Human germ cell formation in xenotransplants of induced pluripotent stem cells carrying X chromosome aneuploidies

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Supplementary Table S1: Fibroblast and iPSC line information

Fibroblast Line	iPSC Line	Source	Age	Karyotype	Reprogramming Method	Subclone
Turner Syndrome Child 1	TSC1	ATCC	3.5 years	45,X + centric fragment	Retroviral	2
Turner Syndrome Child 2	TSC2	Coriell	8 years	45,X	Lentiviral	2
Turner Syndrome Fetal	TSF	Coriell	18 fetal weeks	45,X	Lentiviral	2
Turner Syndrome Neonate	TSN	Coriell	1 day	45,X	Lentiviral	2
Control Female	Control	Skin Biopsy	30 years	46,XX	Retroviral & Lentiviral	3
Premature Ovarian Failure Female	POF	Skin Biopsy	32 years	46,X	Retroviral & Lentiviral	3

Supplementary Table 2: Primer sets used for single cell qRT-PCR

	Forward Primer Sequence	Reverse Primer Sequence
Pluripotency		
DNMT3B	AGGGACATCTCACGGTTCC	CCCTGTGAGCAGCAGAAAC
LIN28A	CATGCAGAAGCGCAGATCAA	GGTGGCAGCTTGCATTCC
SALL4	CACTGGAGAGAAGCCTTTTGTG	CCCCGTGTGTCATGTAGTGA
NANOG	TGCAGAGAAGAGTGTCGCAAA	GCTGGGTGGAAGAGAACACA
ALPL	GTTGACACCTGGAAGAGCTTCA	GAGTTCCGTGCGGTTCCA
Pseudoautosomal		
PLCXD1	GTGCTGAAATGGTCCGTCAC	TCCAGCATGTGGGCTATCC
GTPBP6	TGGACAGGCTTCGCAAGAA	TACCCCACCACGGAGATCA
PPP2R3B	CCAAGATGATAGACAGGATCTTCTCA	TCGGCATAGCTGATCTTCCC
CSF2RA	AGGAGTCACATTTGAGGTTCA	GAAATTCTGAGCAGCGGTAC
SLC25A6	CCGATCGAGCGGGTCAA	GATGCGGACAATGCAGTCC
AKAP17A	TCATGTACAAGGGCGAGGAC	TCACTCAGGTGTTTGGTCGAA
DHRSX	CACCCGTCTGGCGAAGAA	TGCTGCGTAGATGGAAGTCC
ZBED1	TGCATCCTGCAGTGGAAGAA	GTTGGAGGTGTTTCCGGAGTA
CD99	CACCGAACCCACCCAAAC	CGCAAGGTCAGCATCTGAAA
VAMP7	GCTCGAGCCATGTGTATGAA	CACCACAGAGAGGTGAAACA
X-linked		
PRKX	ACTGCTCGTGGTTGACAGAA	GAACCACCGATGATGCTTCAC
NLGN4X	CCGGTCTGCGTCATGTTAAA	TGGCTGTCAATGAGGGTGAA
TBL1X	TCAAATGGGATCCGTCTGGAA	ACCTCCTGTTTCATGCTCCA
TMSB4X	AGACCAGACTTCGCTCGTA	CAGCCATATCGGGTTTGTCA
ZFX	ACTTGCCCTGAGGTCATCAA	CTCTCCACAATGTCTACAGTTCC
USP9X	AAGCAGTGAGTGGCTGGAA	TTGGCCACACATAGCTCCA
DDX3X	AGCTTCTTCAGTGATCGTGGAA	TGCCAATGCCATCGTAATCAC
KDM6A	ACCAATTCCCGCAGAGCTTA	TGTCCACCACTCCAATTGTCA
TSPYL2	AGAAAGGGGCTCCAGGATAAA	TCCATGATCACCACCTCACA
KDM5C	GTGCAGCCTTCCAAGTTCAA	ATGTCTTCCTCTGTGGGTTCC
RPS4X	GTCGCTTTGCTGTACATCGTA	GCGGGCATCATGAGTCAC
RBMX	TGGAGCTTGAACCCATTGTCA	CCTGGGCGATCTGCTTCA
XCI Associated		
XIST	AGCTCCTCGGACAGCTGTAA	GGACACATGCAGCGTGGTA
Germ Layer		
KDR	AGTGGGCTGATGACCAAGAA	CCATGCCACTTCCAAAAGCA
KRT7	CCAAGGTGGATGCCCTGAA	GGGACTGCAGCTCTGTCAA
NES	GCTGCGGGCTACTGAAAA	CTGAGCGATCTGGCTCTGTA
NCAM	CTCCCAGTCCATGTACCTTGAA	GGTTCCCCTCCCAAGTGTAC
ACTC1	GGCTCTGGGCTGGTGAA	AGGAGTCCTTCTGACCCATAC
AFP	GCGGCCTCTTCCAGAAACTA	GGGGCTTTCTTTGTGTAAGCAA
EOMES	CTGTGGCAAAGCCGACAATA	CTCATCCAGTGGGAACCAGTA
FOXA2	ACTGGAGCAGCTACTATGCA	TGTTCATGCCGTTCATCCC
Germ Cell		
BOULE	AATCGCATCTTTGTAGGAGGAA	TCACAGACCCATACTGGGAA
STELLA	CATGTTACTCGGCGGAGTTC	ACTCCCTTAGGCTCCTTGTTT
PRDM1A	CCTGGTACACACGGGAGAAAA	TTGAGATTGCTGGTGCTGCTA
GFR1A1	TTCCTGGCGACCCTGTAC	ACTGGCTTTCACGCAATCC
CKIT	GGATTCCCAGAGCCCACAA	ACATCCACTGGCAGTACAGAA
NANOS3	CCTGACAAGGCGAAGACACA	ACTTCCCGGCACCTCTGAA
DAZL	CCACAACCACGATGAATCCTA	GTGATGACCTGAACTGGTGAA
DDX4 (VASA)	CGTGTTTGCATCAGTTGATACC	CTGGATTGGGAGCTTGTGAA
IFITM3	AAGGGAGGGCTCACTGAGAA	TTCATGGTGTCCAGCGAAGAC
GDF3	GCGTCCGCGGGAATGTA	CTTCTGCAGGCAGGAGGAA
PELOTA	AACAAACTGCTCCTGGAAAACC	AGGGCCTCTTTCAGGGAGTA
PUM1	CTCCTGCCCCAGTCATCATTA	CAGCTGCTCCATTTGCTGAA
Pluripotency/Germ Cell		
PRDM14	CACTCTGGAGACAGACCATACC	GAGTATGCTGGAGGCTGTGAA
SALL4	CACTGGAGAGAAGCCTTTTGTG	CCCCGTGTGTCATGTAGTGA
OCT4	GGGGACCAGTGTCCTTTCC	GGGAAAGGGACCGAGGAGTA

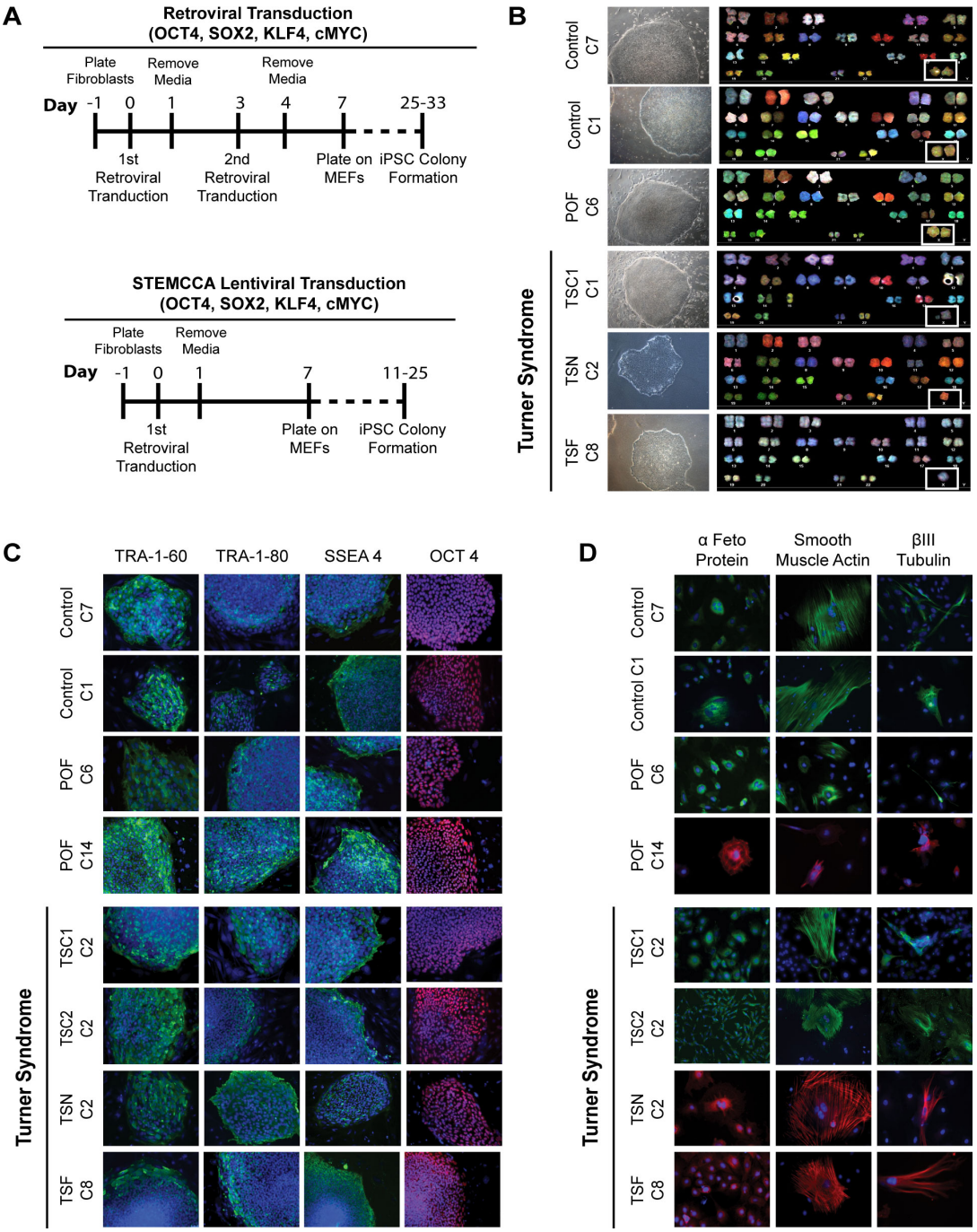
Supplementary Table 3: RNAseq Reads

	# of Reads	# of Reads Mapped	% mapped
Control c7 iPSC	23330829	21139610	90.6080534
Control c8 iPSC	32357096	29304332	90.56539561
Control GFP-	26049335	23979380	92.0537127
Control GFP+	18963035	17287623	91.16485309
H9 hESC	27467071	25046225	91.18637004
H9 GFP-	22299891	20344285	91.2304235
H9 GFP+	20454034	18607504	90.97229427
TSC1 c1 iPSC	31547635	28404049	90.03543055
TSC1 c2 iPSC	39110831	35149362	89.87117149
TSC1 GFP-	29312969	26886076	91.72075336
TSC1 GFP+	33955023	30664415	90.3089213
Triple X iPSC	42850128	39294051	91.70112864
Triple X GFP+	32586396	29371187	90.13327832

Supplementary Table 4: Log sheet and counts for xenotransplantation of pluripotent stem cells

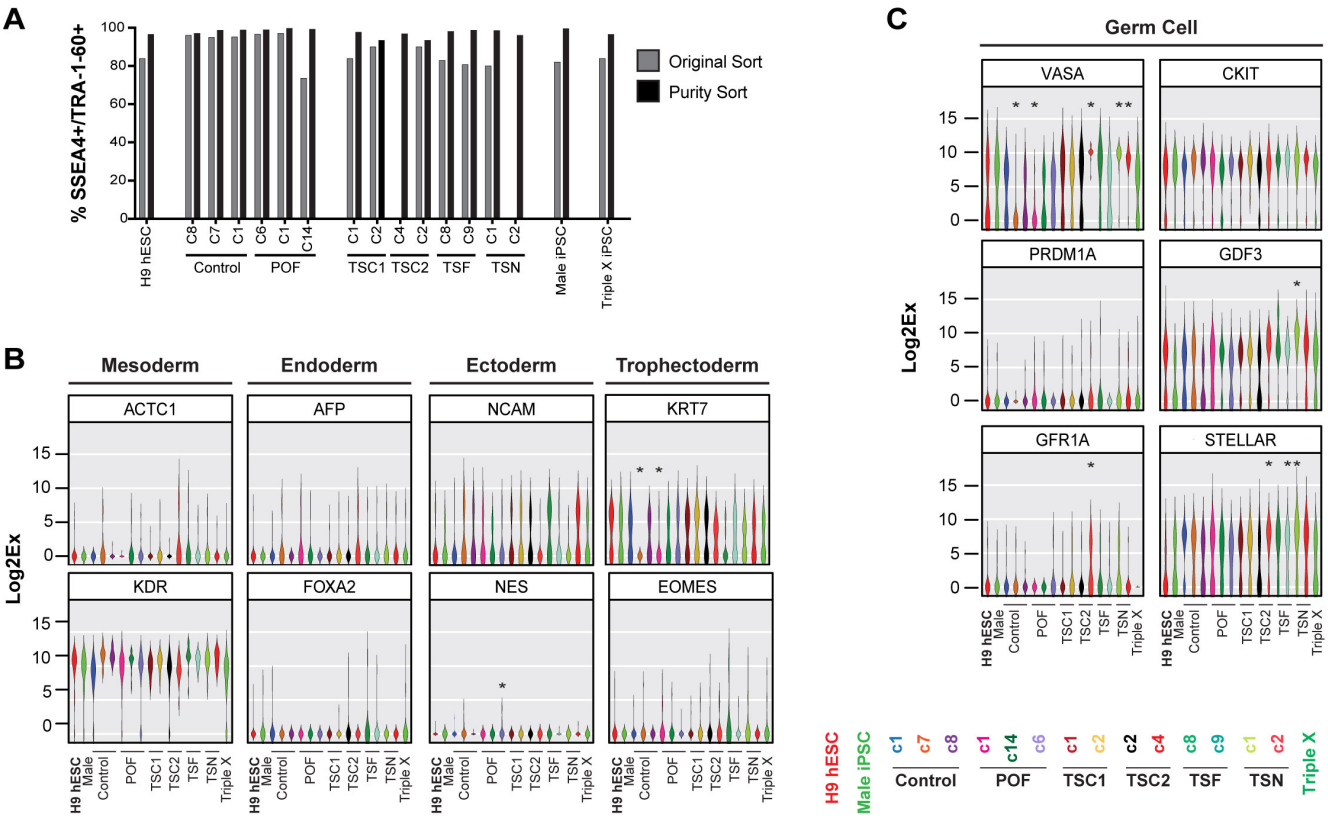
L = Left testis, R = Right testis

Cells	Mouse ID	Testis Site	Injected Cell #	Weight of testis	# Tubules /section	NUMA+ Cells /tubule	% NUMA/VASA + tubules	Total NUMA/VASA + Cells	NUMA/VASA + cells /tubule
Triple X iPSC Passage 28	3956	R	792411	31.56	-	-	-	-	-
		L	1109376	54.52	-	-	-	-	-
	3957	R	1088832	29.20	-	-	-	-	-
		L	933284	28.42	-	-	-	-	-
	3858	R	1088832	25.21	-	-	-	-	-
		L	1088832	27.89	-	-	-	-	-
	4228	R	1324240	33.12	138.5	4.5	3.25	85.17	16.61
		L	1324240	31.43	173.5	4.5	2.59	113.17	22.38
	4229	R	1135063	41.99	237.33	25.66	10.81	272.33	10.59
		L	945886	44.02	196	18.5	9.44	264.5	14.2
	4230	R	1324240	48.55	258	58.33	22.61	736.83	12.58
		L	1135063	45.35	264.67	88.67	33.50	1152.33	14.45
Control iPSC Clone 8 Passage 32	3959	R	1388289	60.64	-	-	-	-	-
		L	1189962	120.22	-	-	-	-	-
	3960	R	1388289	52.13	-	-	-	-	-
		L	1388289	44.92	-	-	-	-	-
	3961	R	1388289	44.22	-	-	-	-	-
		L	1388289	144.30	-	-	-	-	-
	4224	R	1430416	35.40	153.83	54.83	35.64	1134.33	22.03
		L	1430416	27.49	208.75	42.75	20.48	759.5	17.73
	4225	R	1430416	95.17	150.33	20.33	13.53	440	21.58
		L	1430416	62.35	116	15	12.93	235	15.63
Control iPSC Clone 7 Passage 22	3962	R	1674836	28.79	-	-	-	-	-
		L	1674836	33.43	-	-	-	-	-
	3963	R	1674836	41.34	-	-	-	-	-
		L	717787	33.76	-	-	-	-	-
	3964	R	1674836	44.05	-	-	-	-	-
		L	1674836	38.00	-	-	-	-	-
	3965	R	1674836	45.76	-	-	-	-	-
		L	1435574	40.37	-	-	-	-	-
TSC1 iPSC Clone 1 Passage 26	3997	R	803689	52.27	-	-	-	-	-
		L	688876	44.40	-	-	-	-	-
	3998	R	1126213	30.96	-	-	-	-	-
		L	1126213	74.91	-	-	-	-	-
	3999	R	803689	54.16	-	-	-	-	-
		L	803689	66.67	-	-	-	-	-
TSC1 iPSC Clone 2 Passage 25	4000	R	1126212	60.64	267.5	56.5	21.12	772	13.67
		L	1126212	120.22	203.5	24.5	12.04	458	18.70
	4001	R	803689	52.13	156.5	17	10.86	220	12.89
		L	803689	44.92	39	2	5.13	53	26.5
	4002	R	1126212	44.22	149.75	63.25	42.24	1670	26.17
		L	1126212	144.30	150	62.25	41.50	1979.75	32.31
	4246	R	1467889	58.56	184.75	13.5	7.31	217.25	16.13
		L	1467889	56.18	140	20	14.29	530.75	27.03
	4247	R	1258190	34.66	138.25	38	27.49	538	14.73
		L	1467889	32.92	258.25	64.25	24.88	927.75	13.88
	4248	R	1467889	28.78	267.5	56.5	21.12	772	13.67
		L	1467889	43.1	203.5	24.5	12.04	458	18.70
H9 hESC P58	4023	R	1232470	23.48	143	5	3.50	133.33	26.39
		L	1232470	22.92	120.75	8	6.60	188.5	22.93
	4024	R	1232470	88.83	188.67	74.33	39.58	1446.33	18.61
		L	1056403	35.84	208.33	86	41.63	187.33	21.89
H1	3764	R	2010680	1520	20	5	25	-	22.4
		L	2010680	336	39	2	5.13	-	32
	3766	L	2010680	1350	98	8	8.16	-	12.25
Control male iPSC	4226	L	941737	39.98	103	13	12.62	-	18.53
		R	1158108	268.39	40	2	5	-	4
	4227	L	941737	76.21	109	49	44.95	-	36.22
		R	1158108	231.33	106	43	40.57	-	43.56



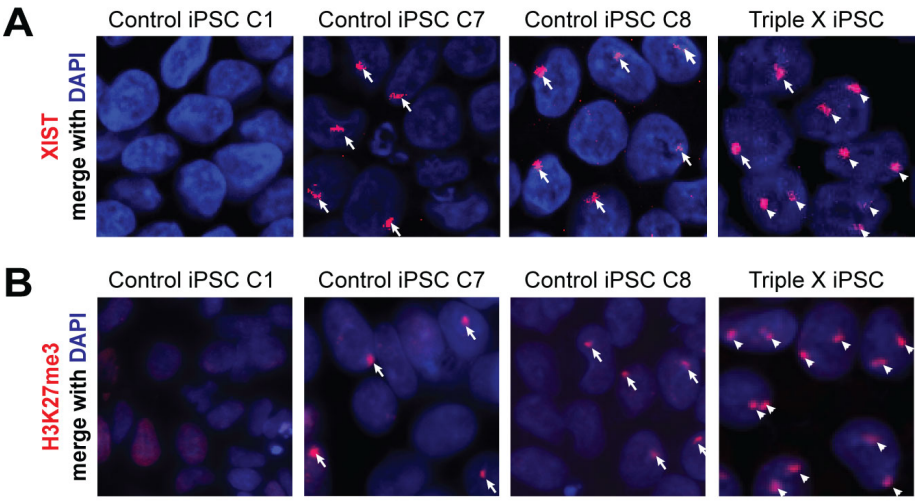
Supplementary Figure 1: Reprogramming methods and additional iPSC subclone characterization

(A) Time course of reprogramming for retroviral transduction of OCT4, SOX2, KLF4 and cMYC and lentiviral transduction of the polycistronic STEMCCA vector. (B) Phase contrast image of one iPSC subclone (C = subclone) colony, grown on MEFs or matrigel, derived from Control, POF and Turner syndrome fibroblasts along with their spectral karyotype. White box indicates the X chromosome and demonstrates two X chromosomes in Control and POF iPSC subclones and only one X chromosome in TSC1, TSN and TSF iPSC subclones. (C) Immunofluorescence for cell surface markers TRA-1-60, TRA-1-80 and SSEA4 (green) along with nuclear marker OCT4 (red) marking pluripotent iPSC subclones. Cell nuclei were costained with DAPI (blue). (D) Immunofluorescence for markers of the three germ layers after spontaneous differentiation of iPSC subclones, demonstrating cells expressing α Feto Protein (endoderm), Smooth Muscle Actin (mesoderm) and βIII Tubulin (ectoderm) (green). Cell nuclei were costained with DAPI (blue).



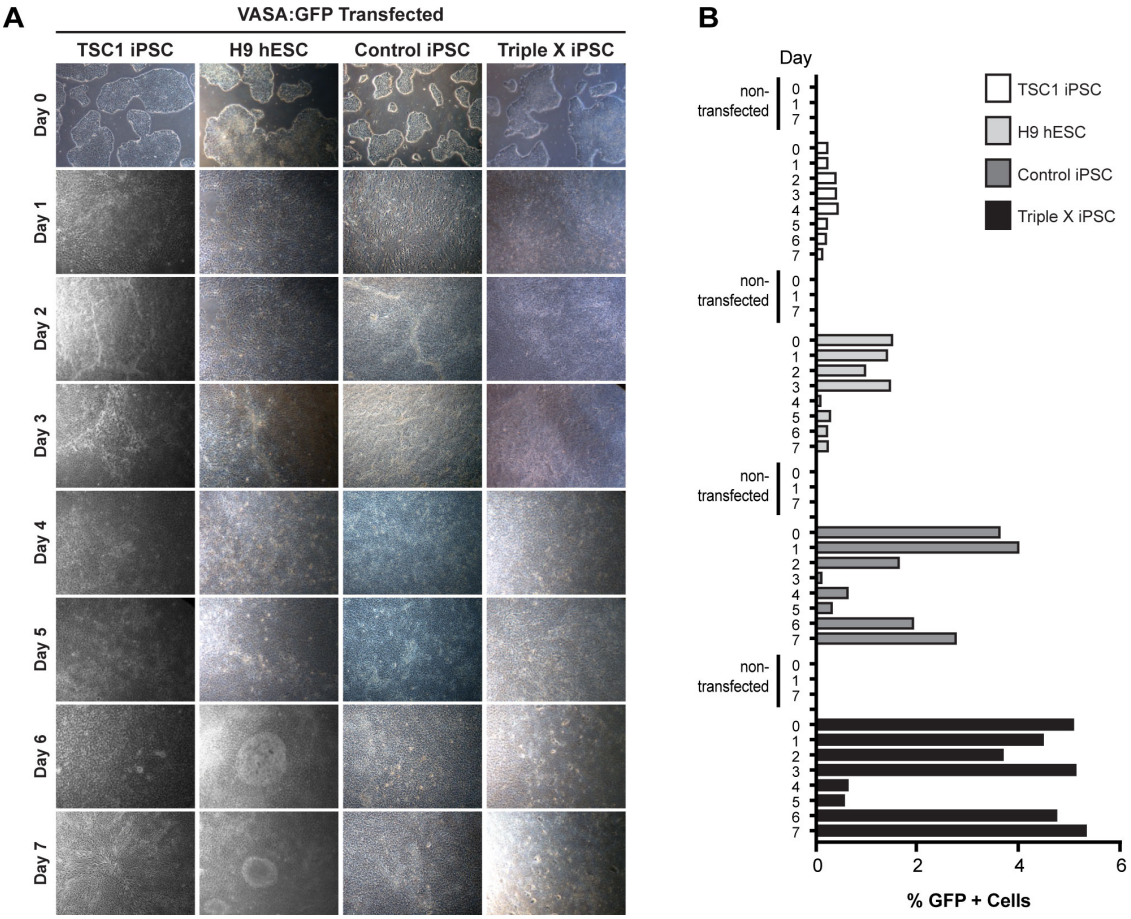
Supplementary Figure 2: Single cell sorting and analysis of iPSCs for a double positive SSEA4/TRA-60 pluripotent population

(A) Percentage of SSEA4/TRA-1-60 double positive single cells from original and purity sorts for each subclone of all pluripotent lines and subclones (C = subclone). (B-C) Violin plot representation of Log_2Ex values (fold change above background) of single cells for (B) mesoderm, endoderm, ectoderm and trophectoderm related genes and (C) germ cell related genes. Asterisk indicates a significant difference in expression (p-value < 0.01) when compared to H9 hESCs. Color-coded subclone information is provided.



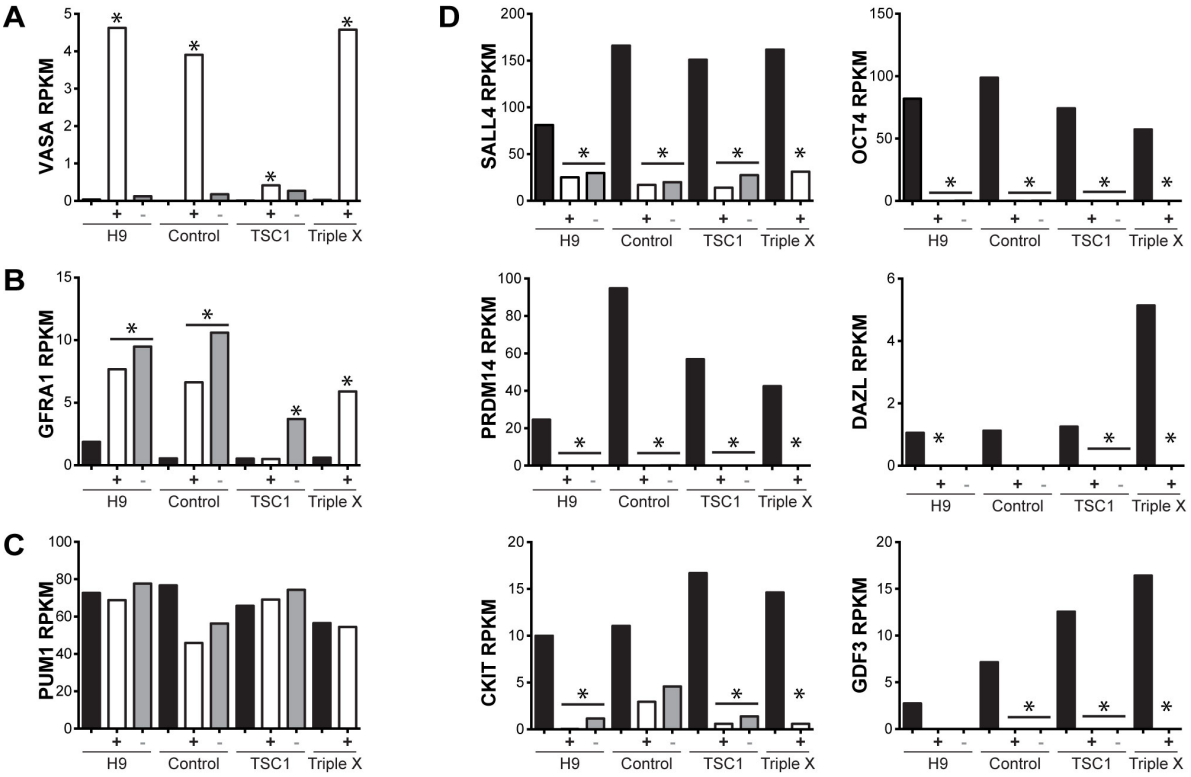
Supplementary Figure 3: Single cell sorting of iPSCs for a double positive SSEA4/TRA-60 pluripotent population

(A) *XIST* RNA FISH marking the inactive X chromosome (red punctate dot) in Control iPSC (subclones 1,7 and 8) and Triple X iPSCs. Arrow indicates a cell with a single *XIST* foci and arrowhead indicates a single cell with two *XIST* foci. (B) Immunofluorescence of H3K27me3 marking the inactive X chromosome (red punctate dot) in Control iPSC (subclones 1,7 and 8) and Triple X iPSCs. Arrow indicates a cell with a single H3K27me3 foci and arrowhead indicates a single cell with two H3K27me3 foci.



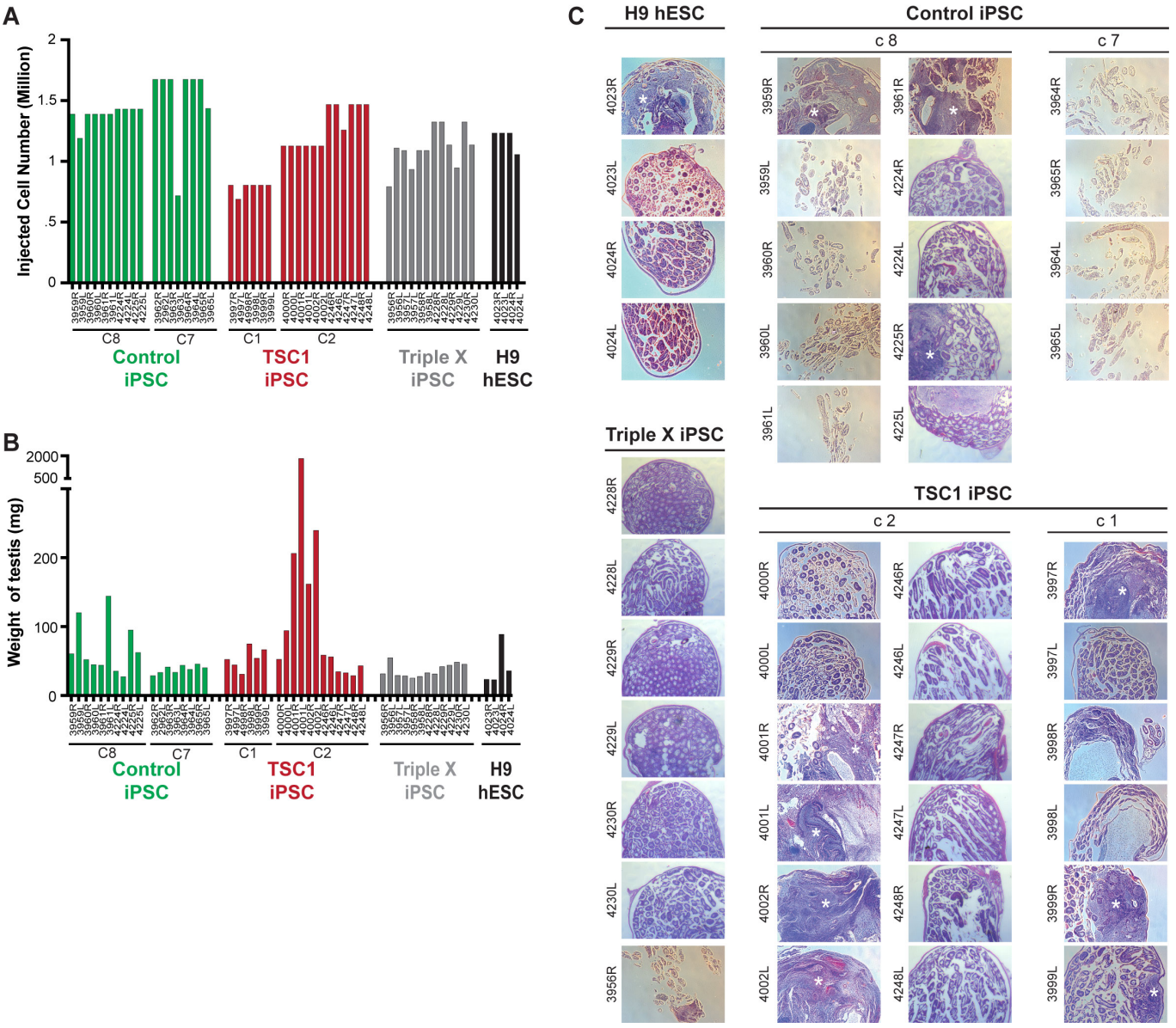
Supplementary Figure 4: Single cell sorting of VASA:GFP positive pluripotent cells differentiated with BMP4/8 and RA.

(A) Phase contrast image of VASA:GFP transfected TSC1 iPSC, H9 hESC, Control iPSC and Triple X iPSCs differentiated for seven days by addition of BMP4/8 and RA. (B) Graph indicating the percentage of GFP+ cells for non-transfected and VASA:GFP transfected TSC1 iPSC, H9 hESC, Control iPSC and Triple X iPSCs throughout the seven day directed differentiation.



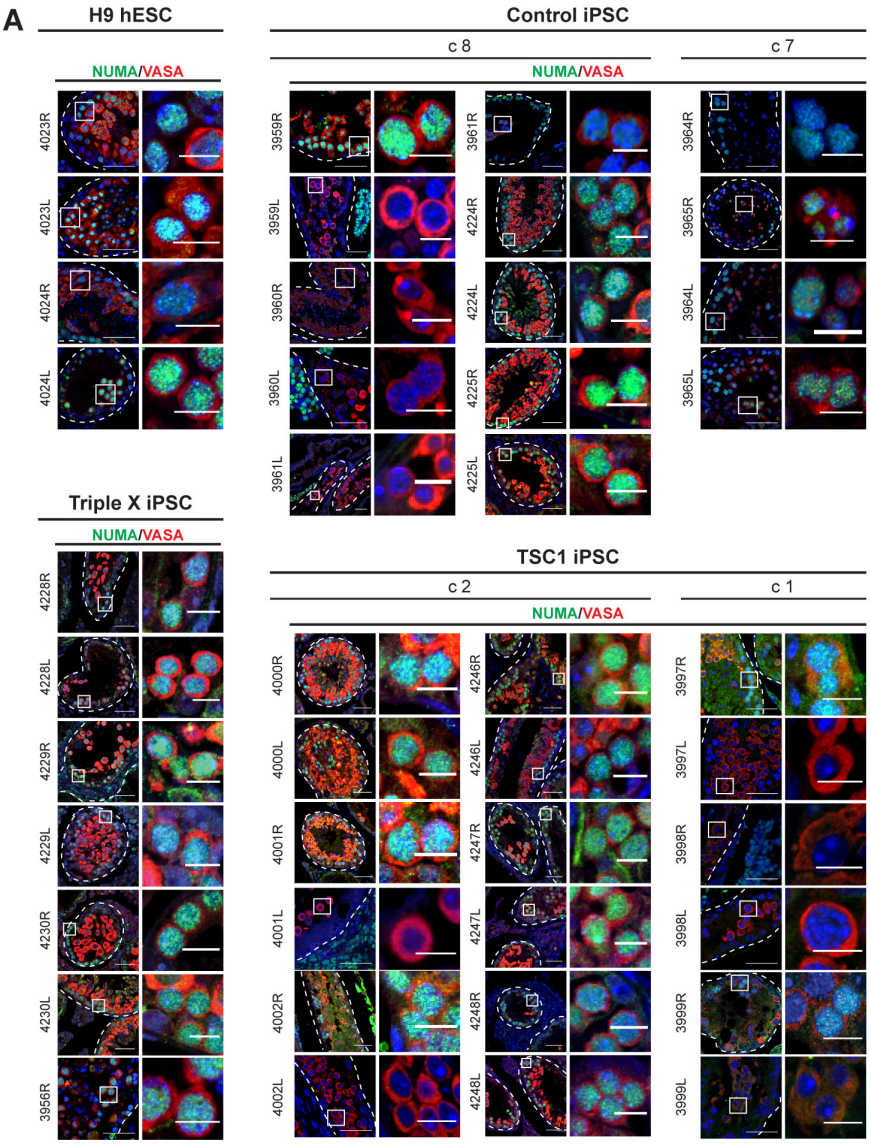
Supplementary Figure 5: RNA-Seq analysis of germ cell-associated genes in pluripotent cells and sorted GFP+ and GFP- populations after directed differentiation.

(A-D) Expression of germ cell associated genes (A) *VASA*, (B) *GFRA1*, (C) *PUM1* and (D) *SALL4, OCT4, PRDM14, DAZL, CKIT*, and *GDF3*, in pluripotent cells (H9 hESC, Control c8 iPSC, TSC1 c2 iPSC and Triple X iPSC; black bar), day 7 GFP+ population (white bar) and day 7 GFP- population (grey bar) were calculated into RPKM (reads per kilobase per million). Asterisks indicate differentiated samples that have more than two-fold difference in gene expression level compared to the pluripotent cell lines it was derived, using GFOLD with significant cutoff for fold change of 0.01 [1].



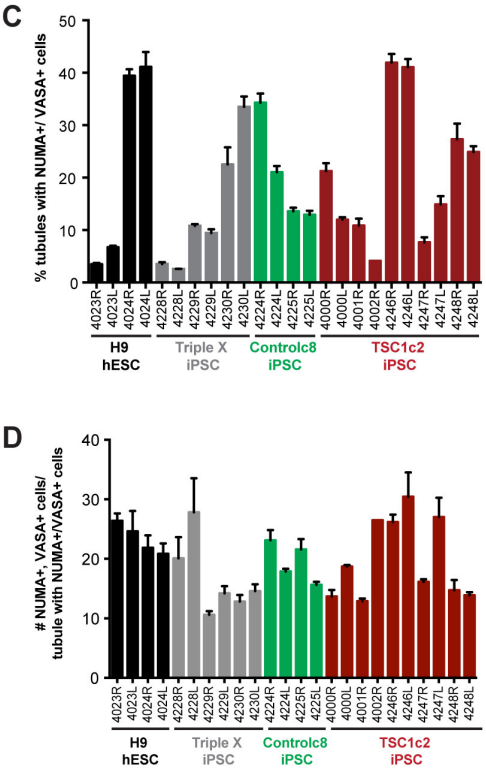
Supplementary Figure 6: Xenotransplantation of control and X chromosome aneuploid iPSCs into busulfan-treated murine testis.

(A) Number of injected cells (million) per testis Control (subclone 8 and 7), TSC1 (subclone 2 and 1), Triple X iPSCs and H9 hESCs. (B) Weight (mg) of testis eight weeks post transplantation of Control (subclone 8 and 7), TSC1 (subclone 2 and 1), Triple X iPSCs and H9 hESCs. (C) Histological analysis of H&E stained cross-sections of H9 hESC, Control iPSCs (subclone 8 and 7), Triple X iPSCs and TSC1 (subclones 1 and 2) injected murine testis and seminiferous tubules. Asterisk indicates areas with teratoma-like formations outside the seminiferous tubules.



B

Transplanted pluripotent cell line	Testis with NUMA+/VASA+ cells / total testis analyzed	Percent NUMA+/VASA+ testis
H9 hESC	4/4	100%
Control iPSC	9/15	60%
Triple X iPSC	5/5	100%
TSC1 iPSC	12/18	66.7%



Supplementary Figure 7: In vivo germ cell formation of control and X chromosome aneuploid pluripotent stem cells within murine seminiferous tubules.

(A) Histological cross sections from left (L) and right (R) murine testis injected with H9 hESCs, Control iPSCs (subclone 8 and 7), Triple X iPSCs, and TSC1 (subclone 2 and 1). Immunohistochemistry using human specific antibody NUMA (green) and germ cell marker VASA (red) co-stained with nuclear DAPI (blue). White dashed line indicates tubule border. Scale bar, 40um. White box demonstrates magnified region (Scale bar, 10um). (B) Table with results of all xenotransplants with number and percentage of testis with NUMA+/VASA+ cells per total testis analyzed. (C & D) Quantification of NUMA/VASA immunohistochemistry results of cross sections from H9 hESC, Triple X iPSC, Control subclone 8 iPSC and TSC1 subclone 2 iPSC injected murine testis. Serial sections were subject to counting (see Materials and Methods). (C) Percentages of tubules with NUMA+/VASA+ cells per total number of tubules within each serial section for each injected murine testis. (D) Number of NUMA+/VASA+ cells per double positive tubule within each serial section for each injected murine testis.

1 Feng, J. *et al.* GFOLD: a generalized fold change for ranking differentially expressed genes from RNA-seq data. *Bioinformatics* **28**, 2782-2788, doi:10.1093/bioinformatics/bts515 (2012).

Supplementary Methods

Isolation and culture of primary human skin fibroblast and Turner syndrome cell lines

Control and POF female primary fibroblasts were isolated and cultured as previously described [52]. Briefly, fibroblasts were derived from a 4 mm skin punch biopsy obtained by the Stanford University Dermatology clinic after adult donor consent. The biopsy was washed in PBS (Life Technologies), minced and plated onto gelatin-coated plates, and expanded in MEF media containing DMEM (Life technologies), 10% FBS (Life Technologies) and 10 IU/ml pen/strep (Life technologies) and cultured at 37°C. Turner syndrome (TS) fibroblast lines consisted of TS child 1 (TSC1; Detroit 525, ATCC), TS child 2 (TSC2; Coriell GM01176), TS Fetal (TSF; Coriell, GM10179), TS Neonate (TSN; Coriell, GM00857). Gm01166, GM00857 and GM10179, Coriell).

Spectral Karyotyping

Karyotyping was performed as described in [38]. Briefly, all cell lines were grown to approximately 90% confluence, KaryoMAX Colcemid (Life technologies) was added for approximately 18 hours at 10 µl/ml. The cells were detached using 0.05% Trypsin (Life technologies), washed and incubated in hypotonic solution containing equal volumes of 0.4% Potassium Chloride (Sigma-Aldrich) and 0.4% Sodium Citrate (Sigma-Aldrich) at 37°C for seven minutes. The cells were then fixed with a methanol: glacial acetic acid solution (Fisher Scientific), 3:1 for five consecutive five-minute incubations at room temperature. The cells were then dropped onto a glass slide for metaphase spreads. Spectral Karyotyping was then carried out as indicated by the human specific SKY Paint DNA kit (Applied Spectral Imaging). Twenty spreads per line were analyzed using SKY view spectral imaging system.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA)/PBS (Sigma-Aldrich and Invitrogen) for 20 min., and washed with PBS + 1% Tween (PBST; Sigma-Aldrich) and blocked with 4% normal goat or donkey serum (Jackson ImmunoResearch) in PBS for 30 min. For nuclear staining, cells were permealized prior to blocking with 1% Triton-X 100/PBS (Sigma-Aldrich). Primary antibodies were added overnight at 4ºC at the following dilutions: SSEA4 (1:200, Millipore), Tra-1-60 (1:200, Millipore), Tra-1-81 (1:200 Millipore), OCT4 (1:100, Santa Cruz), α feto protein (1:150, R&D), smooth muscle actin (1:200, Abcam) and β III Tubulin (1:150, Covance). The cell were then washed with PBST and secondary antibodies were added for one hour at room temperature, all at a 1:500 dilution, Alexa 488-conjugated goat anti-mouse IgG (Invitrogen), Alexa 488-conjugated goat anti-Rat IgM (Invitrogen), Alexa 488-conjugated goat anti mouse IgM (Invitrogen), Alexa 488-conjugated goat anti rabbit IgG (Invitrogen). Lastly, the cells were washed with PBST and DAPI was used to label cell nuclei.

In vitro spontaneous differentiation

Embryoid body (EB) differentiation was conducted by transferring iPSCs to an ultra low attachment plate containing DMEM (Life Technologies) + 20% FBS (Life Technologies). After eight days in suspension, EBs were transferred to a gelatin-coated dish and cultured in the same differentiation media until beating cardiomyocytes were observed. The differentiated cells were then passaged to a 24-well plate for immunocytochemistry for α feto protein, smooth muscle and β III Tubulin.

Fluorescent associated cell sorting (FACS) for SSEA4 and TRA-1-60

One confluent well of a six-well plate was sorted for each iPSC and fibroblast line. Cells were dissociated to single cells by treatment with Accutase (Life technologies) for 3 – 5 min. Cells were then centrifuged for five min. at 1000 rpm and resuspended in FACS buffer (1X PBS, 0.5% BSA, 2mM EDTA) to a concentration of $1x10^6$

cells/100 ul. For single staining, 10 ul of cell suspension was used and for double staining 100 ul of cell suspension was used. Cells were blocked with 0.8% mouse IgG (Invitrogen) for ten min. on ice. The cells were then incubated with TRA-1-60 (BD Pharmigen) and SSEA4 (BD Pharmigen) antibodies for 30 min. on ice and protected from light. The cells were subsequently washed with FACS buffer and resuspended in 200 ul FACS buffer with DAPI. Flow-cytometry analysis was performed on a BD FACSAriaII cell sorter. Compensation beads (BDBiosciences), which went through the same staining protocol, were used to ensure proper staining patterns during data acquisition. PE-TRA-1-60 versus FITC-SSEA4 was then used to identify a double positive population, which was then purity sorted before cells were single cell sorted into individual wells of 12-8 strips with 5ul of CellsDirect 2X reaction mix solution (Invitrogen) with 0.05 units of SUPERNase Inhibitor (Ambion).