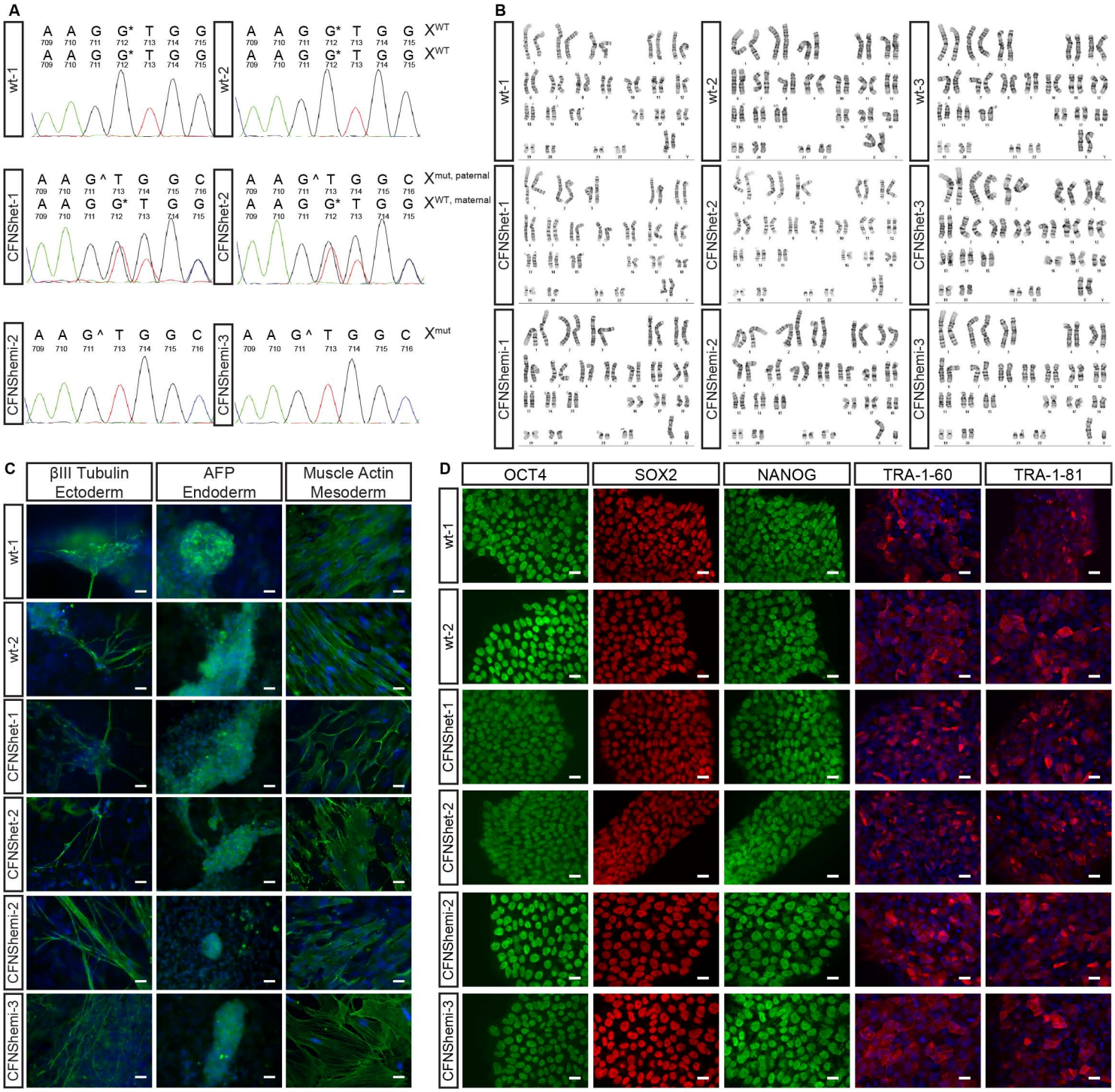

Figure S1. ***Related to Figure 1.*** **Characterization of CFNS patient-derived hiPSCs. (A)** Sequencing chromatograms show retention of G712 in wt hiPSC lines, heterozygous deletion of G712 in CFNShet lines, and deletion of G712 in CFNShemi lines. **(B)** G-banded karyotype analysis shows that all nine hiPSC lines have normal karyotypes (46, XX for female wild type and CFNS heterozygous lines and 46, XY for male CFNS hemizygous lines). **(C)** Upon in vitro embryoid body differentiation, *EFNB1*$^{+/+}$ lines (wt-1, -2), *EFNB1*$^{+/c.712delG}$ lines (CFNShet-1, -2), and *EFNB1*$^{Y/c.712delG}$ lines (CFNShemi-2, -3) demonstrated differentiation potential to ectoderm (βIII tubulin), endoderm (alpha-fetoprotein, AFP), and mesoderm (muscle actin). Samples were counterstained with DAPI (blue). Scale bars, 20 µm. **(D)** Immunocytochemical characterization of *EFNB1*$^{+/+}$ lines (wt-1, -2), *EFNB1*$^{+/c.712delG}$ lines (CFNShet-1, -2), and *EFNB1*$^{Y/c.712delG}$ lines (CFNShemi-2, -3) reveals positive staining for the nuclear factors OCT4, SOX2, and NANOG, as well as the surface antigens TRA-1-60 and TRA-1-81 (counterstained with DAPI (blue)). Scale bars, 20 µm.

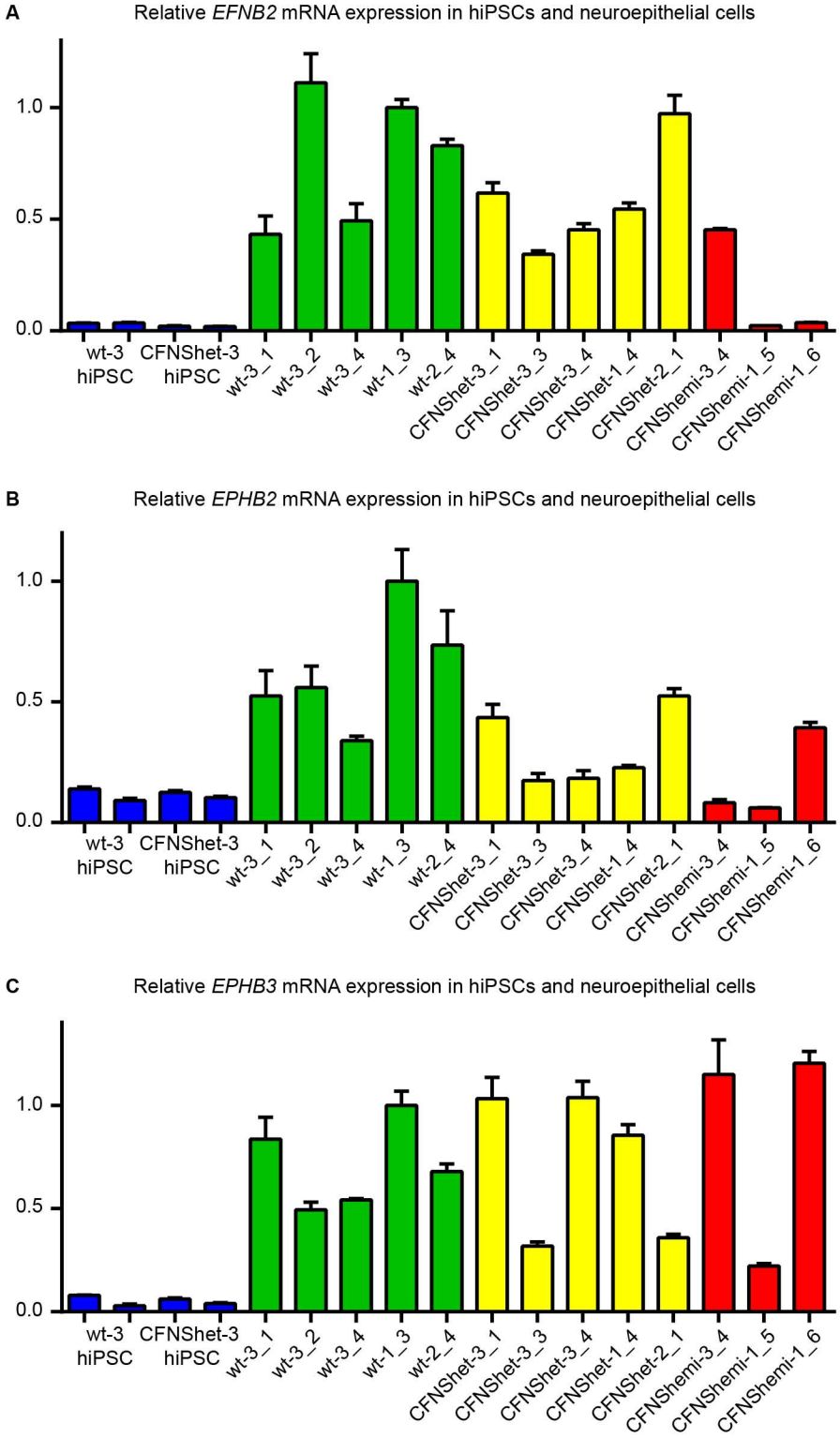

Figure S2. ***Related to Figure 2.*** **Expression of additional EphB/ephrinB signaling family members in hiPSCs and hNE.** qRT-PCR demonstrates expression levels of **(A)** *EFNB2*, **(B)** *EPHB2*, and **(C)** *EPHB3* in hNE cells of all three genotypes. Very low expression of each of these Eph/ephrin signaling family members is seen in hiPSCs. Expression of each Eph/ephrin signaling family member in each sample was normalized to expression of GAPDH. Error bars represent the standard deviation of three technical replicates per hiPSC or hNE line.

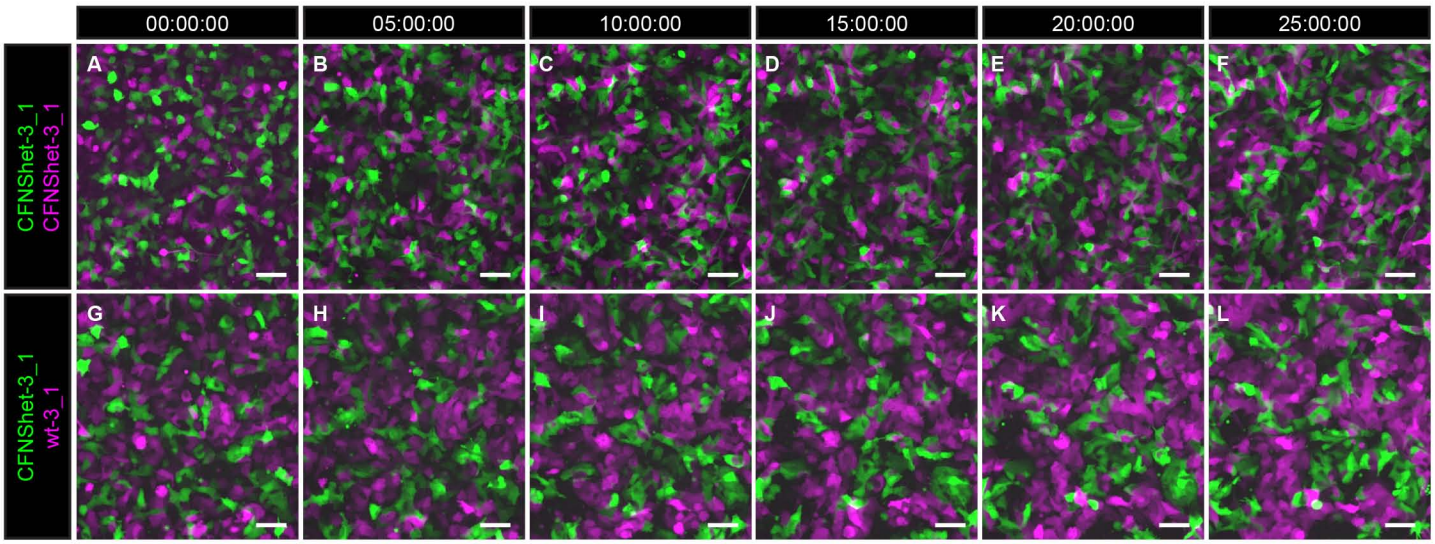

Figure S3. ***Related to Figure 3.*** **Live imaging of hNE cell mixing experiments. (A-F)** Live imaging of a mixture of EPHRIN-B1 non-expressing cells (CFNShet-3+CFNShet-3) over 25 hours demonstrates that EPHRIN-B1 non-expressing cells remain intermixed as they continue to interact with each other, and are not segregating at 25 hours **(F)**. **(G-L)** Live imaging of a cell mixture mosaic for EPHRIN-B1 expression (CFNShet-3+wt-3) shows that cells are intermixed at the beginning of the experiment **(G)**, but segregate from each other over time, and are already forming patches of EPHRIN-B1 expressing and non-expressing cells at 25 hours **(L)**. Scale bars, 50 μm.

Table S1. ***Related to Figure 1.*** **HUMARA demonstrates clonal inactivation of maternal (wild type) X chromosome in each CFNShet hiPSC line.**

	Peak Areas Undigested Samples		**Peak Areas** Digested Samples		**Possible Values of X_A** (Fraction of X activation)		**Ratio of X_A^{wt} to X_A^{mut}** (Average % X activation)	
	Maternal	Paternal	Maternal	Paternal	X_A, maternal	X_A, paternal	X_A, maternal	X_A, paternal
CFNShet HDFs	64276	61770	9880	19316	0.65	0.35	61	39
	51069	43431	16592	24562	0.56	0.44		
					0.63	0.37		
					0.58	0.42		
CFNShet-1 hiPSCs	59768	205805	28403	0	0.00	1.00	0	100
	17428	57018	4606	0	0.00	1.00		
					0.00	1.00		
					0.00	1.00		
CFNShet-2 hiPSCs	69895	230728	183006	0	0.00	1.00	0	100
	41823	157233	173086	0	0.00	1.00		
					0.00	1.00		
					0.00	1.00		
CFNShet-3 hiPSCs	50731	158352	42899	0	0.00	1.00	0	100
	53890	163248	94864	0	0.00	1.00		
					0.00	1.00		
					0.00	1.00		

Supplemental Experimental Procedures

hiPSC generation and culture. A small dermal tissue sample was collected from an excess specimen at the time of a surgical procedure of the 10-month-old female CFNS proband. Punch biopsies were obtained from the proband's father and mother. Primary human fibroblast cultures were established and cultured on plastic culture dishes in DMEM high glucose (Life Technologies) containing 10% FBS (HyClone), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, and penicillin-streptomycin-fungizone. Human iPSCs were generated using episomal reprogramming (Bershteyn et al., 2014; Okita et al., 2011). Briefly, one microgram of each of the Y4 combination of episomal reprogramming factors (Addgene 27078, 27080, 27082) was electroporated into 3 $x10^5$ fibroblasts (passage 5-6) with the Neon Electroporation Device (Invitrogen) using the 100-uL kit and conditions of 1650 V, 10 ms, and three pulses. Cells were detached 6 days after electroporation and seeded at 1.5 x 10^5 cells per 10-cm dish onto irradiated mouse embryonic fibroblasts (Globalstem). On day 7, media was changed from fibroblast media to KnockOut™ ESC/hiPSC culture media containing 4 ng/mL bFGF (Life Technologies), and cells were cultured for a further 18-25 days. Colonies with hiPSC-like morphology were manually selected under a dissecting microscope and subcultured on irradiated MEFs. By passage four, hiPSCs were transferred to feeder-free conditions and cultured in mTeSR1 medium (STEMCELL Technologies) on dishes coated with hESC-qualified Matrigel (Corning).

hiPSC characterization. G-banded karyotype analysis of hiPSC lines was performed after passage 9 by WiCell Research Institute (Madison, WI). To confirm *EFNB1* genotypes, DNA samples were isolated from both HDF and hiPSC lines with a DNeasy Blood and Tissue Kit (Qiagen) and sequenced by SeqWright, Inc. (Houston, TX). To assay for plasmid integration, DNA samples were amplified by PCR with *EBNA-1* plasmid backbone-specific primers and normalized to *GAPDH* as a loading control (see below for primer information). PCR products were resolved against a positive control plasmid diluted to the equivalent of 1 and 0.2 copies plasmid/diploid genome. Female HDF and hiPSC cultures were assayed for relative inactivation of each X-chromosome with the HUMAR assay (Kiedrowski et al., 2011). Briefly, capillary electrophoresis was performed on genomic DNA samples both digested and undigested with the methylation-sensitive restriction enzyme HpaII, which selectively digests the active X chromosome. Each allele is represented as a separate peak in the capillary electrophoresis trace based on the differential number of CAG repeats at the human androgen receptor locus on each X-chromosome. Areas under the peak for each allele were measured on Peak Scanner™ (Applied Biosystems) for both undigested and digested samples, and these peak areas were used to calculate X_A (fraction of expression from a given X chromosome).

hNE cell differentiation and culture. hNE cell differentiations from hiPSCs were performed using a monolayer dual-SMAD inhibition protocol (Chambers et al., 2009), with some modifications. hiPSCs were plated on hESC-qualified Matrigel (Corning)-coated dishes at a density of 2.5 x 10^5 cells/cm^2 (day 0) in STEMdiff Neural Induction Medium (NIM; STEMCELL Technologies) supplemented with penicillin-streptomycin (P/S) and 10 μM Y-27632 (Santa Cruz Biotechnology) to increase cell survival as single cells (Watanabe et al., 2007). Daily media changes were made with NIM supplemented with P/S, 10 μM SB-431542 (Santa Cruz Biotechnology), and 5 μM DMH1 (Sigma) (Neely et al., 2012) on days 1-3 and NIM + P/S alone on days 4 onward. After 8-10 days in culture, cells were dissociated to a single-cell suspension in Accutase and replated on Matrigel-coated dishes at a 1:1 dilution in NIM supplemented with P/S and 10 μM Y-27632. hNE cell cultures were maintained in NIM with or without SB-431542 and DMH1 and split at ratios of 1:1 to 1:3 until experimentation.

hNE cell segregation assays. EPHRIN-B1-expressing and EPHRIN-B1-non-expressing hNE cells were either labeled with CellTracker dye CMFDA (Molecular Probes) for 45 minutes at a concentration of 5μM in NIM supplemented with penicillin-streptomycin (P/S) or infected with adenovirus Ad-CMV-eGFP or Ad-CMV-mCherry (Vector Biolabs) overnight at a concentration of 1-5 x 10^6 IFU/cm^2 in NIM + P/S, followed by incubation in NIM + P/S for 2 additional days. hNE cells from differentially labeled lines were mixed at a concentration of 5 x 10^5 cells/line and plated on Matrigel-coated 24-well glass-bottom dishes (MatTek), for a total of 1 x 10^6 cells per well. Cells were imaged at 48 hours after mixing on a Zeiss Cell Observer spinning disc confocal microscope to assess cell segregation. For live imaging of cell segregation, cell mixing experiments were set up as described above and plated in 4-chamber glass-bottom dishes (Greiner Bio-One), with cell number adjusted to achieve the same density. 15 mM HEPES (UCSF Cell Culture Facility) was added to cell media to facilitate buffering outside the CO_2 incubator during the imaging process. Cell mixtures were imaged at twenty minute intervals over 25 hours after mixing using a Zeiss Cell Observer spinning disc confocal microscope.

Immunocytochemistry. Cells were plated on Matrigel-coated glass coverslips and fixed in 2% paraformaldehyde in PBS at room temperature. The cells were washed with PBS, blocked in 5% normal donkey serum (Jackson ImmunoResearch) and 0.1% Triton-X-100 in PBS, incubated in primary antibody for 1 hour at room temperature or overnight at 4°C, washed with PBS, and incubated in secondary antibody at room temperature (see below for antibody information). Cells were counterstained in 0.1 μg/mL DAPI (Millipore) in PBS for 20 minutes at room temperature and mounted on slides using Aquamount (Thermo Scientific) for imaging.

Immunoblotting. Cells were lysed in NP-40 lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA) supplemented with 1 mM dithiothreitol (Sigma) and the following protease and phosphatase inhibitors: aprotinin, 2 μg/mL; leupeptin, 5 μg/mL; pepstatin, 1 μg/mL; PMSF, 1 mM; NaF, 10 mM; and $NaVO_4$, 1 mM. Protein quantification was performed using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Immunoblotting was performed according to standard procedures using Odyssey® TBS blocking buffer (LI-COR) for blocking and dilution of antibodies (see below for antibody information) and TBS with 0.1%

Tween-20 for washing. Imaging of immunoblots was performed using an Odyssey® Infrared Imaging System (LI-COR), and analysis was carried out using Image Studio™ software (LI-COR).

Antibody Information

ICC - Conjugated Antibodies	Source	Catalog #	Dilution
TRA-1-60, Cy3 conjugate	Millipore	MAB4360C3	1:100
TRA-1-81, Cy3 conjugate	Millipore	MAB4381C3	1:100
NANOG, Alexa Fluor 488 conjugate	Millipore	MABD24A4	1:100
SOX2, Cy3 conjugate	Millipore	MAB4423C3	1:100
OCT4 (POU5FL), Alexa Fluor 488 conjugate	Millipore	MAB4419A4	1:100
ICC - Primary Antibodies	**Source**	**Catalog #**	**Dilution**
βIII-tubulin (TUJ1)	Sigma	T8660	1:1000
α-fetoprotein (AFP)	Sigma	A8452	1:500
human muscle actin	DAKO	M0635	1:50
SOX1	R&D Systems	AF3369	1:150
PAX6	Covance	PRB-278P	1:200
OTX2	Millipore	AB9566	1:250
ICC - Secondary Antibodies	**Source**	**Catalog #**	**Dilution**
Donkey anti-rabbit Alexa Fluor 488	Jackson ImmunoResearch	711-165-152	1:400
Donkey anti-mouse Cy2	Jackson ImmunoResearch	715-225-150	1:400
Donkey anti-goat Cy3	Jackson ImmunoResearch	705-165-003	1:400
IB - Primary Antibodies	**Source**	**Catalog #**	**Dilution**
EPHRIN-B1	R&D Systems	AF473	0.2 μg/mL
HSP70	BD Transduction	610607	1:1000
IB - Secondary Antibodies	**Source**	**Catalog #**	**Dilution**
Donkey anti-goat IRDye® 800CW	LI-COR Biosciences	926-32214	1:5000
Donkey anti-mouse IRDye® 680RD	LI-COR Biosciences	926-68072	1:5000

Primer Sequences

qRT-PCR Primer Sequences		
Target	**Forward Primer Sequence (5' to 3')**	**Reverse Primer Sequence (5' to 3')**
EFNB1	GTATCCTGGAGCTCCCTCAACC	GTAGTACTCATAGGGCCGCC
PAX6	TCGGTGGTGTCTTTGTCAACG	ACTACCACCGATTGCCCTGG
GAPDH	TCTTCACCACCATGGAGAAGG	CATGGATGACCTTGGCCAGG
EFNB2	AATCCAGGTTCTAGCACAGACG	GTGCTTCCTGTGTCTCCTCC
EPHB2	CCATCAAGCTCTACTGTAACGGG	GCTCTGTAGTAGCCATTGCG
EPHB3	TGGGTAACATCTGAGTTGGC	CTTGAGCTCCACGTAGACCC
Plasmid PCR Primer Sequences		
Target	**Forward Primer Sequence (5' to 3')**	**Reverse Primer Sequence (5' to 3')**
EBNA-1	ATCAGGGCCAAGACATAGAGATG	GCCAATGCAACTTGGACGTT
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Supplemental References

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