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Supplemental Information

Mesenchymal to Epithelial Transition Mediated by CDH1 Promotes Spontaneous Reprogramming of Male Germline Stem Cells to Pluripotency

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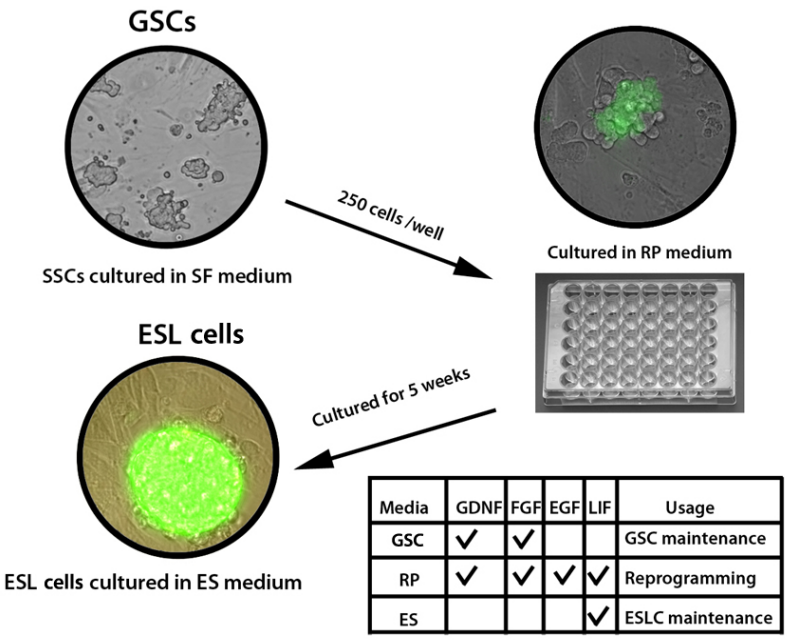
Figure S1

A



Media	GDNF	FGF	EGF	LIF	Usage
GSC	✓	✓			GSC maintenance
RP	✓	✓	✓	✓	Reprogramming
ES				✓	ESLC maintenance

B

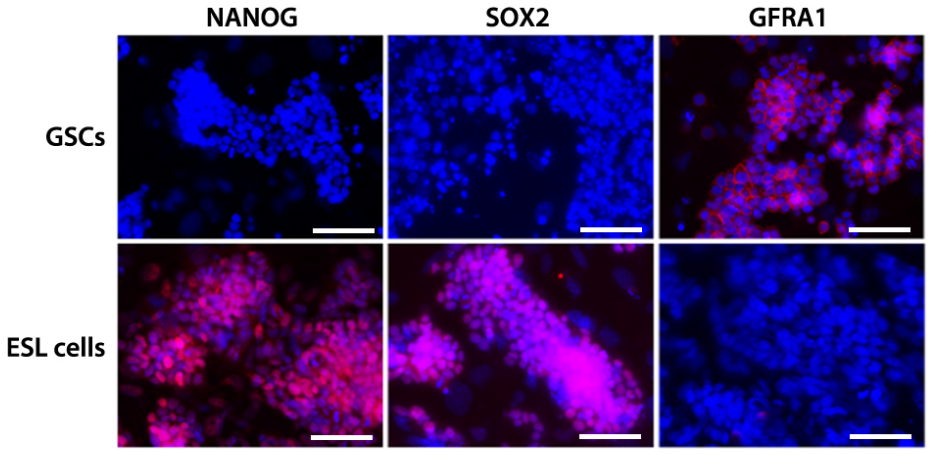


Figure S1. (Related to Figure 1) GSC reprogramming and ESL cell identification. (A) Schematic diagram of GSC reprogramming. Green color indicates Oct4-GFP expression. Image of ESL cells is intentionally equivalent to the data shown in Figure 1. (B) Immunofluorescence analysis of GSCs and ESL cells. Images of NANOG, SOX2 or GFRA1 immunostaining, shown in red, and DAPI (DNA) counterstaining, shown in blue, are merged. Bar=150 um.

Figure S2

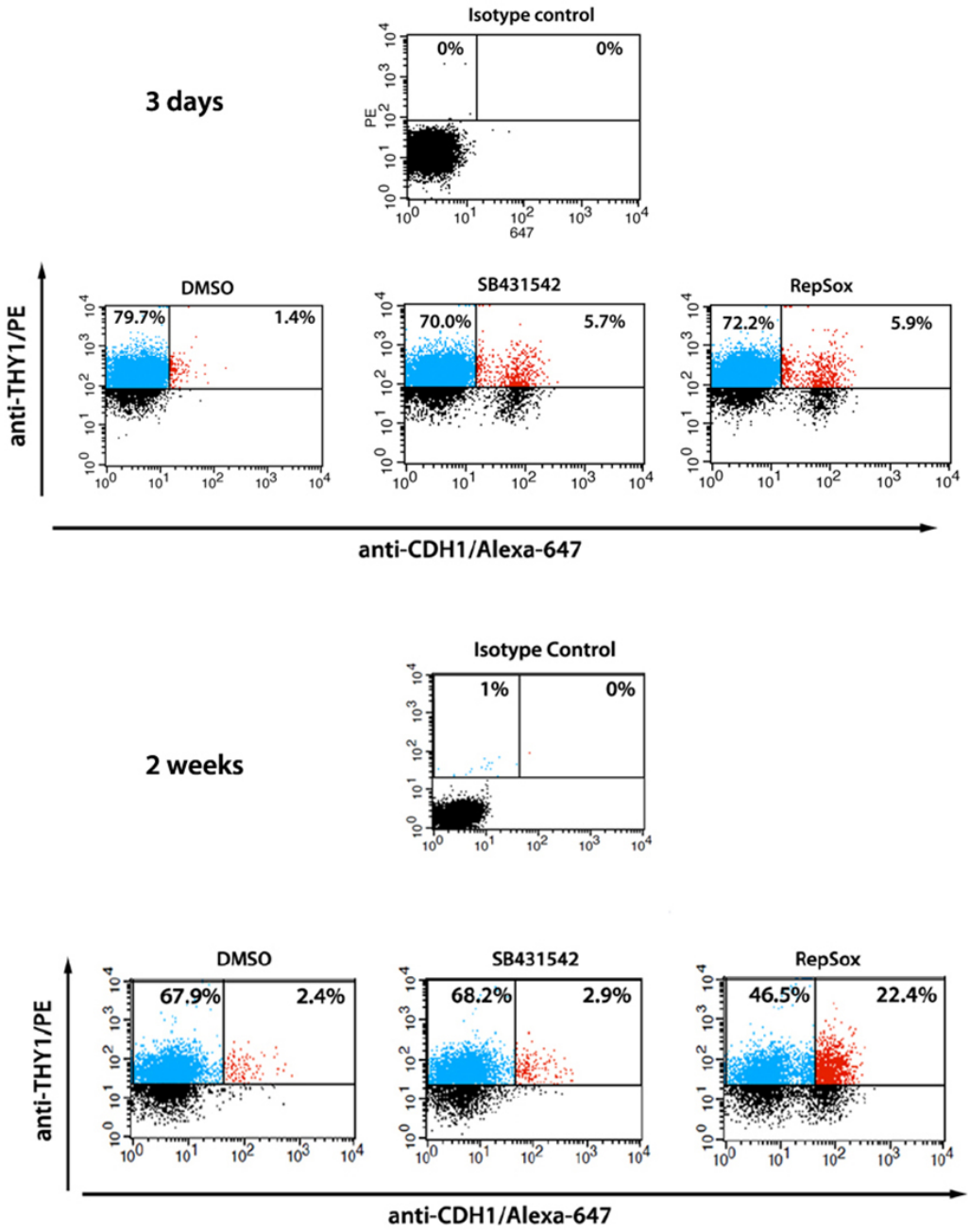


Figure S2. (Related to Figure 3) CDH1 and THY1 expression during reprogramming with TGFb inhibitor treatments. Dot plot showing CDH1 and THY1 expression in Oct4-GFP/Dox-Oct4 GSCs cultured in RP medium with DMSO (control), SB431542 (25μM) and RepSox (25μM) for 3 days (top) and 2 weeks (bottom). Trypsin was used for cell dissociation. Parent gates (not shown) include forward and side scatter to select singleton, viable cells. Percentages of the corresponding populations from GSCs are shown in parenthesis. Isotype controls were used to define background fluorescence for CDH1 and THY1 antibodies. The data shown are representative from three repeats of the same experiment.

Figure S3

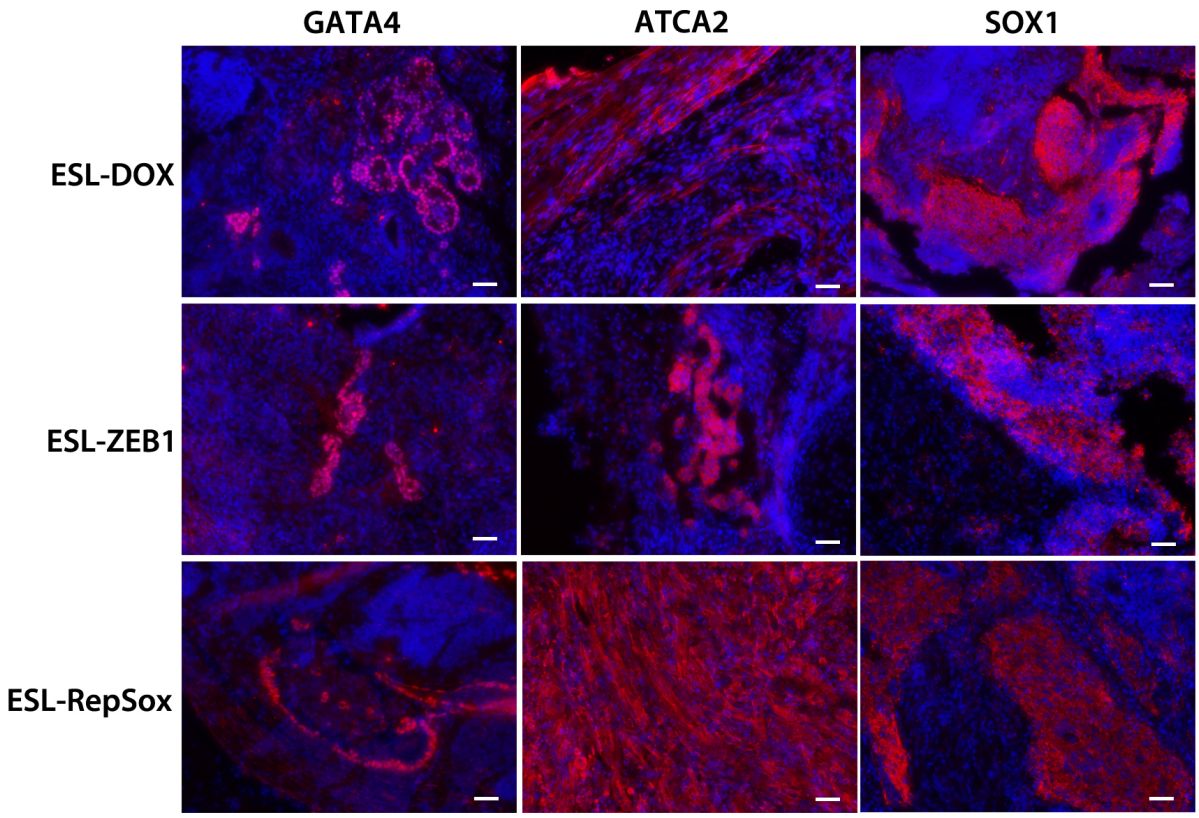


Figure S3. (Related to Figures 2, 3 and 4) Immunofluorescence analysis of teratomas derived from different ESL cells. ESL cells from doxycycline treated Dox-Oct4 GSCs (ESL-DOX), *Zeb1* siRNA treated GSCs (ESL-ZEB1) and RepSox treated GSCs were used for injections into NOD/SCID mice. GATA4, ACTA2 or SOX1 immunostaining, shown in red, and DAPI (DNA) counterstaining, shown in blue, are merged. Bar=150 um.

Figure S4

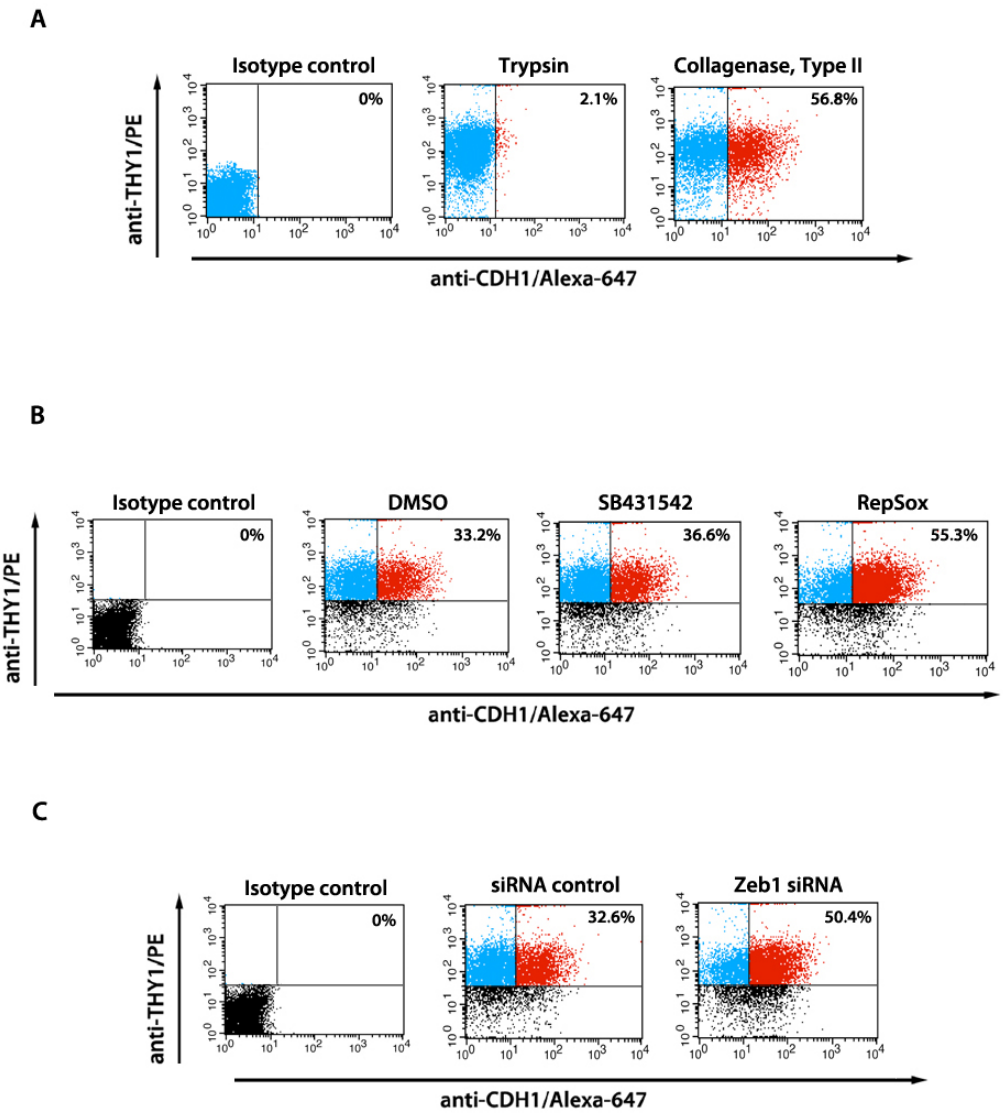
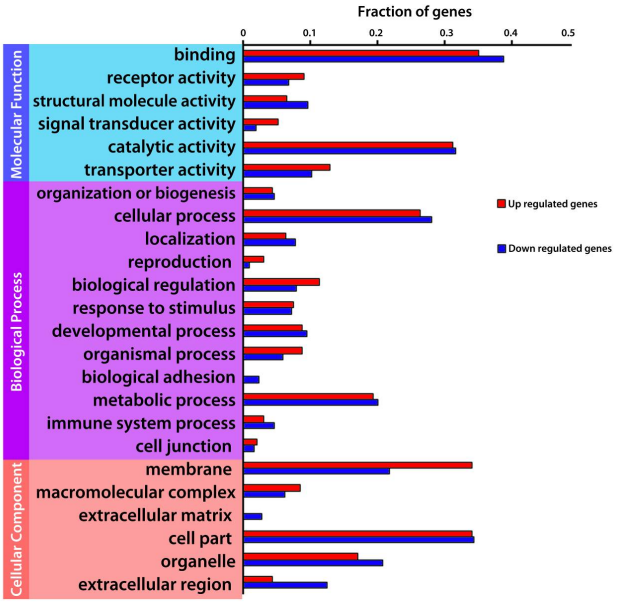


Figure S4. (Related to Figure 5) CDH1 and THY1 expression after collagenase type II digestion. (A) CDH1 and THY1 expression of Oct4-GFP/Dox-Oct4 GSCs after trypsin or collagenase, type II digestion. Dot plot showing CDH1 and THY1 expression in Oct4-GFP/Dox-Oct4 GSCs treated with TGF beta inhibitors (B, 25μM) and Zeb1 siRNA (C, 25nM) for 2 weeks. Collagenase type II was used for cell dissociation. Parent gates (not shown) include forward and side scatter to select singleton, viable cells. Percentages of the corresponding populations from GSCs are shown in parenthesis. Isotype controls were used to define background fluorescence for CDH1 and THY1 antibodies. The data shown are representative from three repeats of the same experiment.

Figure S5

A



B

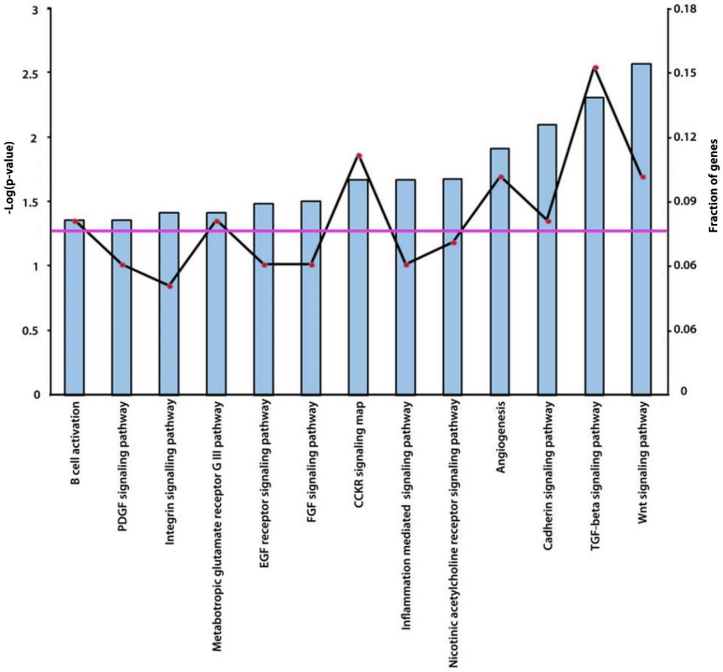


Figure S5. (Related to Figure 5) GO and signaling pathways analysis. (A) Gene Ontology (GO) enrichment analysis of differentially expressed genes ($Log_2FC>2$ or <-2) between CDH1+/THY1+ and CDH1-/THY1+ GSCs. Vertical axis displays the GO annotation corresponding to the three categories. Horizontal axis displays the fraction of significantly up regulated (red) or down regulated (blue) genes in each category. (B) Analysis of canonical signaling pathways in differentially expressed genes ($Log_2FC>2$ and <-2) between CDH1+/THY1+ and CDH1-/THY1+ GSCs. Major Y axis on the left shows the significance (-log (P-value)) of the canonical pathway (blue bar). Secondary Y axis on the right shows the percentage of differentially expressed genes involved in the canonical pathway (red point). The pink line shows the significance threshold cut off of -log (P-value = 0.05).

Figure S6

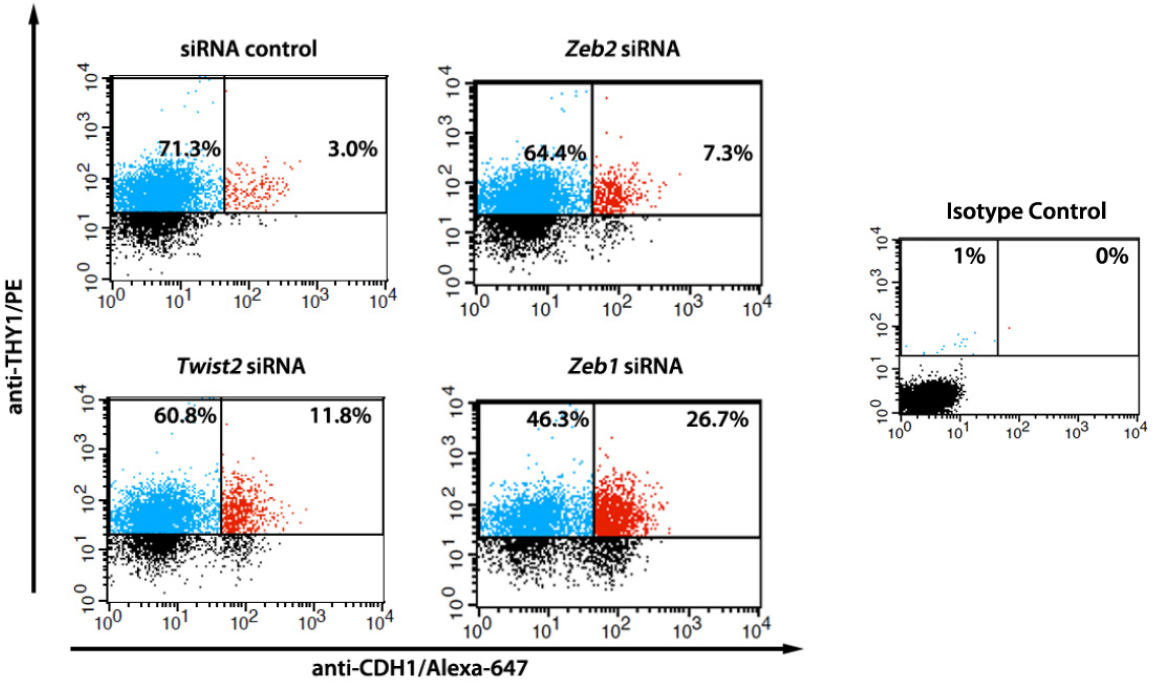


Figure S6. (Related to Figure 5) CDH1 and THY1 expression during reprogramming with knockdown of MET associated factors. Dot plot showing CDH1 and THY1 expression in Oct4-GFP/Dox-Oct4 GSCs cultured in RP medium with *Zeb1* siRNA, *Zeb2* siRNA, *Twist2* siRNA and siRNA control for two weeks. Trypsin was used for cell dissociation. Parent gates (not shown) include forward and side scatter to select singleton, viable cells. Percentages of the corresponding populations from GSCs are shown in parenthesis. Isotype controls were used to define background fluorescence for CDH1 and THY1 antibodies (not shown). The data shown are representative from three repeats of the same experiment.

Figure S7

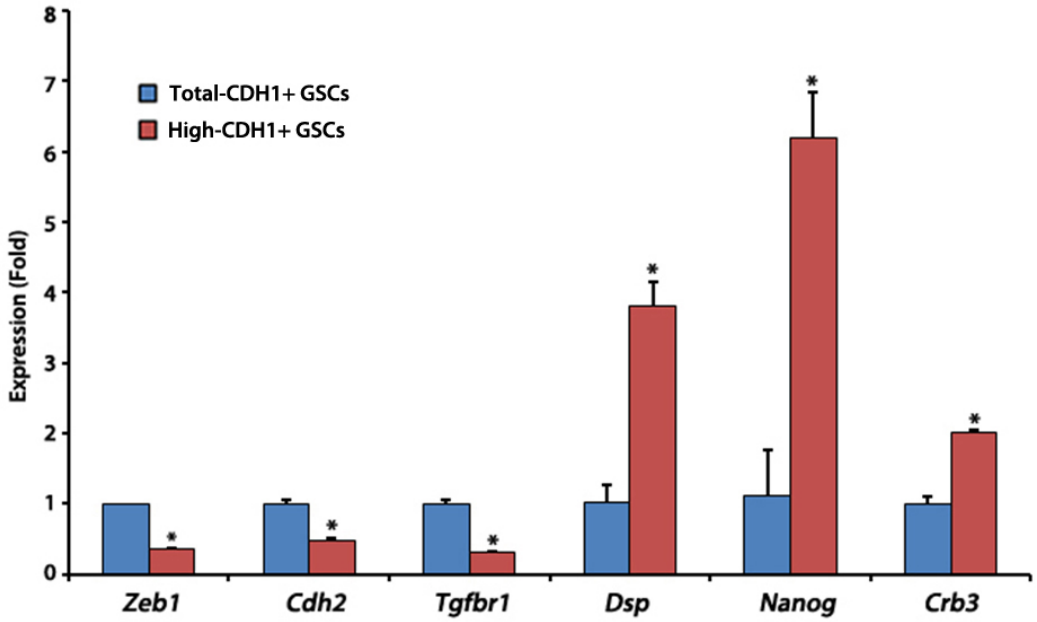


Figure S7. (Related to Figure 5) RT-QPCR analysis of indicated genes in high-CDH1+ cells and total-CDH1+ cells after collagenase type II digestion. High-CDH1+ Oct4-GFP/Dox-Oct4 GSCs (3% highest expressing CDH1 cells) and total-CDH1+ Oct4-GFP/Dox-Oct4 GSCs were sorted and used for RNA anlysis. Mean and SD for three biological replicates are shown. * p-value <0.01.

Table S1. (Related to Figure 1) Comparison of reprogramming media

	Shinahara (2004)	**Seandel (2007)**	**Ko (2009, 2010)**	**Guan (2009)**	**Takashima (2013)**
Base Meduim	**StemPro**	**StemPro**	**StemPro**	**#a-MEM**	**StemPro**
FBS	1%	1%	1%	1%	1%
Anti-biotic	1×	1×	1×	1×	1×
Insulin	25 μg/ml	25 μg/ml	5 μg/ml*	2 5 μg/ml*	25 μg/ml
transferrin	100 μg/ml	100 μg/ml	100 μg/ml*	100 μg/ml*	100 μg/ml
putrescine	60 μM	60 μM	100 μM*	60 μM*	60 μM
sodium selenite	30 nM	30 nM	30 nM*	30 nM*	30 nM
D-(+)-glucose	6 mg/ml	6 mg/ml	6 mg/ml	1 mg/ml#	6 mg/ml
pyruvic acid	30 μg/ml	30 μg/ml	30 mg/ml	n/a#	30 μg/ml
DL-lactic acid	1 μl/ml	11 μM	1 μl/ml	n/a#	1 μl/ml
bovine albumin	5 mg/ml	5 mg/ml	5 mg/ml	2.5 mg/ml#, *	5 mg/ml
l-glutamine	2 mM	2 mM	2 mM	2 mM#	2 mM
M 2-mercaptoethanol	50 μM	50 μM	50 μM (55 μM)	100 μM	50 μM
MEM vitamin solution	1×	1×	1×	1×	1×
NEAA	1×	1×	1×	1×	1×
ascorbic acid	100 μM	100 μM	n/a#	284 μM#	100 μM
d-biotin	10 μg/ml	10 μg/ml	n/a#	0.1 μg/ml#	10 μg/ml
β-estradiol	30 ng/ml	30 ng/ml	30 ng/ml		30 ng/ml
progesterone	60 ng/ml	60 ng/ml	6.3*+60 ng/ml	60 ng/ml*	60 ng/ml
EGF	20 ng/ml	20 ng/ml	20 ng/ml	n/a#	n/a#
FGF	10 ng/ml	10 ng/ml	10 ng/ml	n/a#	10 ng/ml
LIF	10^3 U/ml	10^3 U/ml	10^3 U/ml	10^3 U/ml	n/a#
GDNF	10 ng/ml	10 ng/ml	10 ng/ml *1× N2	4-20 ng/ml *1× N2-1	15 ng/ml
Mouse Age	0-2 days	3 weeks-8 months	5-8 weeks	2-5 weeks	0-2 days

*** an ingredient added as part of the N2 supplement; # notably different component**

Table S2. (Related to Figures 1-5) RT-qPCR and ChIP primers

Gene Name	Forward Primer (5' to 3')	Reserve Primer (5' to 3')
Cripto	ATGGACGCAACTGTGAACATGATGTTC GCA	CTTTGAGGTCCTGGTCCATCACGTGAC CAT
Utf1	GATGTCCCGGTGACTACGTCT	TCGGGGAGGATTCGAAGGTAT
Esg1	ATAAGCT TGATCTCGTCTTCC	CTTGCTAGGATGTAACAAA GC
Eras	TCAGATCCGCCTACTGCC	TTACCAACACCACTTGCACC
Zeb1	GCTGGCAAGACAACGTGAAAG	GCCTCAGGATAAATGACGGC
Zeb2	ATTGCACATCAGACTTTGAGGAA	ATAATGGCCGTGTCGCTTCG
Twist1	GGACAAGCTGAGCAAGATTCA	CGGAGAAGGCGTAGCTGAG
Twist2	CGCTACAGCAAGAAATCGAGC	GCTGAGCTTGTCAGAGGGG
Thy1	TGCTCTCAGTCTTGCAGGTG	TGGATGGAGTTATCCTTGGTGTT
Dsp	GGATTCTTCTAGGGAGACTCAGT	TCCACTCGTATTCCGTCTGGG
Cdh2	AGCGCAGTCTTACCGAAGG	TCGCTGCTTTCATACTGAACTTT
Pkp1	AACCACTCTCCGCTCAAGAC	CTTCTGCCGTTTGACGGTCAT
Crb3	CACCGGACCCTTTCACAAATA	CCCACTGCTATAAGGAGGACT
Cdh1	CAGGTCTCCTCATGGCTTTGC	CTTCCGAAAAGAAGGCTGTCC
Snai1	CACACGCTGCCTTGTGTCT	GGTCAGCAAAAGCACGGTT
Snai2	TGGTCAAGAAACATTTCAACGCC	GGTGAGGATCTCTGGTTTTGGTA
TgfbrI	TCTGCATTGCACTTATGCTGA	AAAGGGCGATCTAGTGATGGA
Smad7	GGCCGGATCTCAGGCATTC	TTGGGTATCTGGAGTAAGGAGG
Oct4	CCTGCAGAAGGAGCTAGAACAGT	TGTTCTTAAGGCTGAGCTGCAA
Nanog	TGGTCCCCACAGTTTGCCTAGTTC	CAGGTCTTCAGAGGAAGGGCGA
actinB	GGCTGTATTCCCCTCCATC	TGCCAGATCTTCTCCATGTC
	Primers for ChIP	
	Forward Primer (5' to 3')	**Reserve Primer (5' to 3')**
E-Box1&2	GACAGGGGTGGAGGAAGTTG	CTGATTGGCTGGGGACGC
E-Box3	CCCAGCCAATCAGCGGC	GGGAACTCAGTAGTGCGCC

Table S3 (Related to Figures 1-5) Antibodies and dilutions used

Antibody (clone)	Company & Catalog No.	Dilution*
Goat anti-GFRA1	R&D systems, AF560	IF: 1:400
Mouse anti-OCT4 (C10)	Santa Cruz, sc-5279	WB: 1:250
Mouse anti-TUB (DM1A)	Biogenex, MU121-5UC	WB: 1:5000
Rabbit anti-pSMAD3 (C25A9)	Cell Signaling Technology, 9520	WB: 1:1000
Rabbit anti-NANOG	Millipore, ABD88	IF:1:100; WB:1:1000
Goat anti-CDH1	R&D systems, AF748	WB:1:500
Mouse anti-beta3 TUB (TU-20)	Cell Signaling Technology, 4466	WB:1:1000
Mouse anti-SSEA1-Cy3 (MC-480)	Millipore, MAB4301C3	IF:1:200
Mouse anti-SOX2 (20G5)	Thermo scientific, MA1-014	IF:1:200; WB:1:1000
Mouse anti-ACTA2 (1A4)	Calbiochem, 113200	IF:1:200
Goat anti-SOX1	R&D systems, AF3369	IF:1:200
Goat anti-GATA4 (C-20)	Santa Cruz, sc-1237	IF:1:200
Rabbit anti ZEB1 (H-102)	Santa Cruz, sc-25388	WB: 1:250

*** WB=Western Blot; IF= Immunofluorescence**

Table S4. (Related to Figure 1) Media composition

Ingredient	Company	F12KB medium	GSC medium	RP medium	ES medium
DMEM/F12	SIGMA	Base	n/a	n/a	n/a
Knock-Out DMEM/F12	GIBCO	n/a	n/a	n/a	Base
Stem Pro-34	GIBCO	n/a	Base	Base	n/a
Stem Pro supp	GIBCO	n/a	1×	1×	n/a
MEM vitamin	GIBCO	n/a	1×	1×	n/a
NEAA	GIBCO	1×	1×	1×	1×
Antibiot/myc	GIBCO	1×	1×	1×	1×
L-glutamine	HYCLONE	1×	1×	1×	1×
estradiol	SIGMA	n/a	30 ng/ml	30 ng/ml	n/a
progesterone	SIGMA	n/a	60ng/ml	60ng/ml	n/a
FBS	HYCLONE	n/a	1%	1%	15%
transferrin	SIGMA	n/a	100 μg/ml	n/a	n/a
insulin	GIBCO	n/a	25 μg/ml	n/a	n/a
Rat GDNF	PEPROTECH	10 ng/ml	10 ng/ml	10 ng/ml	n/a
human FGF	PEPROTECH	10 ng/ml	10 ng/ml	10 ng/ml	n/a
mouse EGF	GOLDBIOTECH	n/a	n/a	20 ng/ml	n/a
ESGRO	MILLIPORE	n/a	n/a	10^3 Unit/ml	n/a
putrescine	SIGMA	n/a	60 μm	n/a	n/a
selenite	SIGMA	n/a	30 nM	n/a	n/a
pyruvic acid	SIGMA	n/a	30 μg/ml	30 μg/ml	n/a
lactic acid	SIGMA	n/a	0.06%	0.06%	n/a
BME	SIGMA	50 μM	50 μM	50 μM	100 μM
ascorbic acid	SIGMA	n/a	100 μM	n/a	n/a
D-biotin	SIGMA	n/a	10 μg/ml	n/a	n/a
D-glucose	SIGMA	n/a	6 mg/ml	6 mg/ml	n/a
BSA	CALBIOCHEM	2 mg/ml	5 mg/ml	5 mg/ml	n/a
KSR	GIBCO	1%	n/a	n/a	n/a
B27 supp	GIBCO	1×	n/a	n/a	n/a

Supplemental Experimental Procedures

GSC line derivation

GSC lines were derived and maintained following published procedures with minor modifications (Dann, 2013; Falciatori et al., 2008; Heim et al., 2012; Kanatsu-Shinohara et al., 2003). Testes were collected from 2 to 8 weeks old transgenic or wild type mice. Testicular cells were dissociated with a standard two-step enzymatic procedure using 1 mg/ml type IV collagenase (Sigma) and then 0.25% Trypsin (Hyclone). Dissociated cells were plated on gelatin coated wells in F12KB medium (Supplementary Table 4), a GSC growth formulation based on findings of Aoshima et al (Aoshima et al., 2013), to remove a portion of the somatic cells through differential adherence (Supplementary Methods). Non-adhering cells were collected and cultured in F12KB medium on a feeder layer of mitotically inactivated DR4 MEFs (ATCC) for approximately one week. Use of F12KB permitted the enrichment of SSCs by trituration prior to subsequent culturing on DR4 MEFs in Stem Pro based medium (GSC medium) containing 10 ng/mL GDNF and 10 ng/mL FGF2 and 19 other supplements as described (Supplementary Table 4) (Heim et al., 2012; Kanatsu-Shinohara et al., 2003). SSCs/spermatogonia cultured in this manner are referred to as "GSCs" (Kanatsu-Shinohara et al., 2003).

Cell culture and cell sorting

GSCs were cultured on DR4 MEFs. GSC medium (Supplementary Table 4) was used for GSCs maintenance. ES-D3 cells (ATCC, CRL-11632) and ESL cells were maintained in ES medium (Supplementary Table 4). To obtain sufficient cells for sorting, GSCs were expanded at standard plating density in GSC medium for two weeks prior to trysinization. GSCs were trypsinized and incubated for 25 min at 4°C in PBS with 2% FBS and Alexa 647 anti-mouse CDH1 antibody (147307, Biolegend), PE anti-mouse THY1 antibody (105307, Biolegend) or the appropriate isotype controls (Biolegend). Sorting was performed on an Aria II (BD Bioscience).

For SSC sorting, testes were collected from 5 to 8 weeks old mice. Testicular cells were dissociated with a standard two-step enzymatic procedure using 1 mg/ml type IV collagenase (Sigma) and then 0.25% Trypsin (Hyclone). Dissociated cells were incubated for 25 min at 4°C in PBS with 2% FBS and Alexa 647 anti-mouse CDH1 antibody (147307, Biolegend), PE anti-mouse THY1 antibody (105307, Biolegend) or the appropriate isotype controls (Biolegend). Sorting was performed on an Aria II and cells were directly sorted to the 48-well plate for reprogramming (BD Bioscience).

siRNA transfection and inhibitor treatment

siRNAs were transfected into GSCs with Lipofectamine 2000 (ThermoFisher) according to the manufacturer's protocol. siGENOME SMARTpool siRNAs were from Dharmacon for mouse *Zeb2* (D-059671), *Twist2* (D-044881) and *Cdh1* (D-041028); Stealth siRNA was from Invitrogen for targeting *Zeb1* (MSS210696). Scrambled non-targeting siRNA was used as a control (SIC001, Sigma). All siRNAs were used at a concentration of 25 nM. For reprogramming experiments, fresh RP medium was applied to cells six hours after transfection with siRNAs and transfections were repeated every 7 days during the reprogramming period (generally four weeks). For RNA or protein isolation cells were lysed 72 h after transfection. For CDH1 cell analysis transfection was conducted every 7 days for a total of 14 days prior to flow cytometry.

RepSox (508158, EMD Millipore) and SB431542 (616464, EMD Millipore) were dissolved in DMSO. RepSox and SB431542 were used at a concentration of 25 μM. GSCs undergoing inhibitor treatments had medium containing freshly diluted inhibitor refreshed every 3 days. Medium containing DMSO was used as a control.

Flow cytometry for cell analysis

GSCs at different reprogramming time points were used for flow cytometry analysis after trypsin digestion. Inhibitors or siRNAs treated GSCs were digested by trypsin (0.05%, SH30236, hyclone) or collagenase type II (1 mg/ml, 17101015, Gibco) and used for analysis. Collagenase type II was dissolved in F12/DMEM. Cells were incubated with trypsin for 10 min or with collagenase type II for 40 min at 37° before analysis. For the analysis, cells were incubated for 25 min at 4°C in PBS with 2% FBS and Alexa 647 anti-mouse CDH1 antibody (147307, Biolegend), PE anti-mouse THY1 antibody (105307, Biolegend) or the appropriate isotype controls (Biolegend).

Flow cytometry was performed on a Calibur (BD Bioscience). Data was analyzed by CellQuest Pro software (BD Bioscience).

Embryoid body formation and cell differentiation

For embryoid body formation ESL cells were cultivated as embryoid bodies (EBs) in hanging drops in differentiation medium (ES medium without LIF) as described for standard mouse ESC differentiation (Spelke et al., 2011). Briefly, 20 µl drops containing 300 ESL cells were pipetted onto the lid of a culture dish filled with PBS and incubated in hanging drops for 2 days. EBs were collected and cultured in non-tissue culture treated petri dishes for 5 days. For ectodermal, mesodermal and endodermal lineage detection, single EBs were transferred into each well of a gelatin coated 48-well plate for immunofluorescence analyses. For cardiac cell differentiation, EBs were seeded into a gelatin coated 48 well plate and cultured in differentiation medium with 1% DMSO for 10-20 days.

Neuronal lineage differentiation was performed as described (Gaspard et al., 2009). Briefly, ESL cells were cultured on gelatin-coated 60 mm dish for 12 days in DDM medium (DMEM/F12 supplemented with N2 supplement (1X, Gibco), non-essential amino acids (1X, Hyclone), Glutamine (1X, Gibco), 1 mM of sodium pyruvate, 500mg/ml of BSA, 0.1 mM of 2-mercaptoethanol, penicillin and streptomycin (1X Hyclone). Cells were subsequently differentiated in N2/B27 medium (1:1 of DDM and Neurobasal/B27 medium) for 10 days. Cells were fixed in 4% paraformaldehyde (EMS) prior to immunofluorescence analysis.

Teratoma formation

6- to 8-week-old NOD-SCID mice obtained from the *In Vivo* Therapeutics Core at the IU Simon Cancer Center (Indianapolis) were used for injections. ESL cells were cultured on DR4 MEFs in ES medium until they were approximately 60-80% confluent. ESL cells suspended in ES medium at 2 x 10^7 cells/ml and mixed with an equal volume of Matrigel (356237, corning). Each mouse was injected with 2 x 10^6 cells subcutaneously into the flank. Teratomas were observed after three to four weeks and recovered for analysis. Teratomas were fixed in formalin overnight at 4 °C and embedded in paraffin for sectioning and processed for immunohistochemical detection of germ layer markers.

Cell immunostaining and histological analysis (includes cell staining)

Cells in plates were fixed in 4% (v/v) paraformaldehyde for 15 min at room temperature and rinsed three times in TBS for 5 min before staining. For nuclear antibodies, cells were treated with TBS containing 0.1% tritonX-100 for 10 min. Cells were blocked for 2 hours in TBS containing 3% (w/v) bovine serum albumin (Jackson ImmunoResearch). Teratomas were embedded in paraffin and sections were washed in xylene and rehydrated through an ethanol series using standard techniques. Antigen retrieval was performed by boiling in Tris (10mM) /EDTA (1mM) antigen retrieval solution (pH=9.0) for 15 min. Primary antibodies and dilutions are listed in Supplementary Table 3. Primary antibodies were applied overnight at 4 °C. Appropriate secondary antibodies conjugated with Alexa-594 (Life Technologies), Alexa-488 (Life Technologies) or Cy3 (Jackson ImmunoResearch) were diluted (1:500) in TBS and applied for one hour at room temperature. For a negative control the primary antibody was omitted. Images were obtained using a Nikon Eclipse TiS inverted microscope equipped with a Retiga 2000R Fast 1394 camera. Images were acquired with Q-capture Pro software and Image J was used for pseudocoloring and to create overlays of colors.

RNA sequencing and transcriptome assembly

Two Oct4-GFP/Dox-Oct4 GCS lines (#1 and #2) and one wildtype GSC line (#3) were used for sorting CDH1-/THY1+ and CDH1+/THY1+ cells. RNAs were isolated using RNeasy-plus micro kit (74034, Qiagen). Libraries for each sample were constructed using the Illumina TruSeq Stranded Total RNA Library Prep Kit / Ribo-Zero Gold kit as per manufacturer's instructions. Libraries were quantified using an Agilent 2200 TapeStation, pooled equimolar and sequenced on an Illumina NextSeq500 instrument using a NextSeq150-v2 Mid Output reagent kit and flowcells. Scatter plot were generated by using core R functions (version 3.3.1). NextSeq read sequences were cleaned using Trimmomatic (version 0.32) to remove adapter sequences and perform quality trimming. The resulting paired reads were mapped against GRCm38.p4 using TopHat2 (version 2.1.1). Tophat uses Bowtie, which is based on the

Burrows-Wheeler transform algorithm, for sequence alignment and allows for mapping across exon junctions. Read counts for each gene were created using featureCount (version 1.5.0-p3) and Gencode m9 as the annotation. Custom Perl scripts were used for estimation of transcript abundances based on Fragments Per Kilobase of exon per Million fragments mapped (FPKM). Differential expression analysis was carried out using the DESeq2 package (version 1.12.3) in R/Bioconductor (R version 3.3.1). The combination of Blast2GO, WEGO, Kyoto Encyclopedia of Genes and Genomes (KEGG) and GO (Gene Ontology) database were used for further analysis. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO Series accession number GSE90712 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE90712).

RT-qPCR

RNA was isolated using the RNeasy plus micro kit (74034, Qiagen) according to the manufacturer's protocol. First-strand cDNA was produced using a qScript cDNA Synthesis Kit (23104, Quanta). For real-time PCR, a MX3000p Real-Time PCR system (Agilent) and Maxima SYBR Green/Rox qPCR Master Mix (ThermoFisher Scientific) were used according to the manufacturer's protocols. Transcript levels were normalized to b-Actin. qPCR conditions were 95°C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s, and then a melt curve analysis was performed to verify the quality of the amplicon. ΔΔCt method was used to calculate relative fold-changes, as described (Livak and Schmittgen, 2001). The PCR primers used in the experiments are shown in Supplementary Table 2.

Western Blotting

Cells were washed in PBS prior to lysis. Cells were trypsinized and resuspended in sample buffer ("SB", Laemmli buffer with 0.1M DTT) and boiled at 100 °C for 10 min. Lysates were electrophoresed through a 10% SDS-PAGE gel and transferred onto PVDF membrane using standard procedures. Following blocking in 5% milk with TBS, the membrane was incubated in primary antibody overnight at 4 °C. After washing, the membrane was incubated in IgG-HRP secondary antibody and washed. Supersignal West Femto (ThermoFisher Scientific) was used for detection. Blots were stripped using standard procedures and re-probed with anti-Tubulin (DM1A clone) to verify even loading.

Chromatin Immunoprecipitation (ChIP)

GSCs were transfected with *Zeb1* siRNA or control siRNA at 25 nM final concentration, using Lipofectamine 2000 (Invitrogen). After 3 days culture, ChIP was conducted according to the manufacturer's instructions by using EZ-ChIP Kit (Millipore). Briefly, cells (1×10^7) were cross-linked with 1% formaldehyde for 10 min at room temperature. Glycine was added to stop cross-linking, and cells were washed with cold PBS and harvested in SDS lysis buffer plus Protease Inhibitor Mixture II. Cell lysates were sonicated on ice by using a sonicator (Misonix S4000) for 8 cycles of 20 sec pulse at 40% power with 40 sec rest between each cycle. 1 % of each sample was used for gel analysis and confirmed to be sheared with DNA fragment sizes ranging from about 500 to 1000 bp. Immunoprecipitations were performed using 10 μg each of Anti-Zeb1 (Santa Cruz) or 10 ug Rabbit IgG overnight at 4 °C with rotation. Samples were mixed with Protein G Agarose and rotated at 4 °C for 1h. After washing, samples were eluted and purified. Maxima SYBR Green mix (Thermo Scientific) was used to perform real-time PCR on an MX3000p real-time PCR system (Agilent). The relative proportions of immunoprecipitated gene fragments were determined on the basis of the threshold cycle (Ct) for each PCR product. Data sets were normalized to ChIP input values. For every gene fragment analyzed, each sample was quantified in duplicate and from at least three independent ChIP experiments.

Reference

Aoshima, K., Baba, A., Makino, Y., and Okada, Y. (2013). Establishment of Alternative Culture Method for Spermatogonial Stem Cells Using Knockout Serum Replacement. Plos One *8*.
Dann, C.T. (2013). Transgenic modification of spermatogonial stem cells using lentiviral vectors. Methods in molecular biology (Clifton, NJ) *927*, 503-518.
Edgar R, Domrachev M, Lash AE (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. *30*, 207-10.
Falciatori, I., Lillard-Wetherell, K., Wu, Z., Hamra, F.K., and Garbers, D.L. (2008). Deriving mouse spermatogonial stem cell lines. Methods Mol Biol *450*, 181-192.
Gaspard, N., Bouschet, T., Herpoel, A., Naeije, G., van den Ameele, J., and Vanderhaeghen, P. (2009). Generation of cortical neurons from mouse embryonic stem cells. Nat Protoc *4*, 1454-1463.
Heim, C.N., Fanslow, D.A., and Dann, C.T. (2012). Development of Quantitative Microscopy-Based Assays for Evaluating Dynamics of Living Cultures of Mouse Spermatogonial Stem/Progenitor Cells. Biology of Reproduction *87*.
Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., and Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod *69*, 612-616.
Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods *25*, 402-408.
Spelke, D.P., Ortmann, D., Khademhosseini, A., Ferreira, L., and Karp, J.M. (2011). Methods for embryoid body formation: the microwell approach. Methods Mol Biol *690*, 151-162.