Supplemental Fig. S1 Real-time PCR analysis of spermatogonia markers. Cells were cultured for 2 days under hypoxia (n = 3). Asterisk indicates statistical significance (p < 0.05).
Supplemental Fig. S2 Long-term culture of GS cells under hypoxia. (A) Growth curve of GS cells under hypoxia. (B) Appearance of recipient testes after transplantation of GS cells under hypoxia. (C) Colony counts (n = 18). (D, E) Appearance (D) and lectin (PNA)-immunostaining (E) of recipient testis. Testes were recovered 77 days after transplantation. The testis on the right side is the untransplanted control (D). Bar = 1 mm (B, D), 20 µm (E). Asterisk indicates statistical significance (p < 0.05).
Supplemental Fig. S3

A  20% O₂
Nox2

B  WT GS
Nox1

C  1% O₂
Nox2

D  1% O₂
Nox3

E  20% WT GS
- Control
- MitoTempo

F  Control
MitoTempo

G  Colony counts
(Colonies/10^5 cells)

Count

250
200
150
100
50
0

ROS

10  10  10  10  10
Supplemental Fig. S3 Impact of oxygen tension on GS cells. (A) Real-time PCR analysis of Nox genes in Nox1 KO GS cells (n = 3). (B) Real-time PCR analysis of Nox1 in WT GS cells (n = 3). (C) Increased number of WT GS cells under hypoxia by supplementation of H2O2. Cells were cultured for 7 days (n = 6). (D) Proliferation of GS cells after MitoTempo treatment. Cells were cultured under normoxia and collected at 5 days (n = 3). (E) Flow cytometric analysis of ROS levels in WT GS cells by CellROX Deep Red 5 days after MitoTempo treatment. (F) Appearance of recipient testes after transplantation of MitoTempo-treated GS cells. (G) Colony count (n = 15-16). Bar = 1 mm (F). Asterisk indicates statistical significance (p < 0.05).
**Supplemental Fig. S4** Defective spermatogenesis in *Top1mt* KO mice. (A, B) Immunostaining (A) and quantification (B) of *Top1mt* KO mouse testis using spermatogonia markers. At least eight tubules were counted for each marker. (C) Proliferation of *Top1mt* KO GS cells (n = 6). Cells were cultured for 6 days. (D) Flow cytometric analysis of GFRA1 expression levels in *Top1mt* KO GS cells. (E, F) Double immunostaining (E) and quantification (F) of HIF1A and spermatogonia markers in *Top1mt* KO mouse testis. At least 10 tubules were counted. Arrows indicate HIF1A-reactive spermatogonia. Control staining was carried out by omitting anti-HIF1A antibody. Stain, Hoechst 33342 (A, E). Bar = 20 µm (A, E). Asterisk indicates statistical significance (p < 0.05).
Supplemental Fig. S5

A  WT GS vs Top1mt KO GS
B 20% O₂ WT GS vs 1% O₂ WT GS
WT GS vs Hif1a KO GS

Color Key
Expression (log2)

WT_GS_1_B
WT_GS_2_B
KO_GS_1_B
KO_GS_2_B
Supplemental Fig. S5 Heatmap of gene expression by RNA-seq. (A) Top1mt KO GS cells. (B) WT GS cells under normoxic and hypoxic conditions. (C) Hif1a KO GS cells under hypoxic conditions. Hif1a KO GS cells under normoxic conditions were omitted because only three genes were differentially expressed between WT and KO cells.
Supplemental Fig. S6 *Hif1a* deficiency in SSCs. (A) Real-time PCR analysis of *Hif1a* and *Epas1* expression in WT GS cells (n = 3). (B-D) Double immunostaining (B) and quantification of HIF1A and spermatogonia markers in *Nox1* KO mouse testis. The numbers of spermatogonia (C) and HIF1A+ spermatogonia (D) were quantified in at least 5 tubules were counted. Arrows indicate HIF1A+ spermatogonia. Control staining was carried out by omitting anti-HIF1A antibody. (E) Evaluation of deletion efficiency by Southern blotting (n = 3). (F) Histological analysis of primary recipient testis. At least 216 tubules were counted. Bar = 20 μm (B). Stain, Hoechst 33342 (B), H &E stain (F). Asterisk indicates statistical significance (p < 0.05).
Supplemental Fig. S7 Decreased MYC/MYCN expression under hypoxia. (A, B) Quantification of \textit{Myc}/\textit{Mycn} and MYC/MYCN in WT GS cells by real-time PCR (A) or Western blot (B) analysis, respectively (n = 3). (C, D) Quantification of MYC/MYCN in \textit{Hif}1\textit{a} KO (C) and \textit{Nox}1 KO (D) GS cells by Western blot analysis (n = 3). Cells were cultured under hypoxia. (E) Quantification of MYC/MYCN in WT GS cells after exposure to \textit{H}_{2}\textit{O}_{2}, apocynin or LPA by Western blot analysis (n = 3). Asterisk indicates statistical significance (p < 0.05).
Supplemental Fig. S8

A

![Bar charts showing relative expression of MAPK7 and MAPK14 under 1% O2 conditions]

B

![Bar charts showing relative expression of Ccnd2 under 20% and 1% O2 conditions, as well as under Nox1 KO and Myc DKO conditions]
Supplemental Fig. S8 Lack of ROS amplification in Hif1α KO GS cells. (A) Quantification of MAPK7 and MAPK14 in Hif1α KO GS cells by Western blot analysis (n = 3). (B) Real-time PCR analysis of Ccnd2 in WT, Myc DKO, Hif1α KO and Nox1 KO GS cells (n = 3). Asterisk indicates statistical significance (p < 0.05).
Supplemental Fig. S9

A

20% O₂
Cdkn1a

1% O₂
Cdkn1a

Control
Myc DKO

Relative expression (fold)

C

1% O₂
Cdkn1a

Control
Cdkn1a KD

Relative expression (fold)

B

20% O₂
CDKN1A

1% O₂
CDKN1A

Control
Myc DKO

Relative expression (fold)

1% O₂
CDKN1A

1% O₂
CDKN1A

Control
Nox1 KO

Relative expression (fold)

Control
Hif1a KO

Relative expression (fold)
Supplemental Fig. S9 CDKN1A-mediated growth suppression of GS cells under hypoxia. (A) Real-time PCR analysis of Cdkn1a in Myc DKO GS cells (n = 3). (B) Quantification of CDKN1A in Myc DKO, Hif1a KO, and Nox1 KO GS cells by Western blot analysis (n = 3). (C) Real-time PCR analysis of Cdkn1a after KD (n = 3). Cells were recovered 3 days after Cdkn1a KD. Asterisk indicates statistical significance (p < 0.05).
Supplemental Methods

Animals and microinjection procedure

Generation of Nox1, Top1mt, Myc\textsuperscript{ff}, and Mycn\textsuperscript{ff} KO mice was reported previously (Knoepfler et al., 2002; Wilson et al., 2004; Matsuno et al. 2005; Khiati et al. 2015). Hif1a\textsuperscript{ff} KO mice were purchased from the Jackson Laboratory (Stock No. 007561; Bar Harbor, ME). Cdkn1a KO mice were kindly provided by Dr. O. Niwa (Radiation Effects Research Foundation, Hiroshima/Nagasaki, Japan). For transplantation experiments, we collected 5- to 10-day-old pup testes, and the cells were dissociated into single cells by a two-step enzymatic procedure using type IV collagenase and trypsin, as described previously (Ogawa et al. 1997). To delete Hif1a gene, the testis cells were cultured overnight with AxCANCre [multiplicity of infection (moi) = 2; RIKEN BRC, Tsukuba, Japan], as described previously (Takehashi et al. 2007). These cells were transplanted into B6 × DBA/2 F1 (BDF1) mice that had been injected intraperitoneally with busulfan (44 mg/kg; Japan SLC, Shizuoka, Japan). The mice were used at least 4 weeks after busulfan treatment.

For spermatogonial transplantation, testis cells were suspended in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum. For colony counting, approximately 10 µl of donor cells (5,000 per testis) were microinjected into the seminiferous tubules of busulfan-treated BDF1 mice via the efferent duct (Ogawa et al. 1997). For serial transplantation experiments, recipient testes were dissociated into single cells and a portion of the total cell suspension from one primary recipient testis was microinjected into two secondary recipient testes. For production of offspring, cells were microinjected into the seminiferous tubules of 5- to 10-day-old W mice (Japan SLC). Each injection filled 75–85% of the seminiferous tubules. The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

Flow cytometry

To analyze ROS levels, GS cells were dissociated into single cells using Trypsin/EDTA (Nacalai Tesque, Kyoto, Japan) for 5 min. Cells were next incubated with CellROX Deep Red reagent (5 µM; Thermo Fisher Scientific, Waltham, MA) for 30 min in GS cell culture medium at 37°C. Mitochondria-derived ROS were detected with MitoSox. Red (5 µM; Thermo Fisher Scientific). Cells were incubated for 10 min at 37 °C. For
analysis of GFRA1 expression, GS cells were dissociated with Cell Dissociation Buffer (Invitrogen, Carlsbad, CA). For pimonidazole staining, pimonidazole was administered (120 mg/kg; Hypoxy probe, Burlington, MA) for 3 h, and samples were collected. Intracellular adducts were detected with indicated an Alexa647-conjugated anti-Pimo antibody (Calbiochem-Merck, Darmstadt, Germany) and indicated antibodies (Supplemental Table S8). All flow cytometric analyses were carried out three times and representative data are shown.

*Western blot analysis*

Samples were separated by SDS-PAGE and transferred to Hybond-P membranes (Amersham Biosciences, Buckinghamshire, UK), and incubated with the primary antibodies. For HIF1A analysis, nuclear extract was prepared, as described previously (Schreiber et al. 1989). Band intensity was measured using Image Gauge (Fuji Film, Tokyo Japan). The antibodies used are listed in Supplemental Table S8.

*RNA sequencing*

Total RNA was extracted from GS cells using the RNeasy Plus Micro Kit (Qiagen, Valencia, CA) and cDNA libraries were generated from 200 ng total RNA using a TruSeq stranded mRNA library preparation kit (Illumina, San Diego, CA). Sequencing was performed using NextSeq550 (Illumina) with a single-read sequencing length of 76 bp (Kanatsu-Shinohara et al. 2019).

The sequenced reads were mapped to the mm10 mouse reference genome using STAR (version 2.6.0c)(Dobin et al. 2013), with the GENCODE M21 annotation gtf file after trimming adaptor sequences and low-quality bases with cutadapt-1.16 (Martin et al. 2011). To identify hypoxia-regulated genes, the uniquely mapped reads were counted and normalized to calculate the gene expression levels using HTSeq (version 0.11.2)(Anders et al. 2014), with GENCODE M21 annotation gtf file (protein-coding genes) and DESeq2 (version 1.22.2) after filtering low expressed genes (cpm =< 5) across paired conditions in each experiment (Love et al. 2014). An IPA (Qiagen; https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis) was performed to identify upstream regulators that could explain the changes in gene expression. The upstream regulators categorized as transcription regulators in the molecular type were
chosen for the upstream analysis.

**Immunostaining**

For immunostaining, samples were fixed in 4% paraformaldehyde for 2 h. After immersion in blocking buffer (0.1% Tween 20, 1% bovine serum albumin, and 10% donkey (for GFRA1 or BCL6B staining) or goat (other antibodies) serum in phosphate-buffered saline for > 1 h, the samples were incubated with the primary antibodies at 4 °C overnight. Next, the secondary antibodies were added, and the samples incubated for 1 h at room temperature. The samples were counterstained with Hoechst 33342 (Sigma), and visualized using a confocal microscope (Fluoview FV1000D; Olympus, Tokyo, Japan). The antibodies used are listed in Supplemental Table S8.

**Analysis of recipient testes**

Recipient mice were killed 8 weeks post-transplantation. Donor cell colonies were counted under UV light. Donor cell clusters were defined as colonies when the entire basal surface of the tubule was occupied and measured at least 0.1 mm in length (Nagano et al. 1999).

For quantification of tubules with spermatogenesis, the sections were viewed at a magnification of × 400 to determine the extent of spermatogenesis. The number of tubule cross-sections with spermatogenesis (defined as the presence of multiple layers of germ cells in the seminiferous tubule) or no spermatogenesis was recorded for one section from each testis.

**Southern blot analysis**

Genomic DNA was collected from cultured cells by phenol/chloroform extraction and ethanol precipitation on the day following AxCANCre infection. To estimate the deletion efficiency, a 400-bp fragment of Hif1a was amplified by PCR using the primers 5’- TTACCTTTACTAGCCACCTGC-3’ and 5’-TGAAAAACATCTGCTTTGGAAATG-3’, which was used as a hybridization probe. Eighteen micrograms of DNA were digested with EcoRI and PstI, and separated on a 1.0% agarose gel. Hybridization was performed, as described previously (Takehashi et al. 2007). The intensity of bands was estimated using
Staining of testes

For immunostaining, testis samples were fixed in 4% paraformaldehyde for 2 h at 4°C. After being embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan), the samples were processed for cryosectioning. Sections of 10-μm thickness were prepared. Rhodamine-labeled PNA (Vector Laboratories, Burlingame, CA) was used to visualize the acrosome. The antibodies used are listed in Supplemental Table S8. Hoechst 33342 (Sigma) was used for counterstaining.

Real-time PCR analysis

For total RNA collection, samples were dissolved in TRIzol (Invitrogen). In experiments using GS cells, dissociated cells were incubated on gelatin-coated plates for 2 h for removal of MEFs. For reverse transcription, first-strand cDNA was produced using a Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). For real-time PCR, the CFX Connect Real-Time System (BioRad, CA) and FastStart Universal SYBR Green Master (ROX) (Roche Applied Science, Mannheim, Germany) were used. Transcript levels were normalized to that of Hprt. The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each PCR was performed in least triplicate. The PCR primers used are listed in Supplemental Table S9.

Microinsemination

Testes were collected and refrigerated overnight before microinsemination, as described previously (Ogonuki et al. 2006). Germ cells were collected by mechanically dissociating the seminiferous tubule segments. These cells were microinjected into BDF1 oocytes using a Piezo-driven micropipetter (PrimeTech, Ibaraki, Japan). Embryos at the two-cell stage after 24 h in culture were transferred to the oviduct of ICR recipient females.

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