Schmidt et al., SUPPLEMENTAL MATERIALS AND METHODS

Isolation, Culture, and Transplantation of SSC-Enriched Testis Cell Fractions
Cultures of EpCAM+ clump forming germ cells enriched for SSCs were established from cells isolated from three to four 8-9 day rat pups as previously described with few modifications [1]. Briefly, in contrast to previous reports [1], collagenase and trypsin digestion were conducted simultaneously, decapsulated testis tissue was digested with 10 ml 0.5% trypsin (Invitrogen, Carlsbad, CA) containing 1 mg/ml collagenase for ten minutes to generate a single cell suspension. At several points during the digestion the tissue was gently pipetted and 1 ml of 7 mg/ml DNAse was added to facilitate break up of the tissue and to remove DNA. After addition of 1 ml FBS (Hyclone, Logan, UT) to inactivate the digestion, cells were strained over a 40 μm cell strainer, centrifuged, and re-suspended in MEMα (Invitrogen) with 1% FBS and counted using a standard hemacytometer. The single cell suspension was then gently layered on top of 30 % Percoll gradients (2 ml each, ~ 2 gradients per testis) such that no more than $2X10^7$ cells passed through any one gradient. After centrifugation, the resulting pellets were pooled and washed once with DPBS-S (PBS containing 1% FBS, 1 mM sodium pyruvate, 5.6 mM glucose, 10 mM Hepes, 50 units/ml penicillin, and 50 mg/ml streptomycin [2]) and finally resuspended in 5 ml of DPBS-S with primary antibody (mouse anti-rat EpCAM, 1:150 dilution of 1 mg/ml antibody, Pickcell, Amsterdam, The Netherlands). The cells were maintained at 4°C for 20 minutes with one mixing after 10 minutes. No more than $5X10^7$ cells were used in the primary antibody incubation. Cells were centrifuged, washed 2 times with DPBS-S in order to remove as much un-attached primary antibody as possible, and finally resuspended in 0.4 ml DPBS-S. 0.1 ml of MACS secondary antibody (goat anti-mouse IgG MicroBeads, Miltenyi, Auburn, CA) was added, and cells were incubated for 10 minutes at 4°C with a mixing half way through the incubation. After incubation, cells were pelleted by centrifugation and resuspended in 2 ml DPBS-S. Isolation of EpCAM+ cells was accomplished by sorting using two MACS columns (1 ml of cell suspension per column) as directed. The EpCAM+ cells were washed two times and approximately 200,000 cells were seeded per well of a 12 well culture dish containing 100,000 mitotically inactivated STO feeders. Cells were maintained in a humidified incubator at 37°C in the presence of 5% CO_2.

The EpCAM+ clump forming germ cells were maintained in a defined serum free media (rSFM). The base media consisted of 90% MEMα and 10% water. Additional components were; 44.25 units/ml penicillin (Invitrogen), 44.25 units/ml streptomycin (Invitrogen), 0.531% BSA (MP Biomedicals, Solon, OH), 88.5 μg/ml iron-saturated transferrin, 13.452 μeq/L free fatty acid solution [3-4], $5.31X10^{-8}$ M Na_2SeO_3, 1.77 mM L-glutamine (Invitrogen), 88.5 μM 2-mercaptoethanol, 22.125 μg/ml insulin, 8.85 mM Hepes buffer, and 106.2 μM putrescine. Additional growth factors (GDNF, 20 ng/ml [R&D systems, Minneapolis, MN]; Gfra1, 150 ng/ml [R&D systems]; and bFGF, 1 ng/ml [BD Biosciences, San Jose, CA]) were added to media immediately before it was applied to the cells. Media was changed every 2-3 days, and cells were subcultured onto fresh feeders approximately 1:1.5 every 7 days using trypsin digestion.

The equation utilized to determine colonies per 10^5 cells cultured for all transplantation experiments is as follows:

$$\frac{Colonies}{10^5 Cells\ Cultured} = Number\ of\ Colonies\left(\frac{10^5 Cells\ Isolated}{10^5 Cells\ Transplanted}\right)\left(\frac{1}{10^5 Cells\ Cultured}\right)$$

RNA Isolation

Long-term cultures of wild type Sprague Dawley EpCAM+ clump forming germ cells were utilized for microarray analysis. Seven days after subculture, cultures were washed with growth factor free media, and fresh media without GDNF was added to wells overnight (18 h). After 18 h of GDNF withdrawal, fresh media containing GDNF was added to cultures. Clump cells were removed from wells using gentle mechanical pipetting in order to limit the amount of feeder contamination in RNA samples. Cells were isolated before GDNF withdrawal, after the 18 h withdrawal, and at 2, 4, and 8 h after GDNF replacement and frozen in Trizol. For RNA extraction for samples to be used for microarray, a combination of the Trizol method and RNeasy (Qiagen, Valencia, CA) columns was used. Briefly, after thawing, 200 μl chloroform was added to the Trizol containing the treated cells and samples were centrifuged 12,000XG for 15 min. After centrifugation, the aqueous phase was removed from the tube, retained and combined with an equal volume of ice-cold isopropanol. RNA was then isolated from the samples using RNeasy columns as directed. Concentration, purity, and integrity of the RNA was determined using an Agilent bioanalyzer (Santa Clara, CA) and a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), and only samples with a 260/280 ratio of > 1.8 were used for microarray analysis. For samples that were used for siRNA validation and in vivo gene analysis, RNA was isolated using the Trizol method as directed.

Microarray Processing and Analysis

After RNA isolation from cultured rat EpCAM+ clump forming germ cells maintained with GDNF, and after GDNF withdrawal and replacement for 2, 4, or 8 h (5 treatments, n=3/treatment), 1 μg of RNA was amplified using the Affymetrix one-cycle kit protocol. The resulting cRNA was evaluated for concentration and integrity. Microarray preparation, hybridization, and scanning were conducted by the University of Pennsylvania School of Medicine Microarray Core Facility according to the Affymetrix GeneChip Expression manual (www.affymetrix.com). Briefly, 5 μg of cRNA was converted to first-strand cDNA using Superscript II reverse transcriptase primed by a poly(T) oligomer that incorporated the T7 promoter. Second-strand cDNA synthesis was followed by in vitro transcription for linear amplification of each transcript and incorporation of biotinylated CTP and UTP. The cDNA products were fragmented, heated, and hybridized to Affymetrix Rat Genome 230 2.0 Arrays. Each sample (n=15) was hybridized to its own array. Microarrays were then washed and stained with streptavidin-phycoerythrin and a confocal scanner was used to collect the fluorescence signals after excitation at 570 nm. The average signal from two sequential scans was calculated for each microarray. For initial data analysis, Affymetrix Microarray Suite 5.0 was used to quantitate expression levels for targeted genes. The default values were provided by Affymetrix and were applied to all analysis parameters. For each probe, border pixels were removed, and the average intensity of pixels within the 75th percentile was computed. The average of the lowest 2% of probe intensities in each of 16 microarray sectors was used as background and subtracted from all features in that sector. Probe pairs were scored as positive or negative for

detection of the specific sequence by comparing signals from the perfect match and mismatch probe features. The number of probe pairs meeting the default discrimination threshold (τ = 0.015) was used to assign a call of absent, present or marginal for each assayed gene, and a p-value was calculated to reflect confidence in the detection call.

For microarray analysis, Affymetrix probe level data (.cel files) were imported into ArrayAssist (v3.4, Stratagene, La Jolla, CA) and probeset signal values were calculated using the GCRMA algorithm. The newly generated probeset signal values were exported into GeneSpring (Agilent). Within GeneSpring, the probeset list was filtered such that only those probes that were flagged as present in at least 2 of the 15 data sets were utilized for further analysis. The filtered data set (21935 probesets) was imported into Partek Genomics Suite (v6.3, Partek Inc., St. Louis, MO) where it was Log_2 transformed. In order to generate a list of genes significantly regulated by GDNF, the transformed data was analyzed using ANOVA, and only those genes with a p-value less than 0.05 were considered further (7948 probe sets). This analysis generated a list of genes that was used as a means to prioritize genes for further conformation using functional experimentation. To identify candidate genes, the list was scrutinized by only considering genes that had at least a 2-fold change in signal values between the control and the GDNF withdrawal samples (137 probe sets). Finally, genes that were positively regulated by GDNF and subsequently recovered at least 2-fold in expression after GDNF replacement were evaluated for further analysis (61 probe sets).

The David Bioinformatics Database (http://david.abcc.ncifcrf.gov) was utilized for functional annotation clustering. Two gene lists were uploaded for analysis, genes that decreased at least 2-fold after GDNF withdrawal and genes that decreased 2-fold after GDNF withdrawal or recovered 2-fold after GDNF replacement. These lists were compared to the RATTUS NORVEGICUS background using the Functional Annotation tool with the default parameters. Only categories that had at least 10 genes represented, at least a 2-fold enrichment, and a p-value of ≤ 0.05 were reported.

siRNA Transfection

After trypsin digestion, washing, and quantification of established cultures of EpCAM+ clump forming germ cells, 250,000 cells/well were plated into wells of a 12-well dish without feeder in 1 ml/well of antibiotic free rSFM. Cells were allowed to settle for 2-3 h prior to siRNA treatment. For each well, 75 pmol of siRNA was mixed with 100 µl of OptiMEM (Invitrogen) and 2 µl of Lipofectamine 2000 (Invitrogen) was mixed with 100 µl of OptiMEM. After a 5 min incubation at room temperature, the siRNA and Lipofectamine mixtures were combined, mixed gently, and incubated at room temperature for 20 min. After the incubation, the siRNA-Lipofectamine mixtures were added to the previously plated cells and maintained overnight for 18 h. After the overnight incubation, cells were sub-cultured onto fresh feeders and maintained as previously described in siRNA free media for 7 more days. The efficacy of gene-specific siRNA knockdown, as well as off-target effects was determined for each siRNA prior to transplantation experiments. All gene-specific siRNA transplant experiments were replicated twice using independent primary cell cultures, and where appropriate data from the gene specific siRNAs from different companies was pooled. A negative control siRNA treated experimental unit was always run in parallel to the gene specific siRNA treated experimental unit because of

variation between SSC numbers in individual primary cultures of EpCAM+ clump forming germ cells.

Flow Cytometry

EpCAM+ clump forming germ cells were digested, washed, quantified and 160,000 cells were suspended in 80 μl of nexin buffer, and 10 μl of nexin reagent was added. Cells were incubated on ice for 20 minutes and analyzed. FlowJo flow cytometric analysis software (Ashland, OR) was used to determine the percent nexin negative and percent GFP positive cells in each sample. The percent of GFP positive cells was multiplied by the total number of cells from each well to determine the number of GFP positive cells.

Immunofluorescence and Immunocytochemistry

Immunofluorescence techniques were utilized to demonstrate the presence of the proteins for identified GDNF-regulated genes within the EpCAM+ clump forming germ cells. Briefly, cultures were fixed with 4% PFA for 10 min at 4°C and cells were permeabilized with ice-cold methanol for 2 min at 4°C. After washing three times with PBS, wells were blocked with 10% donkey serum for 1 h at 4°C. Cells were incubated overnight with primary antibodies to Bcl6b (a kind gift from Dr. D. Fearon, 1:100), Bhlhb2/Sharp2 (abcam, Cambridge, MA, ab12049, 1:25), Etv5 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-22807, 1:100), Hoxc4 (Santa Cruz Biotechnology Inc., sc-49986, 1:100), or Tec (Santa Cruz Biotechnology Inc., sc-25427, 1:100) diluted in 0.5% BSA in PBS. Additionally, IgG controls were also conducted. After overnight incubation with primary antibody, wells were washed 3 times with PBS and incubated with Alexa-488 conjugated secondary antibodies (Invitrogen, 1:1000) for 1 h. After secondary antibody incubation, cells were washed 3 times in PBS, stained with DAPI (Santa Cruz Biotechnology Inc), washed 3 times in PBS, and evaluated using fluorescent microscopy.

To confirm that proteins to identified genes were in fact expressed in vivo immunocytochemistry was performed on adult testis tissue. Briefly, tissues were fixed in formalin, blocked in paraffin, and sectioned at 6 μm. After de-waxing and re-hydration, sections were probed for protein expression using a Zymed histostain detection system (Invitrogen). Sections were blocked with 10% serum for 10 min followed by incubation with primary antibody overnight in a humidified chamber at 4°C. Antibodies used were to Bcl6b (Abnova, Taipei City, Taiwan, 1:100), Bhlhb2/Sharp2 (abcam, 1:50), Etv5/Erm (Santa Cruz Biotechnology Inc., 1:50), HoxC4 (Aviva Systems Biology, San Diego, CA, 1:500), and Tec (abcam, 1:100). The following day, sections were washed and a biotinylated secondary antibody was applied and slides were incubated at room temp for 15 min. Slide were then washed, incubated with enzyme conjugate, washed again, developed with AEC chromagen, mounted in GVA, and visualized. Negative controls were IgGs from the same species as the primary antibody.

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