**Stem Cell Reports, Volume *10***

## Supplemental Information

# IGF-1R Promotes Symmetric Self-Renewal and Migration of Alkaline Phosphatase$^{+}$ Germ Stem Cells through HIF-2$\alpha$-OCT4/CXCR4 Loop under Hypoxia

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# Supplemental information

**Supplemental Figures:**

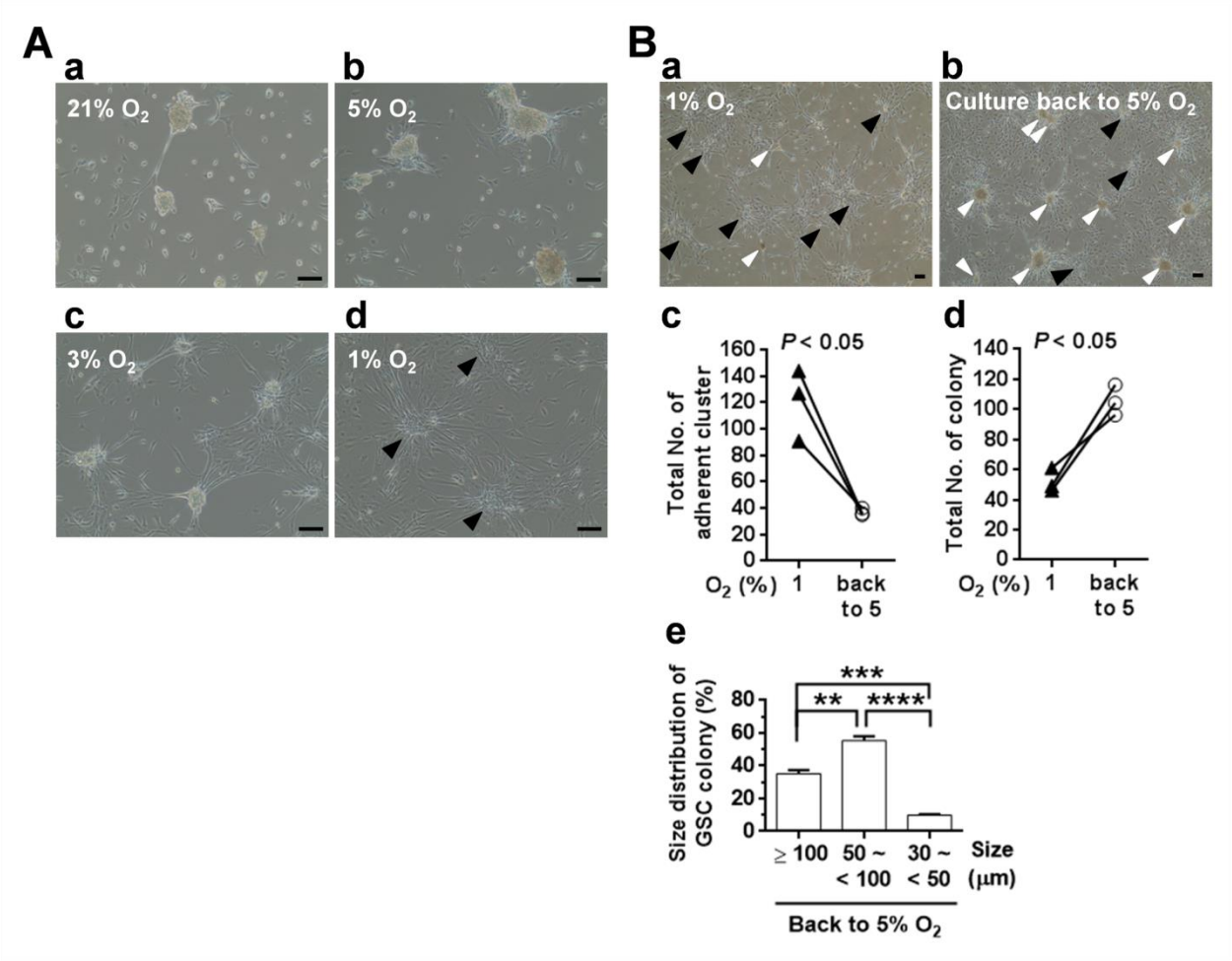


**Figure S1. Effect of oxygen tension on AP$^+$GSC colony formation under a serum-free culture condition. (Related to main Figure 2.)**

**A. (a-c)** Colony morphology of mouse P2 AP$^+$GSCs under normoxia (21% $O_2$) and hypoxia (5% and 3% $O_2$). **(d)** Cluster of mesenchymal-like cells under 1% $O_2$ hypoxia (black arrowhead). Scale bar, 100 μm. **B.** GSC colony re-formation assay. **(a)** The P2 AP$^+$GSCs were cultivated under hypoxia (1% $O_2$) for 7 days and **(b)** re-placed in 5% $O_2$ for another 7 days. Black arrowhead, GSC adherent cluster; white arrowhead, GSC colony. Scale bar, 100 μm. **(c)** Quantification of the GSC adherent cluster number, **(d)** colony number, and **(e)** colony size distribution. Data are mean ± SEM of at least three independent experiments. *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001, ****$P$ < 0.0001. Student's $t$ test **(c and d)** and one-way ANOVA **(e)**.

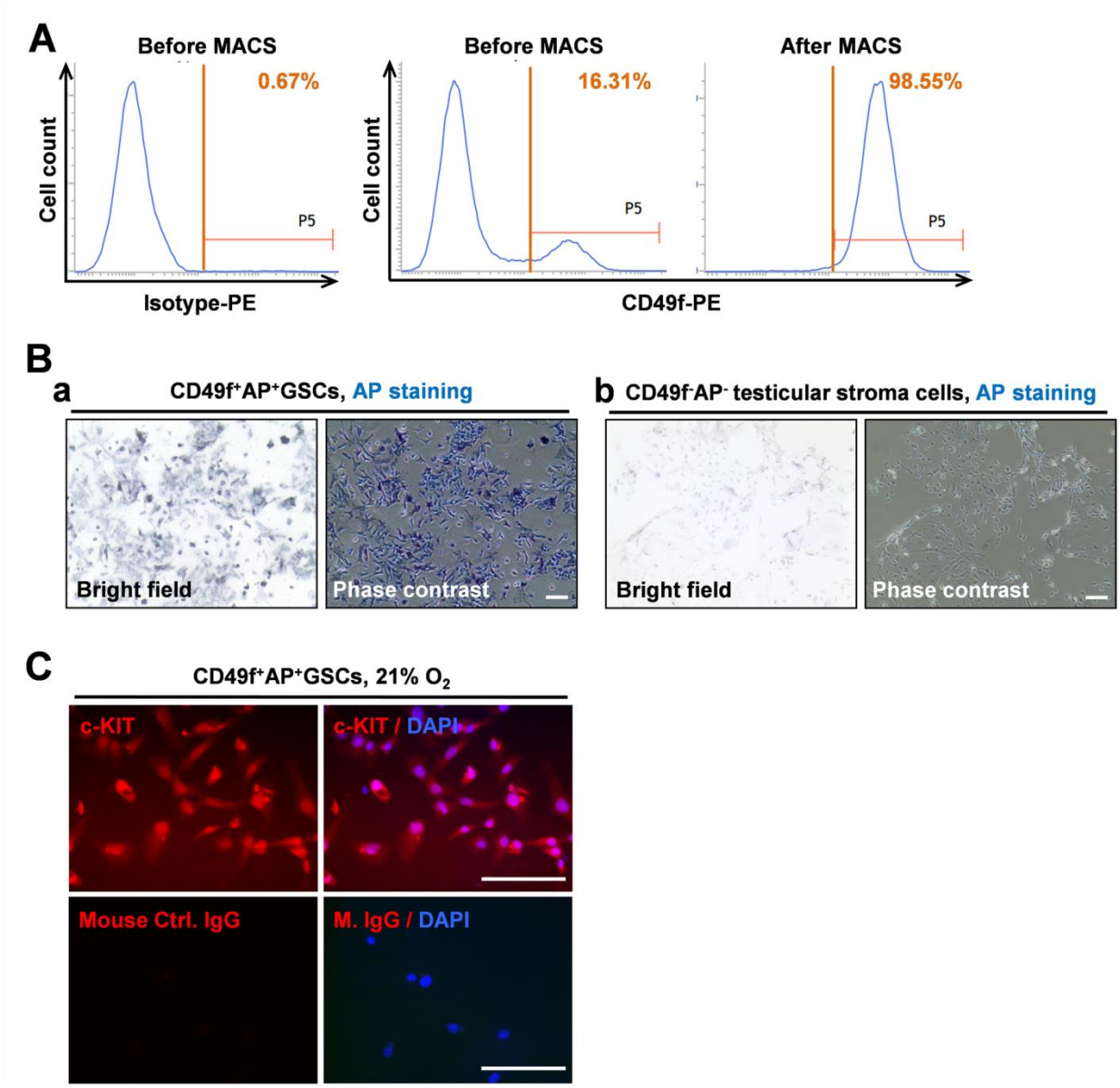


**Figure S2. MACS isolation and characterization of CD49f$^+$AP$^+$GSCs. (Related to main Figure 3.)**

**A.** Purification of CD49f$^+$AP$^+$GSCs using an MACS. The purity of the CD49f$^+$ cell population (P5 region) was analyzed using flow cytometry. **B.** Alkaline phosphatase staining (in blue) of **(a)** CD49f$^+$AP$^+$GSCs and **(b)** CD49f$^-$AP$^-$testicular cells cultivated under 5% $O_2$ hypoxia. Scale bar, 100 μm. **C.** Immunofluorescence staining of germ cell-specific proteins in CD49f$^+$AP$^+$GSCs under normoxia. Scale bar, 100 μm.

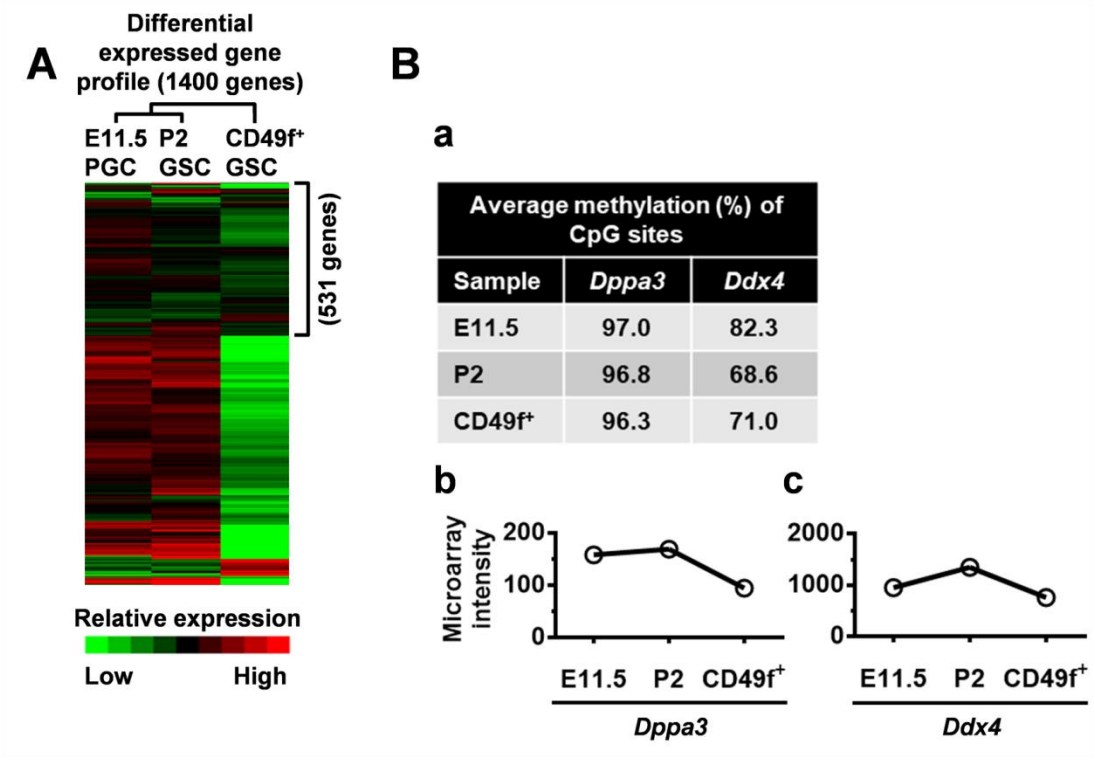


**Figure S3. Transcriptome and epigenetic expression patterns of E11.5 PGCs, P2 AP$^+$GSCs, and CD49f$^+$AP$^+$GSCs. (Related to main Figures 1 and 3.)**

**A.** Heatmap of global gene expression pattern of E11.5 PGCs, P2 AP$^+$GSCs, and purified AP$^+$CD49f$^+$GSCs. **B.** Epigenetic and gene expression analysis of *Dppa3* and *Ddx4*. **(a)** Bisulfite genomic sequencing. **(b and c)** Gene expression levels from a microarray analysis.

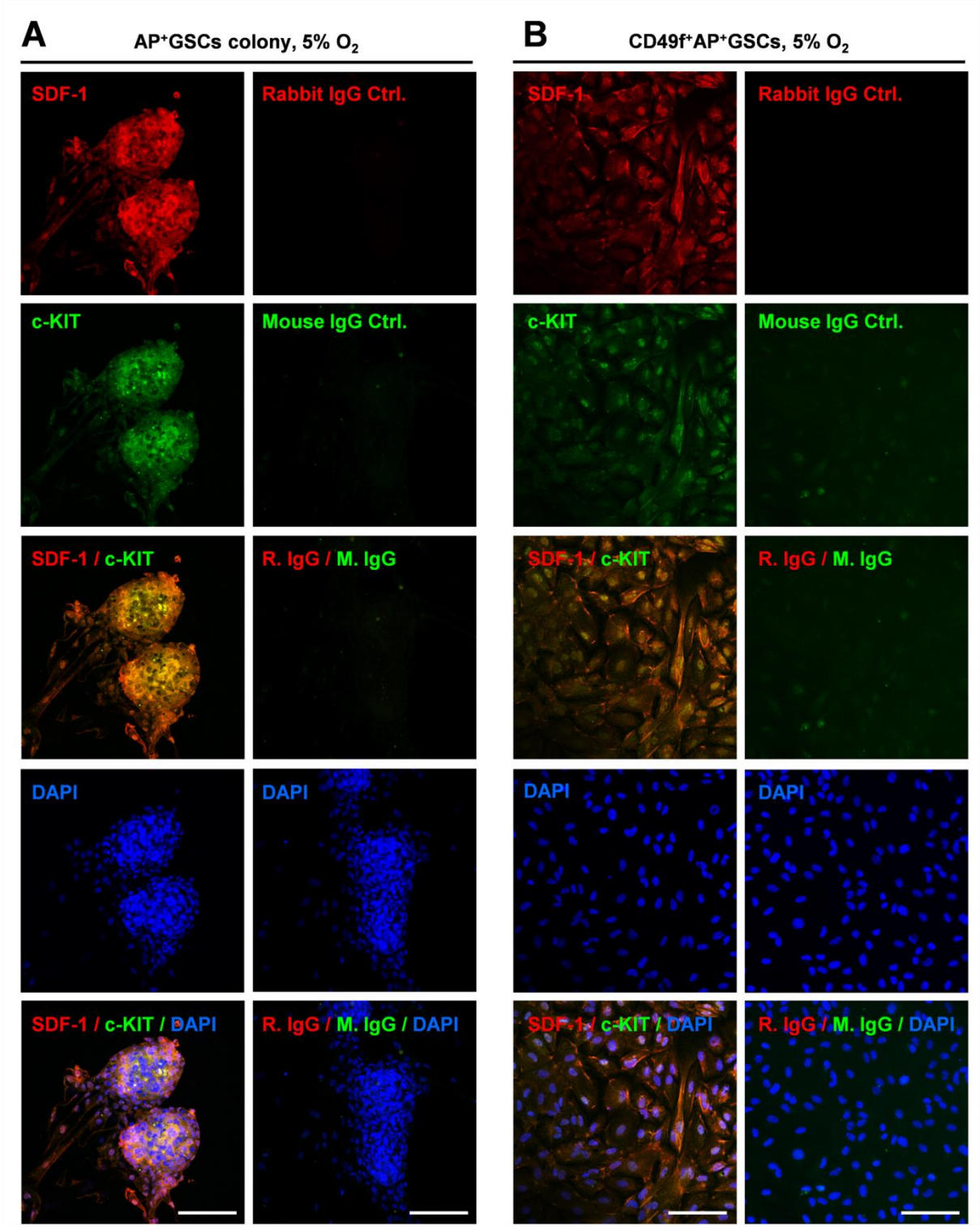


**Figure S4. Immunostaining of SDF-1 and c-KIT in AP$^+$GSC colonies and CD49f$^+$AP$^+$GSCs. (Related to main Figure 3.)**

Immunofluorescence staining of SDF-1 and c-KIT in **(A)** AP$^+$GSC colonies and **(B)** CD49f$^+$AP$^+$GSCs under 5% $O_2$ hypoxia. Negative immunostaining controls (IgG) are shown. Scale bar, 100 μm.

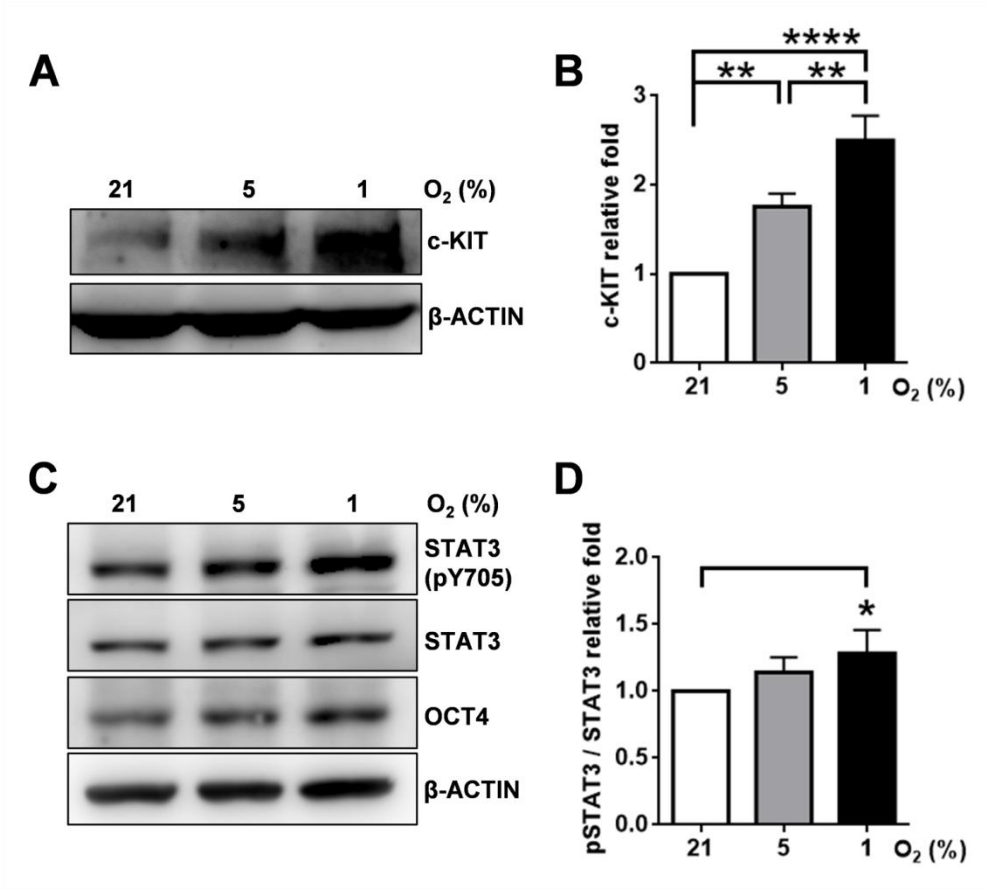


**Figure S5. Effect of hypoxia on c-KIT, phospho-STAT3, and OCT4 expression in CD49f$^+$AP$^+$GSCs. (Related to main Figures 2 and 3.)**

**A.** Effect of different oxygen tension (21%, 5%, and 1% $O_2$ for 24 h) on c-KIT expression levels of CD49f$^+$AP$^+$GSCs (Western blot analysis). **B.** Quantitative analysis of **(A)**. Data are the means ± SEM of at least three independent experiments. One-way ANOVA. $^{**}P < 0.01$, $^{****}P < 0.0001$. **C.** Cells were cultivated under different oxygen tension of 21%, 5%, and 1% $O_2$ for 24 h. The protein levels of STAT3, phospho-STAT3 (pY705), and OCT4 of CD49f$^+$AP$^+$GSCs are shown. Western blot analysis. **D.** Quantitative analysis of **(C)**. Data are the means ± SEM of at least three independent experiments. One-way ANOVA. $^{*}P < 0.05$.

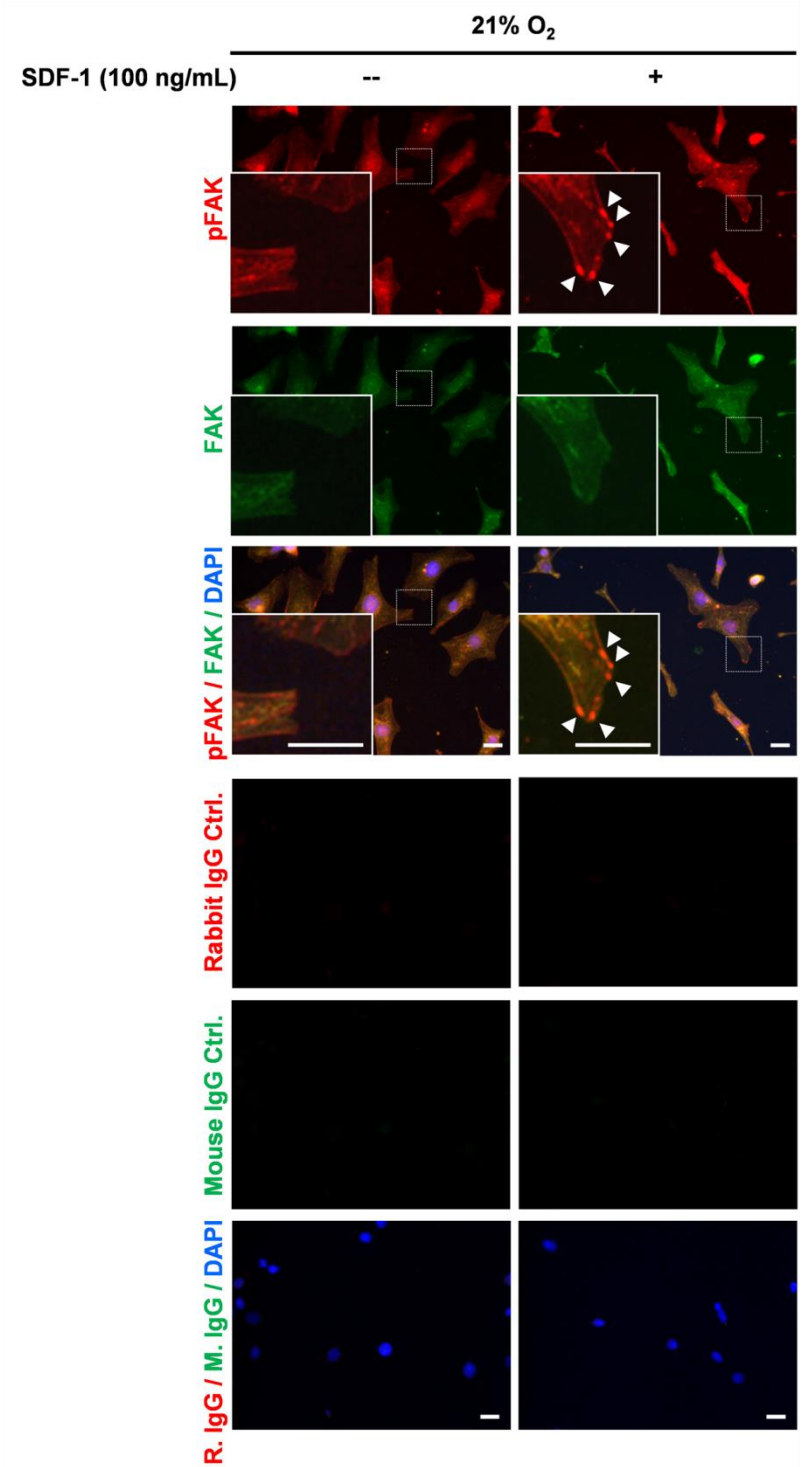


**Figure S6. Effect of SDF-1 on phospho-FAK levels in CD49f$^{+}$AP$^{+}$GSCs under normoxia. (Related to main Figure 3.)** The CD49f$^{+}$AP$^{+}$GSCs were treated with or without SDF-1 (100 ng/mL) for 48 h under normoxia (21% $O_2$). The levels of pFAK and FAK were detected by immunofluorescence staining. Negative immunostaining controls (IgG) are shown. Arrowheads indicate the specific cellular protein localization of pFAK. Scale bar, 20 μm.

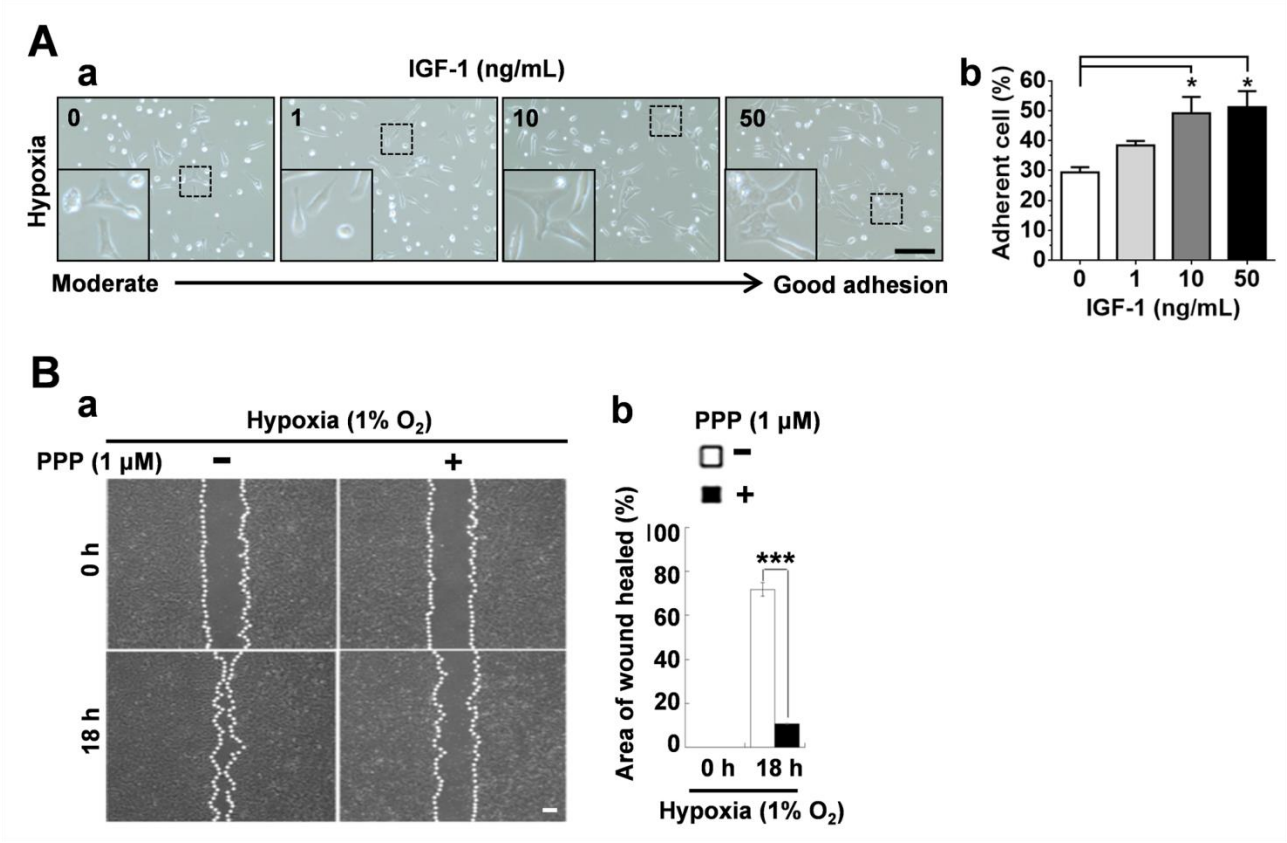


**Figure S7. Effect of IGF-1/IGF-1R activation on the cell adhesion and migration of mouse CD49f$^{+}$AP$^{+}$GSCs. (Related to main Figures 4 and 5.)**

**A.** Effect of IGF-1 (0, 1, 10, and 50 ng/mL) on the cell morphology of CD49f$^{+}$AP$^{+}$GSCs under hypoxia (5% $O_2$). **(a)** Cell adhesion and morphology. Scale bar, 100 μm. **(b)** Quantitative analysis of **(a)**. Data are the means ± SEM of at least three independent experiments. *$P$ < 0.05. One-way ANOVA. **B.** Migration assay of CD49f$^{+}$AP$^{+}$GSCs under hypoxia (1% $O_2$, 18h) with or without PPP treatment. **(a)** Wound closure assay. **(b)** Quantitative analysis of **(a)**. Data are the means ± SEM of at least three independent experiments. ***$P$ < 0.001. Student's $t$ test. Scale bars, 100 μm.

**Supplemental Tables:**

**Table S1. List of protein classes upregulated in hypoxic $AP^{+}$GSCs. (Related to main Figure 2)**

| Protein class | Accession No. | Proportion (%) |
|---|---|---|
| Receptor | PC00197 | 10.9 |
| Extracellular matrix protein | PC00102 | 10.7 |
| Protease | PC00190 | 8.4 |
| Cell adhesion molecule | PC00069 | 6.6 |
| Defense/immunity protein | PC00090 | 4.8 |
| Calcium-binding protein | PC00060 | 4.3 |
| Chaperon | PC00072 | 3.5 |
| Surfactant | PC00212 | 2.3 |
| Kinase | PC00137 | 1.8 |
| Ligase | PC00142 | 1.5 |
| Phosphatase | PC00181 | 1.0 |
| Membrane traffic protein | PC00150 | 0.8 |
| Transmembrane receptor regulatory/adaptor protein | PC00226 | 0.3 |

**Table S2. List of proteins upregulated under hypoxia. (Related to main Figure 2)**

| Protein | Accession No. | Ratio of Hypo/Normo# | Biological functions and processes |
|---|---|---|---|
| CDH11 | Mm.1571 | 88.42 | Calcium ion binding, metal ion binding, cell adhesion, corticospinal tract morphogenesis, etc. |
| DCN | Mm.56769 | 29.54 | Extracellular matrix binding, glycosaminoglycan binding, regulation of autophagy, etc. |
| CXCL12 | Mm.303231 | 11.60 | CXCR chemokine receptor binding, growth factor activity, chemokine activity, regulation of cell migration, regulation of cell proliferation, germ cell development, etc. |
| CST3 | Mm.4263 | 6.52 | β-amyloid binding, cysteine-type endopeptidase inhibitor activity, identical protein binding, protease binding, response to oxidative stress, regulation of programmed cell death, etc. |
| LOX | Mm.172 | 6.32 | Oxidoreductase activity, protein-lysine 6-oxidase activity, copper ion binding, metal ion binding, response to steroid hormone, blood vessel development, etc. |
| CLU | Mm.200608 | 6.08 | Misfolded protein binding, ubiquitin protein ligase binding, intrinsic apoptotic signaling pathway, regulation of cell differentiation, etc. |
| VEGFA | Mm.282184 | 5.55 | Chemoattractant activity, growth factor activity, VEGF receptor binding, PDGF receptor binding, extracellular matrix binding, angiogenesis, cell differentiation, etc. |
| MMP2 | Mm.29564 | 5.02 | Metalloendopeptidase activity, fibronectin binding, metal ion binding, zinc ion binding, angiogenesis, cell migration, response to hypoxia, embryo implantation, etc. |
| SPARC | Mm.291442 | 4.69 | Extracellular matrix binding, calcium ion binding, metal ion binding, endothelial cell migration, cell morphogenesis, cell proliferation, cellular response to growth factor stimulus, etc. |
| CSTB | Mm.6095 | 4.59 | Cysteine-type endopeptidase inhibitor activity, protease binding, negative regulation of proteolysis, etc. |
| GPC4 | Mm.1528 | 4.57 | Heparan sulfate proteoglycan binding. |
| BGN | Mm.2608 | 4.36 | Extracellular matrix binding, glycosaminoglycan binding, peptide cross-linking via chondroitin 4-sulfate glycosaminoglycan |
| IGFBP3 | Mm.29254 | 4.09 | Insulin-like growth factor binding, fibronectin binding, protein tyrosine phosphatase activator activity, regulation of cell growth, regulation of glucose metabolic process, etc. |
| THBS2 | Mm.26688 | 3.87 | Heparin binding, protein binding, synapse assembly, angiogenesis, cell adhesion. |
| FBLN2 | Mm.249146 | 3.83 | Extracellular matrix binding, protein binding, calcium ion binding, regulation of cell-substrate adhesion. |
| LAMA1 | Mm.303386 | 3.80 | Extracellular matrix structural constituent, receptor binding, glycosphingolipid binding, cell adhesion, cell migration, embryonic development, etc. |
| CDH2 | Mm.257437 | 3.71 | α-, β- ,γ-catenin binding, nitric-oxide synthase binding, phosphatase binding, kinase binding, calcium-dependent cell-cell adhesion, cell migration, neuronal stem cell maintenance, etc. |
| BMP1 | Mm.27757 | 3.43 | Cytokine activity, growth factor activity, metalloendopeptidase activity, protein binding, cartilage development, cell differentiation, multicellular organismal development, etc. |
| COL4A2 | Mm.181021 | 3.42 | Extracellular matrix structural constituent, angiogenesis, etc. |
| VCAN | Mm.158700 | 3.32 | Carbohydrate binding, hyaluronic acid binding, protein phosphatase binding, cell adhesion, osteoblast differentiation, etc. |
| COL6A1 | Mm.2509 | 3.22 | PDGF binding, cell adhesion, osteoblast differentiation, protein heterotrimerization. |
| SERPINF1 | Mm.2044 | 3.20 | Serine-type endopeptidase inhibitor activity, neurogenesis, angiogenesis, regulation of endothelial cell migration, regulation of inflammatory response, etc. |
| HSPG2 | Mm.273662 | 3.08 | Protease binding, protein binding, metal ion binding, embryonic skeletal system morphogenesis, endochondral ossification, extracellular matrix organization, angiogenesis, etc. |
| VCL | Mm.279361 | 3.05 | Rho GTPase binding, α-catenin binding, ubiquitin protein ligase binding, adherens junction assembly, lamellipodium assembly, regulation of cell migration, cell-matrix adhesion, etc. |

#, The secreted-protein expression levels in hypoxia condition were relative to normoxia condition.

**Table S3. Antibody information (Related to main Figures 2, 3, 4, 5, and 6)**

| WB | | | | | |
|---|---|---|---|---|---|
| Protein | Cat. No. | Company | Origin | Dilution | Incubation Time |
| Akt | Sc-8312 | Santa Cruz | Rabbit | 1:1000 | 4˚C, overnight |
| Akt (pSer473) | #9271 | Cell signaling | Rabbit | 1:1000 | 4˚C, overnight |
| c-Kit | Sc-365504 | Santa Cruz | Mouse | 1:500 | 4˚C, overnight |
| Cxcr4 | Ab2074 | Abcam | Rabbit | 1:1000 | 4˚C, overnight |
| Cxcr4 (pSer339) | Ab74012 | Abcam | Rabbit | 1:1000 | 4˚C, overnight |
| Hif-2α | NB100-122 | NOVUS | Rabbit | 1:1000 | 4˚C, overnight |
| Igf-1rβ | Sc-713 | Santa Cruz | Rabbit | 1:1000 | 4˚C, overnight |
| Igf-1rβ (pTyr1135/1136) | #3024 | Cell signaling | Rabbit | 1:1000 | 4˚C, overnight |
| Oct4 | Sc-5279 | Santa Cruz | Mouse | 1:500 | 4˚C, overnight |
| Stat3 | #9132 | Cell signaling | Rabbit | 1:2000 | 4˚C, overnight |
| Stat3 (pTyr705) | #9145 | Cell signaling | Rabbit | 1:2000 | 4˚C, overnight |
| β-Actin | A5441 | Sigma-Aldrich | mouse | 1:500 | 4˚C, overnight |
| ICC | | | | | |
| Arp3 | Ab181164 | Abcam | Rabbit | 1:150 | 4˚C, overnight |
| BrdU | M0744 | Dako | Mouse | 1:200 | 4˚C, overnight |
| c-Kit | Sc-365504 | Santa Cruz | Mouse | 1:100 | 4˚C, overnight |
| DAZL | NBP2-23663 | NOVUS | Rabbit | 1:200 | 4˚C, overnight |
| Fak | Sc-1688 | Santa Cruz | Mouse | 1:50 | 4˚C, overnight |
| Fak (pTyr397) | Sc-11765-R | Santa Cruz | Rabbit | 1:50 | 4˚C, overnight |
| Sdf-1 | TP-201 | Torrey Pines Biolabs Inc | Rabbit | 1:100 | 4˚C, overnight |
| Flow Cytometry | | | | | |
| Cxcr4-FITC | #551967 | BD Pharmingen | Rat | 1:25 | 4˚C, 90 min |
| Rat-IgG-FITC | #556923 | BD Pharmingen | Rat | 1:25 | 4˚C, 90 min |

**Table S4. Primer information (Related to main Figures 2 and 3)**

| Gene | Accession number | Sequence | Size (bp) | Annealing Temp (˚C) |
|---|---|---|---|---|
| | | qRT-PCR | | |
| *Sox2* | NM_011443 | 5' GAGTGGAAACTTTTGTCCGAGA 3'<br>5' GAAGCGTGTACTTATCCTTCTTCAT 3' | 151 | 60 |
| *Oct-4* | NM_013633 | 5' TGAAGTTGGAGAAGGTGGAACCAAC 3'<br>5' CCAAGGTGATCCTCTTCTGCTGCTTCAG 3' | 111 | 60 |
| *Hif-2α* | NM_010137 | 5' AGCTTCAGATTCATTTTCAGAGCA 3'<br>5' CCTTCGGACACATAAGCTCCTG 3' | 54 | 60 |
| *Nanog* | NM_028016 | 5' CCTGTGATTTGTGGGCCTG 3'<br>5' GACAGTCTCCGTGTGAGGCAT 3' | 78 | 60 |
| *Sdf1-α* | NM_021704 | 5' CGCTCTGCATCAGTGACGGTA 3'<br>5' GTTCTTCAGCCGTGCAACAATC 3' | 152 | 60 |
| *Igf-1rβ* | NM_010513 | 5' GGAGTGTCCCTCAGGCTTCA 3'<br>5' CATCGCCGCAGACTTTGG 3' | 88 | 60 |
| *Cxcr4* | NM_009911 | 5' AGCCTGTGGATGGTGGTGTTTC 3'<br>5' CCTTGCTTGATGACTCCCAAAAG 3' | 242 | 60 |
| *Igf-1* | NM_010512 | 5' AAGCAGCCCGCTCTATCC 3'<br>5' TTCTGAGTCTTGGGCATGTCA 3' | 55 | 60 |
| *Cdh11* | NM_009866 | 5' TCTTCTGGTCATCGTTGTGC 3'<br>5' AGGGTGGCTATGTCGAAGG 3' | 156 | 60 |
| *Lox* | NM_010728 | 5' GACATTCGCTACACAGGACATC 3'<br>5' CCAGGTACGGCTTTATCCAC 3' | 98 | 60 |
| *Vegfa* | NM_001025250 | 5' GACGGGCCTCCGAAACC 3'<br>5' GCAGCCTGGGACCACTTG 3' | 97 | 60 |
| *Igfbp-3* | NM_008343 | 5' TCAAAGCACAGACACCCAGAAC 3'<br>5' GATGATTCAGTGTGTCCTCCATTTC 3' | 95 | 60 |
| *Lama1* | NM_008480 | 5' CAGAGACCTTGAAAATGTTAGAAACG 3'<br>5' TCGGAGATCAAGTCTGTCTTATTCG 3' | 147 | 60 |
| *Col4a2* | NM_009932 | 5' AGTGGGTACAGCCTGCTATATTTTG 3'<br>5' CCGGATTGCAGTACAGGAAAG 3' | 118 | 60 |

| | | | | |
|---|---|---|---|---|
| *β-2m* | NM_009735 | 5’ CCGAACATACTGAACTGC 3’ | 185 | 60 |
| | | 5’ AGAAAGACCAGTCCTTGC 3’ | | |
| | | Methylation Specific PCR and Pyrosequencing | | |
| *Dppa3* | NM_139218 | 5’ GGTGGTTATTATTGTTAGGTTTGAAGT 3’ | | |
| | | 5’ ACCCAATCTACCCCCAAACTACTTTA 3’ | | |
| | | Pyro seq. 5’ GGAATTGGTTGGGAT 3’ | | |
| *Ddx4* | NM_001145885 | 5’ GAATTGATGAGTTTTTGGAGAGAGAA 3’ | | |
| | | 5’ ACCTCTCCCCTCCAAACTCCCC 3’ | | |
| | | Pyro seq. 5’ GGTTTAGGTTTTAATAAAGGTGG 3’ | | |
| *Nanog* | NM_028016 | 5’ GGATTTTGTAGGTGGGATTAATTG 3’ | | |
| | | 5’ CGCCAGGGTTTTCCCAGTCACGACCTACCCTACCCACCCCCTATTCT 3’ | | |
| | | Pyro seq. 5’ TGAATTTATAGGGTTGGTG 3’ | | |
| *Oct4* | NM_013633 | 5’ TTAAGGTTAGAGGGTGGGATTG 3’ | | |
| | | 5’ CGCCAGGGTTTTCCCAGTCACGACTCTAAAACCAAATATCCAACCATA 3’ | | |
| | | Pyro seq. 5’ GGAGGGAGAGGTGAAAT 3’ | | |
| *Prdm14* | NM_001081209 | 5’ GTTTAGGTTTAGGGAGGGTAGAATGTA3’ | | |
| | | 5’ CCCTTTAAAACCCCATAAACTAATTCT 3’ | | |
| | | Pyro seq. 5’ AGGGTAGAATGTATTGTT 3’ | | |

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**Supplemental Experimental Procedures:**

**Alkaline phosphatase activity assay**

Clumped germline stem cell (GSC) colonies cultivated under 21%, 5%, 3%, or 1% $O_2$ atmosphere were fixed with 4% paraformaldehyde for 30 min at room temperature. The alkaline phosphatase (AP) activity of these GSCs was examined using an AP detection kit (Chemicon, Hampshire, UK), and Nitro Blue Tetrazolium/ 5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

**Identification of protein expression profiles using LC-MS/MS and the Mascot Distiller search engine**

Peptide mixtures obtained from the conditioned medium of $AP^+$GSC colonies cultivated under 21% $O_2$ (normoxia control) and 5% $O_2$ (hypoxia) was separated using a BioBasic $C_{18}$ column (150 mm × 2.1 mm, particle size 5 μm) with a gradient from 5% to 70% acetonitrile in 0.1% formic acid over 90 min at a flow rate of 200 μL/min. The MS/MS analysis was performed using a LTQ Velos system (Thermo Fisher Scientific) in the positive electrospray ionization mode with a survey scan for the precursor ion that ranged from m/z = 400–1600. The MS/MS raw data acquired from either the control or hypoxia-treated sample were processed into an MGF file using Mascot Distiller v2.3.2.0 (Matrix Science, London, UK). The resulting MGF files of the control or hypoxia-treated samples were searched using the Mascot search engine v2.3 (Matrix Science) with the following search parameters: (1) the protein database was set to Swiss-Prot; (2) the taxonomy was set as *Mus musculus* (house mouse); (3) one trypsin missed cleavage was allowed; (4) the precursor and product ion mass tolerance were set to 1.20 Da and 0.60 Da, respectively; (5) carbamidomethyl (C) was chosen for fixed modification; (6) oxidation (M) was chosen for the variable modification; and (7) the significance threshold was $P < 0.05$. The relative quantitative analysis of protein expression in the control or hypoxia-treated samples was performed using Mascot Distiller v2.3.2.0 and the label-free quantitation mode. The parameters of the Mascot Distiller were set as follows: (1) report ratio: C1/Ref (C1 and Ref represent hypoxia-treated and control samples, respectively); (2) protocol: replicate; (3) integration method: Simpson's; (4) integration source: survey (precursor peak area from survey scan); (5) correlation threshold: 0.6; (6) Std. Err. threshold: 999 (threshold on the standard error for a straight line fit of the component intensities from each of the scans in the XIC peak); (7) XIC threshold: 0.1; (8) max XIC width (upper limit on the number of survey scans in an XIC peak): 250; (9) XIC smoothing: 3; (10) peptide threshold type: at least identity; and (11) outlier method: auto (using Dioxon's or Rosner's outlier method according to the number of values). The relative quantitation result was then normalized using the ratio of the internal standard (β-ACTIN).

**Trypsin digestion of proteins extracted from PGC-like $AP^+$GSCs with and without hypoxia treatment**

Either hypoxia-treated or control PGC-like $AP^+$GSCs proteins (100 μg) dissolved in deionized water (60 μL) were denatured and reduced at 95°C for 5 min using 7.5% SDS (9.3 μL) and 1M 1,4-dithiothreitol (0.7 μL). Subsequently, 0.5 M iodoacetamide (8 μL) was added to the protein mixture, which was then incubated at room temperature in the dark for 30 min. Afterward, 50% trichloroacetic acid (52 μL) was added and the precipitate was washed with deionized water (200 μL × 3). The resulting protein precipitate was dissolved in 50 mM ammonium bicarbonate (100 μL) and 2%

N-octyl-β-D-glucopyranoside (5 μL). Sequencing-grade trypsin (2 μg) was added and the mixture was incubated at 37°C for 18 h. The resulting tryptic digest was acidified with 2% formic acid (10 μL) to stop the enzymatic reaction, and the resulting digest was lyophilized and maintained at -20℃.

**Gene ontology (GO) analysis**

The differential protein secretion profiles of mouse $AP^+$GSCs were analyzed by the PANTHER classification system (Mi et al., 2016) for gene ontology secretome analysis. Four selected items of the signaling pathway category were pluripotency regulation of stem cells (KEGG pathway map No.: mmu04550), PI3K-AKT signaling pathway (mmu04151), Chemokine-associated signaling pathway (mmu04512), and HIF signaling pathway (mmu04066). STRING program (Szklarczyk et al., 2015) was used for protein network analysis.

**RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction**

The $AP^+$GSC colonies and $CD49f^+AP^+$GSC cultivated under 21% and 5% $O_2$ atmosphere were collected. Total RNA was extracted using the Trizol Kit (Invitrogen, Frederick, MD, USA), and complementary DNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the FastStart Universal SYBR Green Master Mix (Roche, Indianapolis, IN, USA) in a LightCycler 480 instrument (Roche), and the qRT-PCR results were recorded and analyzed using the instrument's application software. Gene expression levels were normalized by *β-2 microglobulin* (*β-2m*). Primer sequences are listed in Table S4.

**Transcriptome analysis**

Total RNA was extracted from the E11.5 PGC colony, P2 $AP^+$GSC colony, and $CD49f^+AP^+$GSCs using the Trizol reagent (Invitrogen). The quality of the total RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Fluorescent aRNA targets were prepared from 1 μg of total RNA samples using a OneArray® Amino Allyl aRNA Amplification Kit (Phalanx Biotech Group, Hsinchu, Taiwan) and Cy5 dye (GE Healthcare). Fluorescent targets were hybridized to a Mouse Whole Genome OneArray® with Phalanx hybridization buffer using the Phalanx Hybridization System. The Mouse Whole Genome OneArray® v2 (Phalanx Biotech Group) contains 27,307 DNA oligonucleotide probes. Among the probes, 26,423 correspond to the annotated genes in the RefSeq v42 and Ensembl v59 databases. In addition, 884 control probes are included. Briefly, after hybridization and washing, the slides were scanned using a DNA Microarray Scanner (Model G2505C, Agilent Technologies). The Cy5 fluorescent intensity of each spot was analyzed using the GenePix 4.1 software (Molecular Devices). Each single sample was analyzed at least twice in terms of technical or biological replicates, with a reproducibility of more than 0.975. The signal intensity was loaded into the Rosetta Resolver System® (Rosetta Biosoftware, WA, USA) to perform data preprocessing and applied to a 75 percentile centering normalization. The errors of the sample were estimated using an error-weighted approach simultaneously. Both the fold change and *P* value for the pair-wise sample comparison were calculated to evaluate the differentially expressed genes. A fold change of $\geq 2$ or $\leq -2$ and a *P* value of $<0.05$ are strongly recommended for further analysis.

**Bisulphite conversion, methylation-specific PCR, and pyrosequencing**

Total genomic DNA was isolated from the E11.5 PGC colony, P2 $AP^+$GSC colony, and $CD49f^+AP^+$GSCs. First, 500 ng of genomic DNA was bisulphite-converted using the EpiTect Fast bisulphite conversion kit according to the manufacturer's instructions (QIAGEN, Germantown, MD, USA). Bisulfate Specific PCR was performed using a PyroMark PCR kit (QIAGEN), followed by PCR product purification. The purified PCR product was hybridized with a pyrosequence primer using PyroMark Gold Q24 reagent according to the Pyromark Q24 vacuum workstation guide (QIAGEN). The methylation percentage per CpG position was determined using the PyroMark Q24 software (QIAGEN). Briefly, the methylation percentage was assessed by the ratio of real-time incorporated C and T nucleotides into the growing DNA strand, resulting in an enzymatic cascade of pyrophosphate release and the production of a light signal. *Nanog* and *Oct4* pyrosequence primer information was according to a study conducted by Rugg-Gunn et al. (2010). Primer sequences are listed in Table S4.

**Flow cytometry**

For the hypoxia experiment, $CD49f^+AP^+$GSCs were cultivated under 1%, 5%, or 21% $O_2$ atmosphere for 3 days. For the flow cytometry analysis of cell surface CXCR4, the culture medium was replaced every day to eliminate the secreted SDF-1 in the medium, which may lead to cell surface CXCR4 internalization (Signoret et al., 1998). For the IGF-1R signaling inhibition experiment, the cells were treated with or without the IGF-1R phosphorylation inhibitor cyclolignan picropodophyllin (PPP, 1 μM, Enzo Life Sciences, Farmingdale, NY, USA) under 1% $O_2$ atmosphere for 3 days. Cells were detached using Accutase® treatment (Innovative Cell Technologies, San Diego, CA, USA), followed by immunostaining with antibodies against specific surface markers for flow cytometry analysis. Briefly, $2 \times 10^5$ cells were incubated with 1% BSA/PBS blocking solution for 30 min and then interacted with a fluorescein isothiocyanate-conjugated CXCR4 antibody or isotype control (BD Pharmingen, San Jose, CA, USA) at 4℃ for 90 min. After resuspension in PBS buffer, the $CXCR4^+$ cell percentage was analyzed using a BD FACSVerse cytometer and the instrument's application FACSuite software (BD Biosciences, San Jose, CA, USA). The primary antibodies used in the experiment are listed in Table S3

**Western blot analysis**

For oxygen tension experiments, $CD49f^+AP^+$GSCs were cultivated under 1%, 5%, or 21% $O_2$ atmosphere for 3 days; for IGF-1 experiments, the cells were treated with IGF-1 (0–50 ng/mL, PeproTech, Rocky Hill, NJ, USA) under 21% $O_2$ for 3 days. For HIF-2α knockdown experiments, the cells were transfected with a high-efficiency shHIF-2α clone (NM_010137, 5'-ggagacggaggtcttctat-3') (Huang et al., 2014) or shCtrl (TRCN0000072246, National RNAi Core, Taiwan) for 24 h and then treated with or without IGF-1 (10 ng/mL) for an additional 24 h. For receptor crosstalk experiments, the cells were pretreated with or without PPP (1 μM) or AMD3100 (50 μM, Sigma-Aldrich) for 6 h and then treated with IGF-1 (50 ng/mL) or SDF-1 (400 ng/mL, PeproTech) for 0, 2, and 5 min. The cell lysates of $CD49f^+AP^+$GSCs were collected and extracted using reducing 2× Laemmli sample buffer and subjected to 10% SDS-PAGE electrophoresis; western blotting analysis was then performed. The primary antibodies used in the experiments are listed in Table S3, and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:2000) served as the secondary antibody. HRP enzyme activity was detected using an enhanced chemiluminescence system

according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK). Quantifications of the protein bands were performed using the SPOT DENSO software on an AlphaImager2200 instrument (Alpha Innotech Corporation, CA, USA).

**Immunocytochemical staining**

For the BrdU incorporation assay, GSC colonies were cultivated under 5% $O_2$ atmosphere at 37℃ for 7 days, and BrdU (0.1 mM) was added to the medium for further 24 h incubation. Excess BrdU in the medium was removed by three washes with PBS, and the BrdU-incorporated cells were fixed with 4% paraformaldehyde and treated with 2 N HCl, followed by neutralization with 0.1 M sodium borate. For hypoxia experiments, $CD49f^+AP^+$GSCs were cultivated under 1%–21% $O_2$ atmosphere for 3 days, and $AP^+$GSCs were cultivated under a 5% $O_2$ atmosphere for 7 days; for PPP inhibition experiments, the cells were treated with or without PPP (1 μM) under 1% $O_2$ oxygen concentration for 3 days. All experiments were performed using immunocytochemical analysis. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% triton X-100 in PBS, and then blocked with 3% BSA in 0.5% triton X-100 in PBS. Cells were incubated with a primary antibody (Table S3) at 4℃ overnight and then interacted with Alexa 488- or Alexa 594-conjugated secondary antibodies (Invitrogen). The nuclei of all cells were counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich) and analyzed using a fluorescence microscope (Leica, Buffalo Grove, IL, USA) or a laser confocal microscope (Leica).

**Migration assay and wound healing assay**

Transwell assays were performed using an 8-μm pore transwell insert in 24-well plates (Corning, Corning, NY, USA). The upper chambers were seeded with $CD49f^+AP^+$GSCs ($1.5 \times 10^5$/100 μL), followed by treatment with IGF-1 (0–50 ng/mL) in lower chambers as an attractant for 3 days. For hypoxia experiments, the PPP groups of $CD49f^+AP^+$GSCs were pretreated with PPP (1 μM) for 2 h. All experimental cells were treated with PPP (1 μM) or SDF-1 (100 ng/mL) in the upper chamber and then treated with IGF-1 (50 ng/mL) in the lower chamber under 21%, 5%, and/or 1% $O_2$ atmosphere for 3 days. Migrated cells were stained with crystal violet and counted in six random fields under a light microscope. For the wound healing assay, $CD49f^+AP^+$GSCs ($1 \times 10^5$/well) were seeded into a culture insert (Ibidi, Martinsried, Germany). Adherent cells were treated with IGF-1 (50 ng/mL), SDF-1 (100 ng/mL), or PPP (1 μM) under different culture conditions. At the end point, cells were stained with crystal violet. The photos of the cells were taken at each time point, and the gap area was measured and quantified.

**Supplemental References:**

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