Lentivirus production

Lentiviruses were packaged in 293FT cells (Cat# R700-07 Invitrogen) by co-transfecting 5 μg lentiviral expressing vector or shRNA vector together with 3.75 μg Δ8.91 and 1.25 μg pMD VSV-G in a 10 cm cell culture dish using 30 μl FuGene HD transfection reagent (Cat# E2311 Promega). Supernatants were collected 48 and 72 hr after transfection, concentrated by centrifugation at 20,000 g, aliquoted and stored at -80℃ for future use.

Flow cytometry

Single hSKPs were re-suspended in 0.5 ml PBS in an assay tube (Cat# 352054 BD Falcon), and 0.5 ml 4% paraformaldehyde (PFA, Cat#158127 Sigma) in PBS was added into the cell suspension to achieve a final concentration of 2%. Cells were fixed in this 2% PFA/PBS solution for 30 minutes (min) at room temperature (RT), centrifuged, re-suspended in 1 ml 90% methanol, incubated on ice for another 30 min and then stored at -20℃ for later use. On the day of analysis, hSKPs were rinsed twice in the incubation buffer (0.5% bovine serum albumin (BSA, Cat# A2058 Sigma) and 0.1% Tween-20 (Cat# 93773 Sigma) in PBS) and then aliquoted and incubated in different primary antibodies (diluted in the incubation buffer) for 1 hr at RT. Each aliquot contained 0.5-1 $X10^6$ cells. Cells were then rinsed twice as before and incubated in correspondent secondary antibodies for 30 min at RT. After another 2 rounds of wash, cells were re-suspended in 0.1% Tween-20/PBS, strained and analyzed using the BD FACSCalibur™ system. Data were further processed with the Flowjo software. Primary antibodies used were rabbit anti-γH2AX (1:100, Cat# 2212-1 Epitomics), mouse anti-p53 (Cat# SC-126 Santa Cruz) and mouse anti-$p16^{INK4a}$ (Cat# SC-1661 Santa Cruz). FITC conjugated Goat-anti-mouse or goat-anti-rabbit secondary antibodies (1:200) were purchased from ZSGB Biotechnologies (Beijing, China).

SA-β-gal staining and positive cell counting

hSKP spheres or single hSKPs (about 2.5 X 10^4 cells per slide) were cytospinned, and the staining was performed using a SA-β-gal staining Kit (Cat# C0602 Beyotime, Jiangsu, China). Briefly, cells were fixed for 15 min at RT, washed in PBS for three times and then incubated in a staining mixture for 18 hr at 37℃. Cells were then counter-stained with propidium iodide (PI, Cat# P4170 Sigma), dehydrated and then mounted with resin. Slides were observed and photographed under a Nikon Ti microscope. two slides were made for each sample, and four pictures were taken from the upper, lower, left and right parts of each slide. To calculate SA-β-gal+ cell ratio, SA-β-gal+ cell number and the total cell number were counted with the ImageJ software.

Immunostaining and quantitative analysis

Immunostaining process was described before [1]. Briefly, cytospinned cells (about 2.5 X 10^4 cells per slide) were fixed, washed in PBS, blocked with 5% BSA/PBS and incubated in primary antibodies

diluted in 1% BSA/PBS at 4℃ over night. On the next day, slides were washed in PBS, incubated in secondary antibodies, washed again and counterstained with PI or Hoechst 33342 (Cat# B2261 Sigma). Primary antibodies used in immunostaining were mouse anti-Ki67 (1:100, Cat# ZM-0166 ZSGB BIO), rabbit anti-Ki67 (1:100,ZA-0502 ZSGB BIO), rabbit anti-γH2AX (1:100, Cat# 2212-1 Epitomics), mouse anti-p53 (1:100, Cat# SC-126 Santa Cruz), mouse anti-$p21^{CIP1}$ (1:100, Cat# SC-6246 Santa Cruz), mouse anti-$p16^{INK4a}$ (1:100, Cat# SC-1661 Santa Cruz), rabbit anti-phospho-Akt (Thr308) (1:100, Cat# 4056 Cell Signaling), mouse anti-$p27^{KIP1}$ (1:100, Cat# SC-1641 Santa Cruz), rabbit anti-$p15^{INK4b}$ (1:100, Cat#4822 Cell Signaling), rabbit anti-Phospho-RB (Ser807/811) (1:100, Cat# 9308 Cell Signaling) and rabbit anti-FOXO3 (1:100, Cat# 9467 Cell Signaling). FITC or TRITC-conjugated secondary antibodies were purchased from ZSGB BIO and used at a dilution rate of 1:200.

Slides were observed and photographed under the Nikon Ti microscope. For quantitative analysis, two slides were made for each sample, and four fields at the upper, lower, left and right parts of each slide were photographed. Pictures were analyzed using the ImageJ software. The positive cell number indicated by antibody staining and the total cell number indicated by nuclear staining in each picture were counted, and the positive cell ratio was calculated by dividing the former with the latter. An average positive cell ratio was then calculated for each sample.

RNA purification, reverse transcription and quantitative real-time polymerase chain reaction (qPCR)

Single SKPs were collected by centrifugation, and the total RNA was purified with Trizol reagent (Cat# 15596018 Life technologies). The genomic DNA was eliminated using RQ1 RNase-free DNase I (Cat# M6101 Promega), and the cDNA was then synthesized with M-MuLV reverse transcriptase (Cat# M0253S New England Biolabs).

qPCR was performed using GoTaq® qPCR Master Mix (SYBR Green) (Cat# A6001 Promega) in the LightCycler® 480 Ⅱ System thermocycler platform (Roche, USA). The qPCR procedure was 94℃ for 2 minutes followed by 40 cycles of 95℃ for 15 seconds , 55℃ for 15 seconds and 68℃ for 25 seconds. The table below lists the primers used in qPCR reactions. Primers were verified by melting curve examination, and their amplification efficiencies were calculated using a relative standard curve method. mRNA expression of each gene relative to that of GAPDH was calculated using the ΔΔCt method with efficiency correction [2].

Primers used for qPCR experiments

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Cdkn1a	CCTGTCACTGTCTTGTACCCT	GCGTTTGGAGTGGTAGAAATCT
Cdkn1b	GCGCAGGAATAAGGAAGCGA	CTCCACAGAACCGGCATTTG

Cdkn1c		AAGAGGCTGCGGTGAG	CTGCTACATGAACGGTCC
Cdkn2a		AAGGTCCCTCAGACATCCC	TGTAGGACCTTCGGTGACTG
Cdkn2b	(transcript	CTAGTGGAGAAGGTGCGACAG	GCCCATCATCATGACCTGGA
variant 1)			
Cdkn2b	(transcript	GTACAGGAGTCTCCGTTGGC	GGTGAGAGTGGCAGGGTCT
variant 2)			
Cdkn2c		ATTTGGAAGGACTGCGCTGC	AGTTCGGTCTTTCAAATCGGGA
Cdkn2d		AGTCCAGTCCATGACGCAG	ATCAGGCACGTTGACATCAGC
Tp53		AACAACACCAGCTCCTCTCC	CTCATTCAGCTCTCGGAACA

Time-dependent microarray analysis

hSKPs from several donors were trypsinized and collected on Day 3, 6, 9 and 12. Senescence of these hSKPs was examined by SA-β-gal staining, and 2 independent hSKP cultures which had comparable senescence rate were chosen for microarray analysis (Fig. S2A). 1.5 X 10^6 hSKPs at each point were used for RNA extraction. Microarray was performed using GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, www.affymetrix.com). Gene and sample clustering were performed using Cluster 3.0 software. Data were also log normalized and further analyzed using the Short Time-Series Expression Miner (STEM) software [3].

Protein extraction and western blotting

Single hSKPs were centrifuged and collected. Total protein was extracted with RIPA lysis buffer (Cat# P0013C Beyotime) completed with Phenylmethanesulfonyl fluoride (PMSF, Cat# ST505 Beyotime). Protein concentration was measured using absorbance at 280 nm. Total Protein was heated for 5 min at 95℃ with 5X loading buffer (Cat# P0015 Beyotime), and about 20 μg protein was loaded for each sample in sodium dodecyl sulfate polyacrylamide gel and transferred onto polyvinyl difluoride (PVDF) membranes (Cat# IPVH00010 Millipore). After being blocked with 5% non-fat milk in Tris-Buffered Saline Tween-20 (TBST pH 7.4) for 1 hr at RT, membranes were incubated with primary antibodies in blocking solution with slow rocking at 4℃ overnight. For goat-derived primary antibodies, the blocking solution was 5% rabbit serum in TBST. After incubation with horseradish peroxidase (HRP)-conjugated-secondary antibodies (ZSGB BIO) in TBST and several washes, membranes were detected with Immobilon Western HRP substrate (Cat# WBKL S0500 Millipore). GAPDH was used as a loading control. Quantitative gray-scale analysis was performed using Quantity One™ software (Bio-Rad Laboratories).

Primary antibodies used in western blotting were mouse anti-PCNA (1:1000, Cat# ZM0213 ZSGB BIO), rabbit anti-phospho-RB (Ser807/811) (1:1000, Cat# 9308 Cell Signaling), mouse anti-underphospho-RB (1:1000, Cat# 554164 BD Pharmigen), mouse anti-GAPDH (1:3000, MAB374 Millipore), rabbit anti-phospho-Akt (Thr308) (1:1000, Cat# 4056 Cell signaling), goat anti-Akt1/2 (1:1000, Cat# 1619 Santa Cruz), rabbit anti-phospho-FOXO1 (Thr24)/FoxO3a (Thr32) (1:1000, Cat# 9464 Cell Signaling), rabbit anti-FoxO3 (1:1000, Cat# 9467 Cell Signaling), mouse anti-p53 (1:1000, Cat# SC-126 Santa Cruz), mouse anti-$p16^{INK4a}$ (1:1000, SC-1661 Santa Cruz), mouse anti-$p21^{CIP1}$ (1:1000, Cat# SC-6246 Santa Cruz), rabbit anti-$p15^{INK4b}$ (1:1000, Cat#4822 Cell Signaling), rabbit anti-phospho-GSK3β (Ser9) (1:1000, Cat# 9336 Cell Signaling), rabbit anti-GSK3β (1:1000, Cat# SC-9166 Santa Cruz), rabbit anti-phospho-S6 Ribosomal Protein (Ser235/236) (1:1000, Cat#4856 Cell Signaling) and rabbit anti-S6 ribosomal protein (1:1000, Cat# 2217 Cell Signaling).

Statistical analysis

At least 3 independent cultures were used for statistical analysis in qPCR, western blotting and staining experiments unless specifically indicated. Differences were analyzed with independent sample T-test. Data were presented as the means ± SEM. A P value < 0.05 was considered statistically significant.

References

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