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Supplemental Information

Enhanced Fitness of Adult Spermatogonial Stem Cells Bearing a Paternal Age-Associated FGFR2 Mutation

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Figure S1

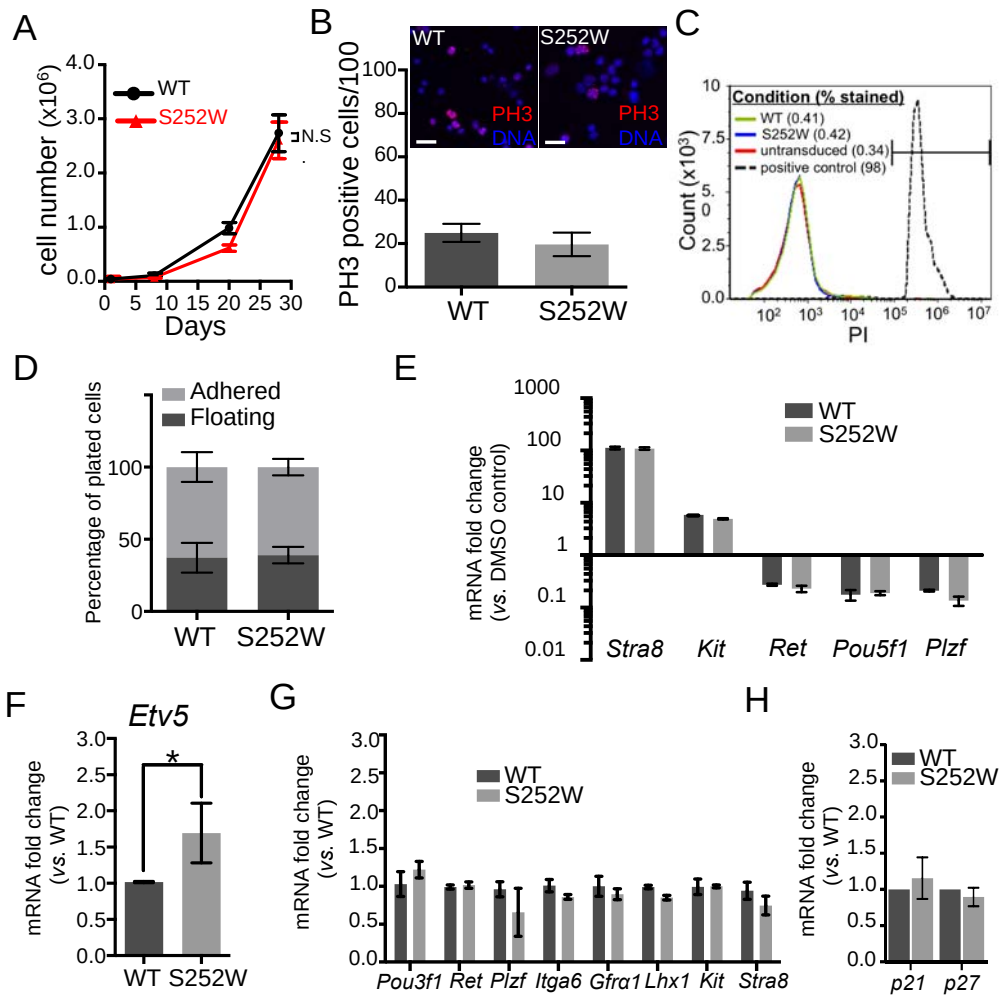

Figure S1, Related to Figure 2. Comparison of Phenotype in WT and S252W SSCs **(**A) Growth curves of WT and S252W SSCs after several weeks *in vitro* in standard culture conditions. Data represent mean ± SD; n=3 wells per time point). N.S., p >0.05 (two-tailed unpaired t-test with Welch's correction). (B) Representative immunostaining (red) for mitosis marker phospho-histone H3 (PH3) for WT and S252W SSCs (Scale bar = 20 μm) and quantification with percentage of PH3-positive cells. Data represent mean ± SD in 5 image fields/genotype (>900 total cells). (C) FACS showing proportions of cell death in trypsinized WT and S252W SSCs stained with propidium iodide (PI). Ethanol-treated SSCs were used as positive control. (D) Cell adhesion to laminin in the presence of growth factors of WT and S252W SSC lines (mean ± SD of three experiments (*i.e.*, different cell lines) with n=3 wells/ experiment. **(**E) Representative QPCR showing changes in expression of differentiation or self-renewal markers in WT and S252W SSC lines after treatment with retinoic acid (RA) for 48 hr (fold change *vs.* vehicle control). Data are mean ± SD (n=3 wells). (F) QPCR showing mean relative *Etv5* expression (± SD; n=4 independent cell lines) in S252W *vs.* WT SSCs. *p< 0.05 (two-tailed, Mann Whitney test). (G-H) QPCR: Relative expression levels (mean ± SD; n=3 wells) for SSC self-renewal and differentiation markers in S252W *vs.* WT SSC lines. Panels A-C, E, G, and H show one out of ≥ two representative experiments performed with independent lines.

Figure S2

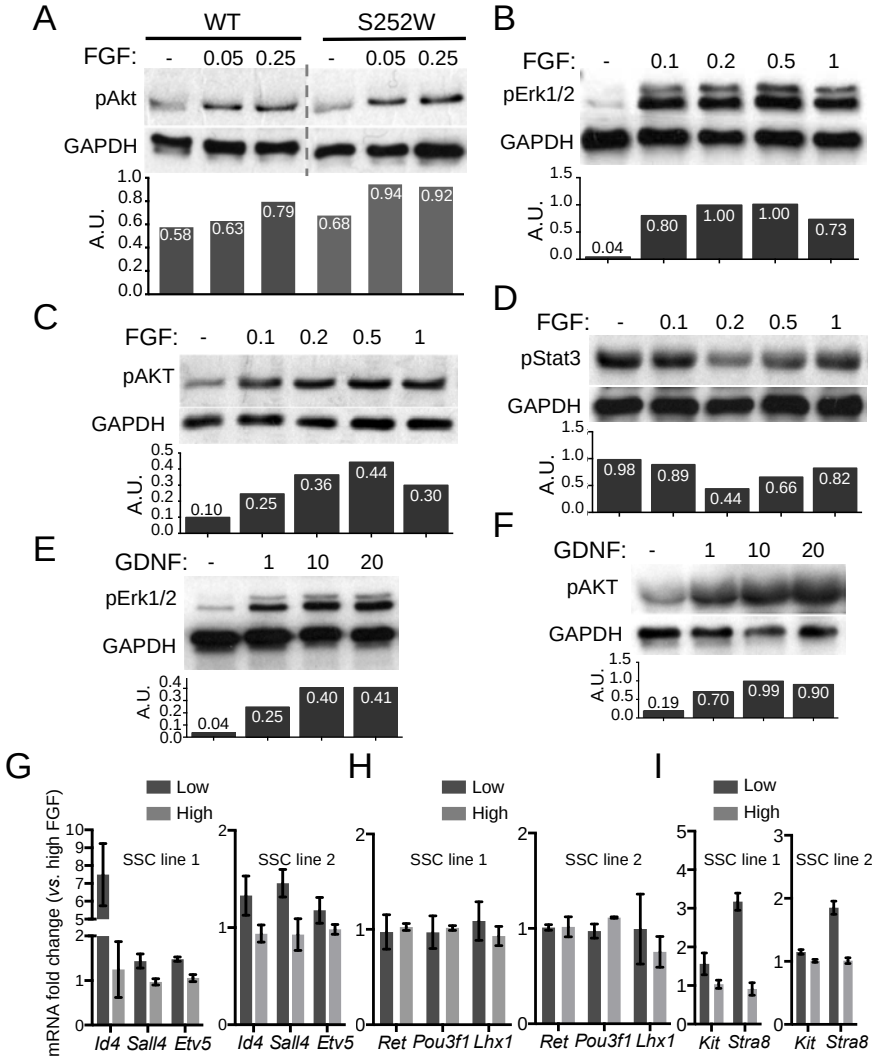

Figure S2, Related to Figure 3. Growth factor dose modulates downstream signaling and expression of self-renewal markers in SSCs. (A) Representative anti-AKT phospho-serine 473 IB in WT and S252W SSCs in response to FGF2 (0.05 and 0.25 ng/ml) after starvation for 16 hr. Grey dashed line denotes a cropped lane. Graphs correspond to densitometric analyses of IB images above each plot for pAkt normalized to loading control (GADPH). Numbers correspond to the quantification for each condition in arbitrary units (A.U.). (B-D) IB and densitometry for changes in pMAPK (*i.e*. pErk1/2), pAKT, and pStat3 in native SSCs (*i.e.,* without hFGFR2) treated with different doses of FGF2 (0-1 ng/ml). (E-F) IB and densitometry for changes in pMAPK (*i.e*. pErk1/2) and pAKT in native SSCs (*i.e.,* without hFGFR2) treated with different doses of GDNF (0-20 ng/ml). Densitometry is normalized to GAPDH. (G-I) QPCR in native SSCs (*i.e.,* without hFGFR2), comparing expression levels of self-renewal and differentiation markers in the presence of FGF2 at low (0.5 ng/ml) *vs* high (10 ng/ml) levels for 1 week in feeder-free conditions. Left and right graphs in each panel correspond to two biological replicates out of ≥2 independent experiments with different SSC lines. Data points represent mean±SD; n=3 technical replicates. (G) Differentially expressed stem cell markers. (H) Stably expressed stem cell markers. (I) Differentiation markers.

Figure S3

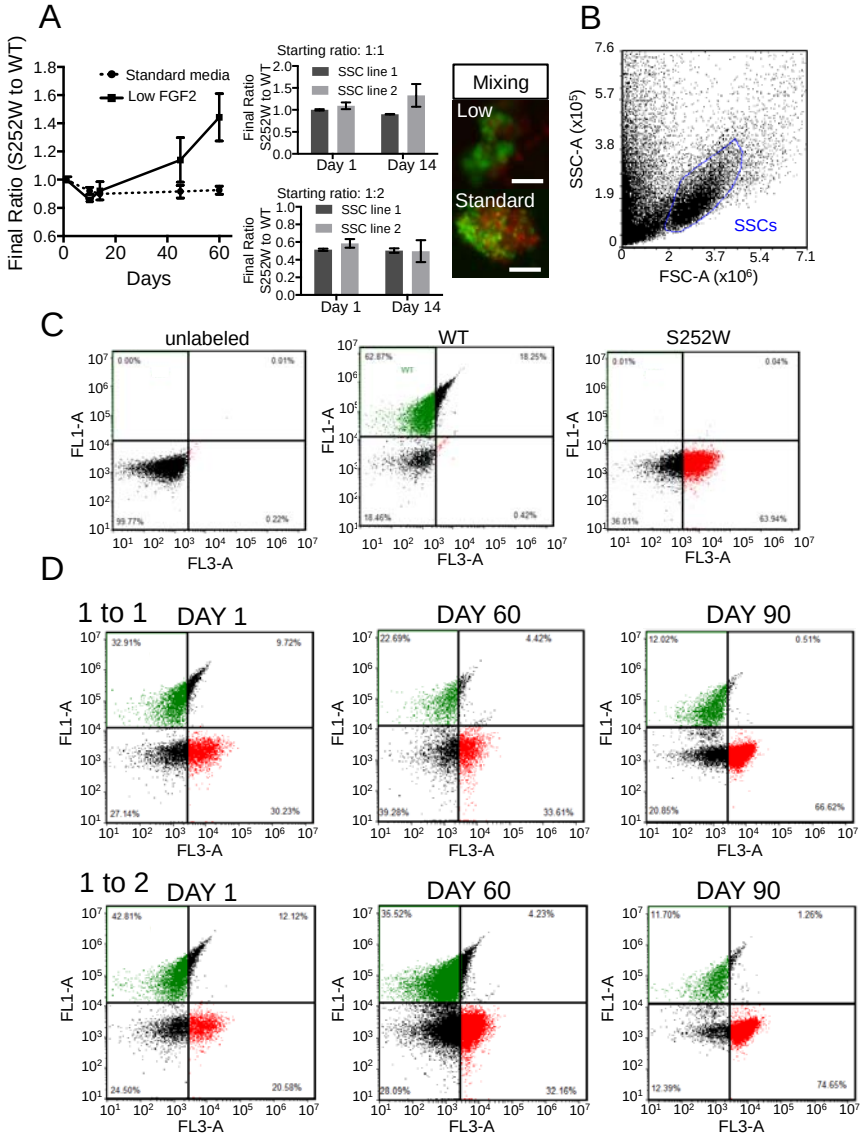

Figure S3, Related to Figure 4. *In vitro* competition assay with representative flow cytometry used for quantification. (A) Left: *In vitro* mixing experiment showing change in ratio (FACS analysis) over time of S252W to WT in standard media or low FGF2 (1 ng/ml). Mutant and WT SSCs were mixed at a 1:1 ratio. Data are from one of two biological replicates (*i.e.*, independent lines), showing the mean±SD (n=3 wells/condition) for each time point. Middle: Bar graphs show results of mixing experiments at day 1 and 2 weeks for 2 independent biological replicates in standard media in both 1:1 (top) and 1:2 (bottom) starting mixing ratios. Error bars correspond to mean±SD (n=3 wells/condition). Right: Fluorescent images correspond to mixed SSC colonies in each condition (scale bar =50 μm) (B-D) Representative FACS data. (B) SSC population (blue) based on forward-scatter (FSC-A) *vs.* side-scatter (SSC-A). (C) Dot plots with quadrants of gated SSCs showing percentage of GFP^+ (green) and $mCherry^+$ (red) SSCs in unlabeled (left), WT (center) and S252W (right) SSC lines cultured in separate wells. (D) Dot plots with quadrants of gated SSCs, showing the percentages of WT-GFP^+ (green) and S252W-$mCherry^+$ (red) SSCs in mixing experiments at different time points for 1:1 (Top) and 1:2 (Bottom) mutant:WT ratios.

Supplemental Experimental Procedures

SSC Lines and Cell Culture

Cell culture media consisted of StemPro-34 (Gibco Cat. No 10640) with nutrient supplement (Gibco Cat No 10641) containing additional supplements and growth factors at the following concentrations: 20 ng/ml GDNF, 10 ng/ml FGF2, 25 μg/ml insulin, and 20 ng/ml EGF. Media changes and culture passages were performed as described previously (Martin and Seandel, 2013). SSC lines stably expressing hFGFR2 alleles driven by the human PGK promoter were generated by lentivirus delivery. Labeling was performed by co-transduction with EGFP or mCherry and hFGFR2 lentivirus. The ratio of EGFP (or mCherry) to FGFR2 was 1:4 by volume. Alternatively, we used pre-labeled SSCs that had been sorted to homogeneity using a BD FACSAria II. Supernatants from 293T cells containing lentiviruses were titrated using p24 ELISA (Lenti-X p24 Rapid Titer Kit, Clontech). Feeder-free SSC cultures were infected with lentivirus over night in SSC media containing polybrene (5 μg/ml). Transduction efficiency was monitored by fluorescence microscopy and by QPCR with specific primers for human FGFR2. FGFR2 protein was detected by immunofluorescence and immunoprecipitation followed by immunoblot as described below. For propidium iodide staining, SSCs were triturated to detach from feeders, trypsinized, treated with 0.5 mg/ml of DNAse I and resuspended in PBS/ 0.1 % bovine serum albumin. Propidium iodide (Calbiochem) was added at 10 μg/ml for 15 min at room temperature, and positive cells were quantified using a BD Accuri C6 Flow Cytometer. Adhesion assays were performed in laminin coated 24-wells (8μg/well overnight at room temperature, Millipore) in the absence of feeders. Specifically, SSCs were triturated off feeders, trypsinized, treated with 0.5 mg/ml of

DNAse I and plated in triplicate at $1x10^3$ cells/mm^2 in standard SSC culture media. Floating and adhered cells were collected two hours after plating and trypsinized prior counting. For co-culture experiments, differentially labeled WT and S252W SSCs were mixed in triplicate wells (at 1:1 or 2:1 WT to S252W ratios) and co-cultured for several weeks *in vitro* in standard cell culture conditions at different FGF2 doses (0.2, 1, and 10 ng/ml). The S252W to WT ratio was measured the day after plating by flow cytometry (BD Accuri C6) and weekly thereafter. Mixed cells were re-plated onto fresh feeders for continuous co-culture.

Retinoic Acid and Growth Factor Treatments

SSCs were plated in gelatin-coated wells without feeders at 2-3 $x10^3$ cells/mm^2 in growth media for retinoic acid (RA) treatment or in starvation media for 12-16 hr. Starvation media consisted of growth media without growth factors (*i.e*. lacking GDNF, FGF2, insulin and EGF) Next, RA or vehicle control (DMSO) was added to 1 μM or 0.5% final concentration, respectively, every 24h for 2 or 3 days. Alternatively, growth factor stimulation was performed for 20 minutes at 37°C in starvation media containing the appropriate growth factor at indicated concentrations.

SSC Transplantation

For *in vivo* evaluation of stem cell activity, differentially labeled WT and S252W SSCs were mixed prior transplantation into host mice or mixed for *in vitro* co-culture for 3 weeks prior to transplantation. SSC transplants were performed as described previously (Seandel et al., 2007). Briefly, adult C57Bl6 male mice at 6-8 weeks of age were treated

with busulfan (40 mg/kg body weight) and used 4-6 weeks later as recipients. SSCs were dissociated with 0.05% trypsin/EDTA, treated with 1 mg/ml DNAse I, and resuspended at a concentration of 8-10 $x10^6$ cells/ml in culture medium containing DNase I and sterile-filtered trypan blue. 10 μl of donor cell suspension was transplanted into each testis by microinjection into the efferent ducts. Two months after transplantation, detunicated recipient testes were analyzed using a fluorescent stereoscope (Zeiss) for the number of GFP or mCherry colonies. Pooled data of transplanted testis included 10 experiments using four independent cell lines at "1 to 1" or "1 to 2" (S252W to WT ratio) in which each genotype was labeled with either GFP or mCherry. For depiction of pooled data, we first normalized the colony number based on the ratio injected. The data were depicted as the percentage of normalized colony numbers for each genotype out of the total. Statistical analyses were performed using GraphPad™.

Immunohistochemistry and Immunofluorescence

Human cadaver testes were obtained from the New York Organ Donor Network. Slides prepared from Bouin's fixed paraffin embedded testis tissue were used for endogenous FGFR2 immunohistochemistry (IHC). Slides were rinsed in xylenes and EtOH to deparaffinize and rehydrate respectively. Next, slides were incubated for 30 min in steamer in Tris-EDTA pH 9 antigen retrieval buffer, cooled for 15 min and incubated in 3% H_2O_2 for 3 min. Slides were blocked with tyramide blocking buffer (TBB; Jacobs et al., 1998) for 30 min and incubated with mouse monoclonal anti-human FGFR2 antibody ([3F8] ab119237, Abcam; 0.1 μg/ml) or control mouse IgG (Jackson ImmunoResearch at 0.1 μg/ml) in TBB over night at 4° C. Slides were then incubated with streptavidin HRP

for 30 min at room temperature (Jackson Immunoresearch 016-030-084 0.5 μg/ml) followed by biotinyl tyramide for 15 min at room temperature (1:200 in PBS with 0.03% H_2O_2). Finally, slides were incubated with streptavidin HRP for 30 min at room temperature and developed for 2.5 min with 0.05% AEC (3-amino-9-ethylcarbazole, Amresco) and 0.015% H_2O_2 in acetate buffer pH 5.5. Hematoxylin was used as the counterstain. For immunofluorescence on cultured cells, SSCs were triturated off from feeders, trypsinized, treated with 1 mg/ml DNAse I and resuspended in growth media. The cell suspension was dried on to glass slides and fixed in 4% PFA for 10 min at room temperature. Cells were blocked using 10% donkey serum before incubation with primary antibodies overnight at 4° C. The following primary antibodies were used: mouse monoclonal anti-human FGFR2 antibodies ([3F8] ab119237, Abcam; and Bek (C-8) sc-6930, Santa Cruz Biotechnology) and anti-phospho-Histone H3 (Ser10) antibody (Millipore 06-570). Detection of primary antibodies was performed with biotin-conjugated secondary antibodies followed by Alexa488-conjugated streptavidin. Slides were counterstained and mounted with ProLong Gold antifade reagent (Invitrogen) and analyzed using Olympus BX50 microscope. For phospho-H3 quantification, images were captured from multiple randomly selected areas counting >900 cells in total for each cell line. Total cell number per area was determined by counting DAPI-stained nuclei, followed by quantification of pH3 positive cells. IH and IF images were captured with Olympus BX50 fluorescence microscope.

Immunoprecipitation and Immunoblot

Feeder-free SSCs were suspended in lysis buffer containing PMSF (Sigma) and protease and phosphatase inhibitors cocktails (Sigma). For immunoprecipitation, 75 μg of protein lysate was incubated overnight at 4° C with anti-human FGFR2 rabbit monoclonal antibody (D4H9, Cell Signaling) followed by incubation with protein A agarose beads for 3 hr at 4° C. Immunocomplexes were washed three times with lysis buffer, and the pellet was resuspended in 2x Laemmli sample buffer, separated by SDS-PAGE, and transferred to a PVDF membrane (Amershan) for detection with mouse monoclonal anti-human FGFR2 antibody ([3F8] ab119237, Abcam) as described next. Immunoblot was performed following standard procedures. Briefly, 10 to 15 μg of total cell lysates were separated by SDS-PAGE and transferred into PVDF membrane (Amershan). Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBST) with 3% BSA and incubated with the following primary antibodies: anti-pAkt (Ser473, Cell Signaling), anti-pAkt (Ser473, R&D Systems), anti-p44/42 MAP Kinase (Cell Signaling), and anti phosphoStat3 (Tyr705) (clone M9C6, Cell Signaling). Anti-GAPDH (mouse monoclonal, Abcam) was used as a loading control. Blots were washed with TBST and incubated at room temperature with the appropriate secondary antibody conjugated to horseradish peroxidase. Chemiluminescent immunodetection was performed with ECL system (Amersham), and membranes were exposed to X-ray film (Hyperfilm ECL, Amersham). Digitized scans of the blots were analyzed using ImageJ for densitometry.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Quantitative Reverse Transcription Polymerase Chain Reaction (QPCR)

Total RNA was extracted from snap-frozen human cadaver testis samples or feeder-free cultured SSCs using RNeasy Plus extraction kit (Qiagen). RNA was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences). PCR amplification was performed with Platinum Taq Polymerase. QPCR was performed in a LightCycler 480II real-time System (Roche) with PerfeCta SYBR Green FastMix (Quanta Biosciences). For quantification, data was normalized to endogenous β-actin and GAPDH and relative transcript expression was calculated using the comparative C_T method ($2^{-\Delta\Delta Ct}$ method).

Molecular Biology

Human FGFR2 cDNA was obtained from GeneCopoeia (clone A0979, catalog no EX-A0979-M02) and used as template to generate the hFGFR2 S252W mutant cDNA using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's protocol. The mutagenesis primers were fwd 5'-TGTTGTGGAGCGATG GCCTCACCGGC-3' and rev 5'-GCCGGTGAGGCCATCGCTCCACAACA-3'. WT and S252W expression vectors for lentivirus production were prepared as follows: full-length WT and S252W cDNAs were amplified using primers containing BamHI and SalI restriction sites at the 5' and 3' end of the PCR product respectively (fwd 5'-CGCGG ATCCACCATGGTCAGCTGGGGTCGTTTC-3' and rev 5'-GCCGACGTCGACTCAT GTTTTAACACTGCCGTTTATG-3'). Digested PCR products were subcloned into pCCL4-PGK expression vector using BamHI and SalI sites. The final constructs were sequenced in the Applied Biosystems Automated 3730xl DNA Analyzer.

Statistics

Results are presented as means ± SD. The number of independent biological or technical replicates is indicated by "n". The statistical significance was determined by two-tailed unpaired t-test, Wilcoxon matched-pairs signed rank test, or the Kruskal-Wallis test as indicated in each figure (GraphPad Prism, version 6.0). Results were considered significant at p-value < 0.05 ($p < 0.05$ is indicated as *; $p < 0.005$ is indicated as ***). NS = not significant.

Primer Sequences for QPCR

Human *FGFR2*
Fwd.1 5'-TGACACCGATGGTGCGGAAGA-3'
Rev.1 5'-CGGCTGGGCAGCGAAACTTG-3'
Fwd.2 5'-GAATATCATAAATCTTCTTGGAGC-3'
Rev.2 5'-TCATAGGAGTACTCCATCCCG-3'
Mouse *Fgfr2*
Fwd.1 5'-TCCAACGCCCACAATGAGGTG-3'
Rev.1 5'-CTGACGGGACCACACTTTCC-3'
Fwd.2 5'-GTCTGTCCTCAACAGCGGAC-3'
Rev.2 5'-CCCACTGCTTCAGCCATGAC-3'
Mouse *Etv5*
Fwd 5'-AGAACCTGGATCACAGCAAC-3'
Rev 5'-ACTATCTCCAGGAACTCCTG-3'
Mouse *Stra8*
Fwd 5'-TCCCAGTCTGATATCACAGC-***3***
Rev 5'-TCCCATCTTGCAGGTTGAAGG-3'
Mouse kit
Fwd 5'- GCCAGTGCTTCCGTGACATT-3'
Rev 5'- TGCCATTTATGAGCCTGTCGTA-3'
Mouse Ret
Fwd 5'- GGCTGTCCCGAGATGTTTATG-3'
Rev 5'- GACTCAATTGCCATCCACTTGA-3'
Mouse P*ou5f1*
Fwd *5'*-AGCAACTCAGAGGGAACCTCC-*3*'

Rev 5’-GGTGATCCTCTTCTGCTTCAG-3’

Mouse *Plzf*

Fwd 5’- TTTGCGACTGAGAATGCATTTAC-3’

Rev 5’-ACCGCATTGATCACACACAAAG-3’

Mouse *Id4*

Fwd 5’-ACTACATCCTGGACCTGCAG-3’

Rev 5’-TGCTGTCACCCTGCTTGTTC-3’

Mouse *Sall4*

Fwd 5’-AGCACTGC TGCACACGGTGTG-3’

Rev 5’- GTCATGTAGTGTACCTTCAGG-3’.

Mouse *Lhx1*

Fwd 5’-AAGCCAAGCAACTGGAGACG- 3’

Rev 5’- GATTCTGGAACCAGACCTGG-3’

Mouse *Pou3f1*

Fwd 5’-AAGCAACGACGCATCAAG C-3’

Rev 5’-TTGCACATGTTCTTGAAGCTC-3’

Mouse *Itga6*

Fwd 5’-CTGCAGCGTCAACGTGAGGTGT-3’

Rev 5’-ACTCGAACCTGAGTGCCCGC-3’

Mouse *Gfrα1*

Fwrd 5’- AGCAACAGTGGCAATGACCTG -3’

Rev 5’- AGTGGTAGTCGTGGCAGTGG -3’

Mouse *p21*

Fwd 5′-GCAGATCCACAGCGATATCC-3′

Rev 5′-CAACTGCTCACTGTCCACGG-3′

Mouse *p27*

Fwd 5’ GGGTTAGCGGAGCAGTGTCC 3’

Rev 5’ GTCTGCTCCACAGTGCCAGC 3’

Supplemental References

Jacobs, W., Dhaene, K., and Van Marck, E. (1998). Tyramine-amplified immunohistochemical testing using "homemade" biotinylated tyramine is highly sensitive and cost-effective. Archives of pathology & laboratory medicine *122*, 642-643.