Supporting Materials and Methods

Cell culture, induction of reprogramming and proliferation assay

AGM fibroblast cell line was established from embryos at around 60 days post-fertilization by E. Sasaki (Tokyo University) and K. Tani (Kyushu University). Lentiviral 293T LTV cells (Cell Biolabs, San Diego, CA, USA), HeLa cells (American Type Culture Collection, Manassas, VA, USA) and mouse embryonic fibroblast (MEFs) feeder cells were maintained in DMEM (Sigma Aldrich) containing 10% FBS. KG-1a (Ozawa, Tokyo University) and HUT (American Type Culture Collection) cells were maintained in RPMI (Sigma Aldrich) containing 10% FBS.

To induce reprogramming of AGM fibroblasts, cells were seeded at 3×10^4 cells per well on 6-well plates (BD Biosciences). At 24 h after seeding, fibroblasts were transduced with lentiviruses (1.2×10^6 IU). One week after transduction, cells were trypsinized and transferred onto MEFs. Colonies formed 1-2 weeks after transfer, and individual colonies were picked manually and transferred to new MEF layers maintained on gelatin-coated 24-well plates (BD Biosciences).

To investigate the proliferation, cells (3×10^4) were seeded on 24-well plates. The following day (day 0), they were cultured in 10% FBS/DMEM in the presence of recombinant human bFGF (PeproTech, Rocky Hill, NJ, USA) at 5-45 ng/ml. Cell numbers were counted on days 2, 4 and 6 using the TC10™ Automated Cell Counter (Bio-Rad, Hercules, CA, USA). bFGF signaling was inhibited using FGFR inhibitor 1-4,BGJ398(3-(2,6-dichloro-3,5-dimethoxyphenyl)-1-(6-(4-(4-ethylpiperazin-1-yl)phenylamino)pyrimidin-4-yl)-1-methylurea ; Selleck Chemicals, Houston, TX, USA) in the presence of bFGF.

Immunocytochemistry and Alkaline phosphatase (AP) staining

Cells were fixed with 4% paraformaldehyde/PBS for 20 min at room temperature, and stained

with primary antibodies against TRA1-60 (Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. The samples were then incubated with secondary antibodies against Alexa 633 anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). The stained cells were visualized by confocal microscopy (Axiovert 135M, Zeiss, Deutschland, Germany). AP staining of ARCs was performed using a Leukocyte Alkaline Phosphatase kit, according to the manufacturer's protocols (Sigma-Aldrich).

Karyotype analysis

CM ARC lines were subjected to karyotype analysis using a modified Q-banding technique[(1)]. Briefly, iPSCs were cultured in medium containing 100 ng/ml colcemid (Invitrogen) for 90 min, washed in PBS, dissociated using trypsin-EDTA solution, and spun down. Chromosome slides were stained with 0.01 µg/ml Hoechst 33258 (Sigma Aldrich) for 5 min followed by 5.0 g/ml quinacrine mustard (Sigma Aldrich) for 20 min. The stained slides were mounted and observed under a fluorescence microscope (DMRXA2, Leica, Wetzlar, Germany) and analyzed using CW4000 software (Leica).

***In vivo* tumorigenicity assay and immunohistochemistry**

All procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Faculty of Medicine, Kyushu University, and were conducted according to the Guidelines for Animal Experiments of Faculty of Medicine, Kyushu University. The tumorigenicity of ARCs and CM DGs was evaluated *in vivo* by transplanting 1×10^6 cells into the testes or subcutaneous tissues of SCID mice (CB17/Icr-Prkdcscid/CrlCrlj; Charles River, Kanagawa, Japan). 6 weeks after injection, tumors were dissected and fixed with 4% paraformaldehyde/PBS, and paraffin-embedded. For pathological analyses, 3-μm tissue sections were cut, deparaffinized and stained with H&E or antibodies to low- and high-molecular weight cytokeratins (cocktail of clone AE1/AE3; Chemicon International,

Temecula, CA, USA), S100, desmin, α-SMA (clone 1A4), and NSE (DKO, Glostrup, Denmark). In the case of cytokeratins, desmin and NSE, sections were subjected to heat-induced antigen retrieval for 20 min at 120 °C in citrate buffer before addition of the primary antibody. Staining was carried out using the REAL™ EnVision™ Detection System, peroxidase/DAB+, rabbit/mouse (DKO). The samples were observed by optical microscopy (AX70, Olympus).

Microarray analysis

The microarrays were scanned using a GeneChip Scanner 3000 7G (Affymetrix), and the raw image files were converted into normalized signal intensity values using MAS5 algorithm in Affymetrix Expression Console software. The fold change in signal intensity between different samples was calculated for each probe. As a threshold to identify candidate differentially expressed probes, the 2-fold change in signal was used in this study, and 4,453 probes were finally selected. Two-dimensional hierarchical cluster analysis of selected probes and samples was performed to generate a heat map, using two hold change The distance between every pair of probes was calculated as 1.0 - (Pearson's correlation coefficient in signal between the 2 probes). The Ward's linkage algorithm was used for cluster analysis.

Reverse transcription PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and subjected to cDNA synthesis using superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). Individual primers were designed for the target genes as follows. The following (forward and reverse) primer pairs were used: β-ACTIN: 5'-TCCTGACCCTSAAGTACCCC-3' and 5'-GTGGTGGTGAAGCTGTAGCC-3', DPPA4: 5'-AGAGGATCAGCAGGCTTCTA-3' and 5'-GTGTCTCAGAACTTTGCAGG-3', SALL1: 5'-GCTGCTGCAGACACAAGATC-3' and 5'-CTCATCATGAATGACAGCGA-3', LIN28: 5'-ATGGGGTTCGGCTTCCTGTC-3' and

5'-GCTTGCAATCCTTGGCATGA-3', CD30: 5' -TGTGCATGGAACTCCTCCC-3' and 5'-TTAGAAGCAGCTTCCTGGGC-3', CD45: 5'-GGAGACCATTGGTGACTTTTG-3' and 5'-CCATACGTTTGCTTTCCTTCTC-3', c-KIT: 5'-TGAAGCATTCCCCAAACCTGAACA-3' and 5'-GGTGTGGGGATGGATTTGCTCTTT-3', end-OCT3/4: 5'-CAAGCGATCAAG CAGTGAC-3' and 5'-CTTCCCTCTAACCAGTTGCC-3', end-SOX2: 5'-AAGATGGCC CAGGAGAACC-3' and 5'-CGTAGCTTTCTTTCCTCCAGAT-3', end-KLF4: 5'-TCCCATCTCAAGGCACACC-3' and 5'-CCACAACTTCCAGTCACCC-3', end-c-MYC: 5'-CTCGGACACCGAGGAGAATG-3' and 5'-CAGCCAAGGTTGTGAGGTTGC-3', exo-OCT3/4: 5'-TGTCTCCGTCACCACTCTGG-3', exo-SOX2: 5'-TGTCCCAGCACTACCAGAG-3', exo-KLF4: 5'-GACCACCTCGCCTTACACAT-3', exo-c-MYC: 5'-CTCCTGGCAAAAGGTCAGAG-3' and IRES2: 5'-TGCTTC CTTCACGACATTCA-3', ZFHX4: 5'- GGCAACCCTGTTCACCTAAA-3' and 5'- CCTTCTGCCTCCTCACTTTG -3', NFIX: 5'- GATGCAGAACTGCCTTCCTC -3' and 5'- GGACTGTACAGAGGGGTCCA -3', HOXC8: 5'- CCGAAGGATGAAGTGGAAAA-3' and 5'- GGGCAAGGAGTGAGACAGAG-3', CXORF67: 5'- TCACCCTGGTAGCTTCTGCT -3' and 5'- CTCATCAGCTGACCCAGACA-3', STMN2: 5'- ACGTCTGCAGGAAAAGGAGA -3' and 5'- GACATGGGGAGCTTCAAAAA -3' .

***In vitro* differentiation of induced pluripotent stem cells(iPSCs)**

Neural cell differentiation from human ESCs or ARCs was performed an *in vitro* differentiation assay based on the protocol[2]. Briefly, single cells were harvested by trypsinization, and 1×10^6 cells were cultured on low-adhesion plates in ESC medium (day 0). Half of the medium was refreshed every day, and embryoid bodies (EBs) were allowed to grow up for 4 days in suspension before culture in DMEM/F12 containing N2 supplement (Gibco, Carlsbad, CA, USA), NEAA and heparin (Mochida Pharmaceuticals, Tokyo, Japan) for days 4–6. EBs were then transferred onto laminin-coated plates in the same medium containing retinoic acid, and

cultured for 4 days (days 6–10). The cells were fixed and stained with antibodies against nestin (Millipore, Tokyo, Japan) on day 10. The samples were observed by immunofluorescence microscopy (BZ-9000, Keyence, Osaka, Japan). Cardiomyocyte differentiation was induced using the PSdif-myocyte Differentiation Kit (StemRD, Burlingame, CA, USA) according to the manufacturer's protocol. Hepatic cell differentiation was performed as described previously [3]. Briefly, after EB formation for 7 days, cells were transferred onto 35-mm plates and cultured for a further 14 days. The cells were then fixed and stained with antibodies against albumin (Bethel Laboratories, Montgomery, TX, USA).

Western blotting

Western blotting was performed as described previously[4]. The primary antibodies used were anti-OCT3/4 antibody (Santa Cruz Biotechnology), anti-SOX2 antibody (Santa Cruz Biotechnology), anti-c-MYC antibody (Santa Cruz Biotechnology), anti-β-ACTIN antibody (Santa Cruz Biotechnology), and anti-KLF4 antibody (Abcam, Cambridge, UK).

Genomic PCR

Genomic DNA was isolated using the DNeasy® Blood and Tissue Kits (Qiagen). Individual (forward and reverse) primer pairs were designed for the target genes as follows: OCT3/4: 5'-GCTCTCCCATGCATTCAAAC-3', SOX2: 5'-CACTGCCCCTCTCACACATG-3', KLF4: 5'-TACACATGAAGAGGCATTTT-3', c-MYC: 5'-CTACGGAACTCTTGTGCG-3' and IRES2: 5'-TGCTTC CTTCACGACATTCA-3'.

Inverse PCR

Inverse PCR was performed as described previously[5]. The following (forward and reverse) primer pairs were used: Forward: 5'-CTCTGGTTTCCCTTTCGCTT-3' and Reverse: 5'-TTGGAGACCTCT CAGCCTGA-3' (OCT3/4), 5'-GACAGTTACGCGCACATGAA-3'

(SOX2), 5'-CCCTACCTCGGAGAGAGACC-3' (KLF4),

5'-CTGGGAGGAGACATGGTGAAC-3' (c-MYC), for PCR using PstI-digested genome as templates, and forward: 5'-CTCTGGTTTCCCTTTCGCTT-3' and 5'-TTCACTGCACTGTACTCCTC-3' (OCT3/4), 5'-CATGTCCCAGCACTACCAGA-3' (SOX2), 5'-GACCACCTCGCCTTACACAT-3' (KLF4), and 5'-TACATCCTGTCCGTCCAAGC-3' (c-MYC), for PCR using EcoRI-digested genome as templates.

MTS assay

The concentration of chemicals causing 50% growth inhibition (IC50) was determined by MTS assay. *In vitro* cell viability of CM DGs was assessed using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA), following the manufacturer's instruction.

References

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