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Partial cellular reprogramming stably restores the stemness of senescent epidermal stem cells

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Abstract. – OBJECTIVE: Adult stem cell senescence and exhaustion are important drivers of organismal age. Restored stem cell self-renewal has revealed novel therapeutic targets for decreasing the incidence of age-associated diseases (AADs) and prolonging the human health span. Transient ectopic expression of the reprogramming factors Oct3/4, Sox2, Klf4 and c-Myc (collectively known as OSKM) in somatic cells can induce partial cellular reprogramming and effectively ameliorate their age-associated hallmarks. However, how this form of rejuvenation is applied to senescent stem cells remains unknown.

MATERIALS AND METHODS: The Integrin-α6^{high}CD71^{high} epidermal stem cells (ESCs) with low self-renewal ability were sorted by flow cytometry and then treated by the interrupted reprogramming induced by transient expression of OSKM. The ability of secondary clones' generation and self-proliferation *in vitro*, as well as stem cell marker p63, were detected to determine their self-renewal ability. Besides, gene and protein of epidermal cell markers were detected to determine whether their cell identities were retained. Finally, DNA methylation age (eAge) and DNA dehydroxymethylase/methyltransferase were analyzed to explore the alternation of their global DNA methylation pattern during this rejuvenation.

RESULTS: The partial reprogramming restored the youthful self-renewal and proliferation in senescent ESCs, including larger secondary clone generation, higher expression of stem cell marker p63 and proliferation marker Ki67, and faster proliferation speed, in each case without abolishing epithelial cellular identity. Moreover, the rejuvenation of adult stem cells could be maintained for 2 weeks after reprogramming factor withdrawal, which was more stable than that of differentiated somatic cells. Additionally, we found that partial reprogramming counteracted the acceleration of eAge in senescent epidermal stem cells and DNA methyltransferase 1 (DNMT1) may play a crucial role in this process.

CONCLUSIONS: Partial reprogramming has high therapeutic potential for reversing adult stem cell age, providing an advanced way to treat AADs.

Key Words:

Partial reprogramming, Epidermal stem cells, Aging, Rejuvenation.

Introduction

With population age, delaying the onset of age-associated diseases (AADs) would undoubtedly be a great challenge[1]. Adult stem cells play important roles in restoring function and are essential for the maintenance of tissue homeostasis and regeneration[2]. Aging is characterized by a gradual loss of function occurring at the molecular, cellular, tissue, and organismal levels. Moreover, there is a quantitative and qualitative decline in stem cell function[3]. Thus, the interest in therapeutically targeting stem cells to treat AADs has been growing rapidly. Recent evidence[4] has shown that intraperitoneal administration of muscle-derived stem cells, isolated from young wild-type mice to progeroid mice, prolonged lifespan and health span extension, which implied senescent stem cell rejuvenation could effectively reserve age-associated phenotypes *in vivo*. Adult stem cell aging is associated[5] with diverse epigenetic alterations. Numerous lines of evidence[6,7] demonstrated that resetting the epigenetic landscapes, obtained by genetic, nutritional, or pharmacological interventions, could reverse the aging hallmarks of stem cells, which means remodeling epigenetic landscapes holds promise as a way to rejuvenate senescent stem cells.

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Ectopic expression of the reprogramming factors Oct3/4, Sox2, Klf4, and c-Myc (collectively known as OSKM) in somatic cells can generate induced pluripotent stem cells (iPSCs)[8]. The process of iPSCs generation is characterized by a complete resetting of the epigenetic landscape of cells[9], suggesting that cellular reprogramming technology may rejuvenate senescent stem cells. However, the continuous expression of reprogramming factors in mice often induces teratomas or tissue dysplasia caused by the loss of original cell identity and can be fatal within days[10], limiting its clinical application in AADs. But the partial reprogramming method through the short-term expression of reprogramming factors can reverse aging symptoms without altering cell identity[11]. Partial reprogramming alleviates the aging symptom in the skin[12] and during liver failing[13] and intervertebral disc degeneration (IDD)[14]. These studies[12-14] demonstrated that partial reprogramming effectively reversed the senescent hallmarks of differentiated somatic cells [i.e., skin fibroblasts[12], hepatocytes[14], endothelial cells[15], and nucleus pulposus cells (NPCs)[14]. However, the role of partial reprogramming in rejuvenating senescent adult stem cells has rarely been reported[9].

The skin, the largest organ of the body, is prone to exhibit a senescent phenotype during aging[16]. Epidermal stem cells (ESCs) not only self-renew but also generate daughter cells undergoing terminal differentiation and maintain a skin self-renewal ability[17]. During aging, ESCs exhibit many obvious and distinguishable changes, such as decreased stemness, which includes the ability to self-renew and proliferate with high expression of Integrin-α6 and CD71 protein[18]. Therefore, we selected senescent ESCs as the research model in the present study. We found that short-term treatment with OSKM restored youthful self-renewal and proliferation, as well as counteracted the acceleration of DNA methylation with age, without losing epidermal cell identity in senescent ESCs. Additionally, we explored the preliminary mechanism underlying this phenomenon.

Materials and Methods

Mice Primary Epidermal Cell Isolation

Male C57BL/6 mice were 6-8 weeks. All mice were purchased from the Guangzhou University of Chinese Medicine and were kept in a specific pathogen-free facility in the Animal Laboratories of Zhongshan Ophthalmic Center. The experiments were approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University. Mouse primary epidermal cells were isolated as previously reported[19]. Briefly, we chopped the tail of C57BL/6J mice and removed its tip, the skin tissue was gently peeled from the tail bone. Then the tail skin was incubated in 0.25% dispase solution for 2 h at 37°C. The single-cell solution was prepared by scraping the epidermis and filtering it with strainers (70 μm filter, followed by 40 μm filter). ESCs were isolated by flow cytometry. Single-cell solutions of epidermal cells were stained with the following antibodies for 30 min on ice: Integrin-α6-PE (0.125 μg/test, Invitrogen, Carlsbad, CA, USA, 12-0495-83) and CD71-Percp-eFlour710 (0.25 μg/test, Invitrogen, Carlsbad, CA, USA, 46-0711-82). The dead cells were excluded by staining with Dye eFlour 450 (1 μg/test, Invitrogen, Carlsbad, CA, USA, 65-0863). Cell isolation was performed using FACS Aria (BD Biosciences, Franklin Lakes, NJ, USA) and the data were analyzed by FlowJo software (BD Biosciences, Franklin Lakes, NJ, USA).

Cell Culture and Induction of OSKM Expression

Epidermal cells and ESCs were cultured in epidermal cell low Ca medium [Ca-free DMEM, 10% chelex-treated FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL), L-glutamine (2 mM), sodium pyruvate (1 mM), hydrocortisone (0.5 μg/ml), cholera enterotoxin (10^{-7} mM), EGF (10 ng/ml), and insulin (5 μg/mL)]. Virus-infected cells were cultured in an induction medium [supplemented epidermal cell low Ca medium with 1 μg/mL doxycycline (Dox)] to induce OSKM expression.

Partial Reprogramming Treatment

A 1:1:1:1 mixture of lentiviruses carrying a single doxycycline-inducible lentiviral vector expressing OSKM factor (http://www.addgene.org/, plasmid 20,321) allied with rtTA (http://www.addgene.org/, plasmid 20,342) was added to cells in the presence of 1 μg/ml polybrene and spinfected for 45 min at 750×g. This procedure was repeated the following day. After replacing with induction medium and incubating for 3 days to induce OSKM expression, cells were changed into reprogramming medium, consisting of embryonic stem cell low Ca medium [Ca-free DMEM, 15% chelex-treated FBS, penicillin/streptomycin, L-glutamine (2 mM), Sodium Pyruvate (1mM),

LIF (1,000 U/mL), nonessential amino acids, β-mercaptothion and 1 μg/mL Dox]. According to our experimental results, these cells were incubated in a reprogramming medium for 4 days, which was considered a partial reprogramming treatment. Finally, the medium was then replaced with an epidermal cell low Ca medium.

Secondary Clone Generation Assay

As previously reported[19], cells were digested with a dispose solution and reseeded in a 35-mm dish coated with Matrigel matrix at a concentration of 10^3 cells/cm^2. Cells were cultured for 3 weeks, and the medium was changed every 2 days. At the end of the experiment, clones were stained with crystal violet and observed under a stereomicroscope. Colonies were counted manually, and were divided into three categories: <1.5, 1.5-2, and >2 mm.

Immunofluorescence

Cells were grown on plastic-covered slide chambers and fixed with 4% paraformaldehyde. The following antibodies were used: antibodies against Oct3/4 (1:50, R&D systems, Minneapolis, MN, USA, JTW0419111), Ki67 (1:50, CST, Danvers, MA, USA, 9129 s), p63 (1:30, Abcam, Cambridge, UK, ab51745), and K14 (1 μg/mL, LSBio, Shirley, MA, USA, LS-C352436). Cells incubated with Phosphate Buffered Saline (PBS) instead of primary antibody were used as negative controls. Then the cells were washed in PBS and incubated at room temperature (RT) for 1 hr with the corresponding secondary antibody. Nuclei were stained with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA). The secondary antibody used was Alexa Fluor Series from Invitrogen, Carlsbad, CA, USA (all 1:500). Images were captured using a confocal microscope (LSM 800, Carl Zeiss, Jena, Germany).

RT-qPCR

Cells were collected and transferred to RNase/DNase-free tubes. RNA isolated from cells using the RNeasy Micro Kit (QIAGEN, Hilden, Germany) was converted to cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). The primer pair sequences used for the PCRs were: 5'-ggcttcagacttcgcctcc-3' and 5'-aacctgaggtccacagtatgc-3' for mice *Oct3/4*, 5'-gcggagtggaaacttttgtcc-3' and 5'-cgggaagcgtgtacttatcctt-3' for mice *Sox2*, 5'-gtccccgactaaccgttg-3' and 5'-gtcgttgaactcctcggtct-3' for mice *Klf4*, 5'-atgcccctcaacgtgaacttc-3' and 5'-cgcaacataggatggagagca-3' for mice *c-Myc*, 5'-tcttcctggtccccacagttt-3' and 5'-gcaagaatagttctcgggatgaa-3' for mice *Nanog*, 5'-agcggcaagagtgagatttct-3' and 5'-cctccaggttattctccaggg-3' for mice *K14*, 5'-atcattgaccgctcctttaggt-3' and 5'-gctcgccttgatggttcct-3' for mice *Ki67*, 5'-gtgcagagcataaagaatgtgttc-3' and 5'-gcaacttctccctgctttctac-3' for mice *Integrin-β1*, 5'- cggaaagatggcgacgatg-3' and 5'-ctcagctgcttctcagggaaag-3' for mice *Integrin-α6*, 5'-aacagacagctggccgtca-3' and 5'-ggaggttggcacactgcttc-3' for mice *K5*, 5'-gtccactctgagcagatccaga-3' and 5'-ccagggcagctttcatgct-3' for mice *K19*, 5'-atgcaaggaggatcttgcca-3' and 5'-cactctccagacatcctgatct-3' for mice *K1*, 5'-tcaagcaatggaccactggg-3' and 5'-tctccatgagctccctgaca-3' for mice Ten-eleven translocation methylcytosine dioxygenase (*TET*) 1, 5'-actcctggtgaacaaagtcaga-3' and 5'-catccctgagagctcttgcc-3' for mice *TET2*, 5'-ccggattgagaaggtcatctac-3' and 5'-aagataacaatcacggcgttct-3' for mice *TET3*, 5'-agatccagaatggagcctcgaatt-3' and 5'-accagtgggctcatgtcctt-3' for mice *DNMT1*, and 5'-gtgatgggtgtgaaccacga-3' and 5'-ggtcatgagcccttccacaa-3' for mice Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). qPCR was performed using SYBR® Premix Ex Taq™ II (Takara, Shiga, Japan) on a 7 LightCycler 480 (Roche, Rotkreuz, Switzerland). Results are reported relative to the expression levels in control group after normalization to *GAPDH* levels using the ΔΔCT method.

Genomic DNA Isolation and Bisulfite Conversion

According to the manufacturer's guidelines, the genomic DNA of each sample was isolated using the QIAamp DNA Mini Kit (50,000 cells per sample; QIAGEN, Hilden, Germany). The concentration of DNA in each sample was quantified by Nanodrop 2,000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Genomic DNA (200 ng) was subsequently bisulfite converted with the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA).

DNA Methylation Age Predictor

DNA methylation analysis by Droplet Digital PCR (ddPCR) was performed with a QX200 ddPCR System (Bio-Rad, Berkeley, CA, USA) as Han et al[20] previously described. We used dual-labeled TaqMan hydrolysis probes, which recognize either methylated or non-methylated target three CG dinucleotides (CpGs) in *Prima1*, *Kcns1*, and *Hsf4* genes. A 20 μl reaction mixture was used for each sample, which consisted of 10 μl of 2× ddP-

CR Supermix (No dUTP; Bio-Rad, Berkeley, CA, USA), 0.9 nM of forward and reverse primers, 250 nM of dual probes, and 25 ng of bisulfite converted DNA. The primer pair sequences used for ddPCR were: 5'-ggagaggtaaattatgaattagg-3' and 5'-actcttacttactcaaacctccct-3' for mice *Prima1,* 5'-aatgttggtatttttggttttgttt-3' and 5'-aaaacttacacctctcccaacaat-3' for mice *Hsf4,* 5'-ttgggagttagtagtagtaggyg-3' and 5'-atacatccacaacctaccra-3' for mice *Kcns1.* The probe pair sequences used for ddPCR were: 6-Fam-tatatttttcgggtggggg-BHQ-1 targeting the methylated sequence for *Prima1* and Hex-tatattttttgggtgggggg-BHQ-1 targeting the non-methylated sequence for *Prima1*, 6-Fam-tgtgtttcggatggtgttttttgt-BHQ-1 targeting the methylated sequence for *Hsf4* and Hex-tgtgttttggatggtgtttttgt-BHQ-1 targeting the non-methylated sequence for *Hsf4,* 6-Fam-agttgaattaagcgatatagtagaagtatttta-BHQ-1 targeting the methylated sequence for *Kcns1* and Hex-agttgaattaagtgatgtagaagtagggga-BHQ-1 targeting the non-methylated sequence for *Kcns1.* Then the mixture and 70 µl of droplet generation oil were subjected to QX200 Droplet Generator (Bio-Rad, Berkeley, CA, USA). 40 µl of the generated droplets were transferred to the ddPCR 96 plate (Bio-Rad, Berkeley, CA, USA). The plate was heat sealed with the PX1 PCR Plate Sealer (Bio-Rad, Berkeley, CA, USA) and subsequently placed in the C1000 Touch Thermal Cycler (Bio-Rad, Berkeley, CA, USA) for PCR runs as follows: 95°C for 10 min, 40 cycles of 94°C for 30 s and 1 min (2.5°C/s ramp rate) at 55°C (*Prima1*, *Kcns1*) or 58°C (*Hsf4)*, followed by 10 min enzyme deactivation step at 98°C and a final hold at 4°C. The PCR plate was read on the QX200 droplet reader (Bio-Rad, Berkeley, CA, USA) and data were analyzed by QuantaSoft 1.7.4 software (Bio-Rad, Berkeley, CA, USA). The percentage methylation of each reaction was determined by Poisson statistics according to the fraction of positive droplets for methylated and non-methylated probes. A multivariable model for age predictions was established for percentage methylation at the CpGs in *Prima1* (a), *Hsf4* (b), and *Kcns1* (c):

Predicted DNA methylation age (weeks) = -11.56 + (-0.26) a + 2.33 b + 0.43 c.

Statistical Analysis

All experiments were performed in triplicate unless indicated otherwise. The measurement data are expressed as means±standard deviation (SD). Statistical tests were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA); one-way ANOVA was used to analyze the data among groups and $p<0.05$ was considered significant.

Results

Modelling the Partial Reprogramming of Epidermal Cells

Epidermal cells were infected with a lentivirus carrying a Dox-inducible vector for mouse OSKM at various multiplicity of infection (MOI) values to determine the optimal MOI. MOI values tested were 2, 4, 8, and 16. Cells were cultured in an induction medium for 3 days, and Oct3/4 protein expression was detected. The 'mock infected' (subject to the transduction process but without lentiviruses) was treated as a negative control. Immunofluorescence results revealed an optimal MOI of 8 (Figure 1A). Therefore, we used a MOI of 8 in the subsequent experiments. We next investigated the expression levels of *OSKM* expression in epidermal cells infected with lentivirus. Virus-infected cells cultured in induction medium for 3 days (+Dox) showed higher *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* mRNA expression than those cultured without Dox (-Dox) (Figure 1B), suggesting that OSKM expression could be controlled by the presence or absence of Dox in epidermal cells.

To detect the optimal conditions of epidermal cells partial reprogramming, virus-infected epidermal cells were cultured in an induction medium for 3 days and then sequentially cultured in the reprogramming medium for 2 days (Dox+2 d), 4 days (Dox+4 d), and 1 week (Dox+1 w), according to previous research[4,21]. Mock-infected epidermal cells were used as a negative control (mock). The mRNA expression showed that Dox+4 d resulted in the highest expression of the proliferation marker *Ki67* among the groups without activation of the pluripotency marker *Nanog* (Figure 1C-D). Thus, transient expression of OSKM for 4 days was the optimal condition for the partial reprogramming of epidermal cells.

Partial Reprogramming Restores the Self-Renewing Capacity of Senescent ESCs

The clonogenic ability, i.e., the ability of cells to form large clones, is a typical feature of cells with a high self-renewing capacity[19]. Tani et al[18] proposed that Integrin-$\alpha6^{high}$CD71^{low} could label cells with inactive division at the base of mouse epidermis, a feature consistent with young ESCs. To verify whether partial reprogramming could

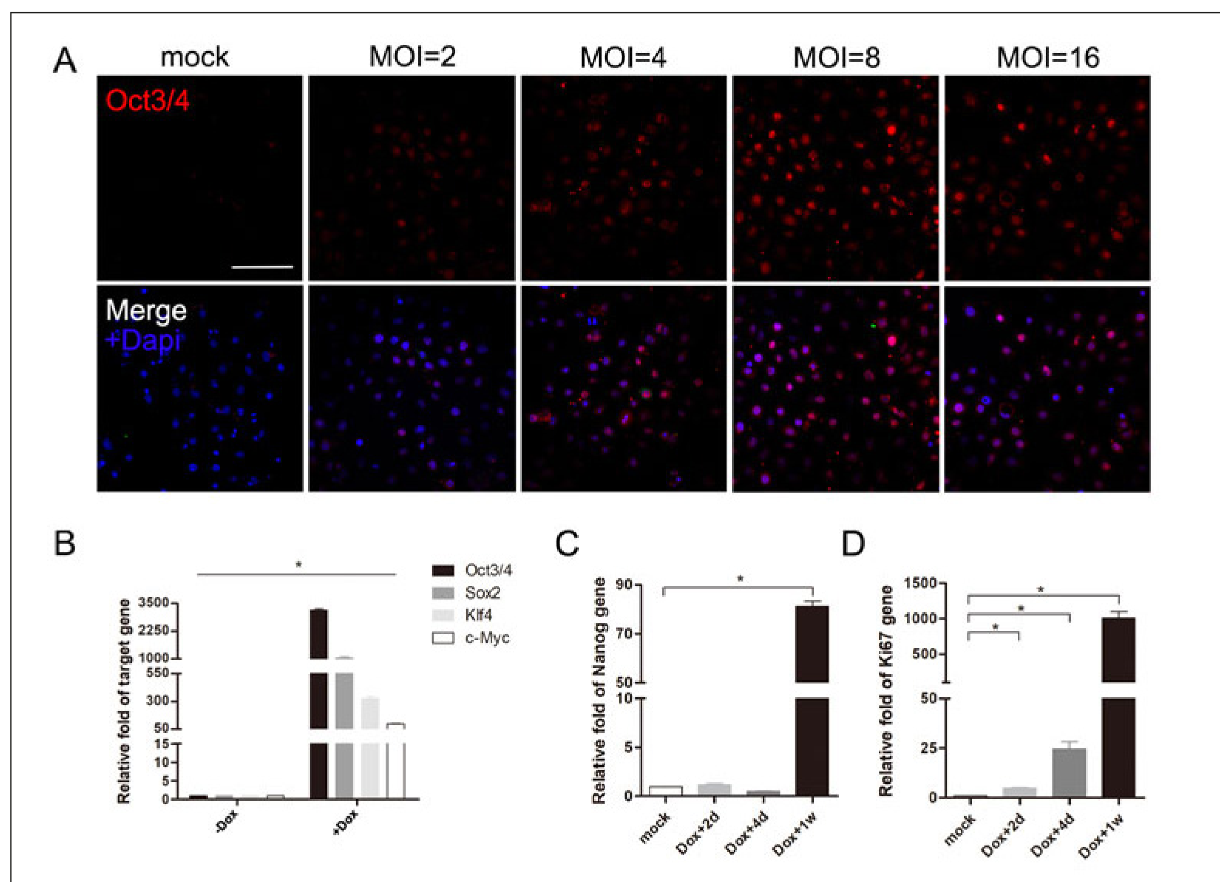

Figure 1. The optimal condition for partial reprogramming of epidermal cells. **A**, Confocal images of indirect immunofluorescence of Oct3/4 in epidermal cells infected with lentiviruses at various MOI. Scale bar=50 μm. **B**, qPCR analysis of *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* expression in virus-infected epidermal cells with and without 3 days Dox addiction as assessed by using RT-qPCR (n=3). **p*<0.05, data are presented as means±SD. **C-D**, qPCR analysis of *Nanog* and *Ki67* in virus-infected epidermal cells with 2 days, 4 days, and 1 week reprogramming and epidermal cells with mock infection as assessed by using RT-qPCR (n=3). **p*<0.05, data are presented as means±SD.

restore the self-renewal capacity of senescent ESCs, we screened out Integrin-α6^{high}CD71^{low} cells (CD71^{low}) and Integrin-α6^{high}CD71^{high} cells (CD71^{high}). Integrin-α6^{high}CD71^{high} cells were subjected to partial reprogramming treatment (CD71^{high}+treated). As a negative control, we simultaneously mock-infected Integrin-α6^{high}CD71^{high} cells (CD71^{high}+mock). We next collected cells from these four groups and performed the following assays (Figure 2A). Firstly, a secondary clone generation was observed. Most clones generated from CD71^{low} cells had a dense, healthy appearance without signs of differentiation. Most clones formed from CD71^{high} and CD71^{high}+mock cells were flat and small in appearance with many differentiated cells. The clone shape of CD71^{high}+treated cells was similar to that of CD71^{low} (Figure 2B-C). Next, we calculated the percentage of colonies of various sizes in all groups. Most clones generated from CD71^{low} were >2 mm in size, by contrast, most clones formed from CD71^{high} and CD71^{high}+mocks were <1.5 mm in size. Significant increases in the percentages of large clones (>2 mm and 2-1.5 mm in size) were observed in CD71^{high}+treated cells compared with CD71^{high} +mock cells (Figure 2D-E). The transcription factor p63 implies the self-renewal capacity of ESCs and is highly expressed in the basal cells of various epithelial tissues[22]. Subsequently, we collected cells from CD71^{low}, CD71^{high}+treated, and CD71^{high} +mock cells to detect p63 protein expression levels. In contrast to CD71^{high}+mock cells, CD71^{high}+treated cells increased the expression level of p63 protein, which was similar to that of CD71^{low} cells (Figure 2F-G). These results suggested that partial reprogramming could improve the self-renewing capacity of senescent ESCs.

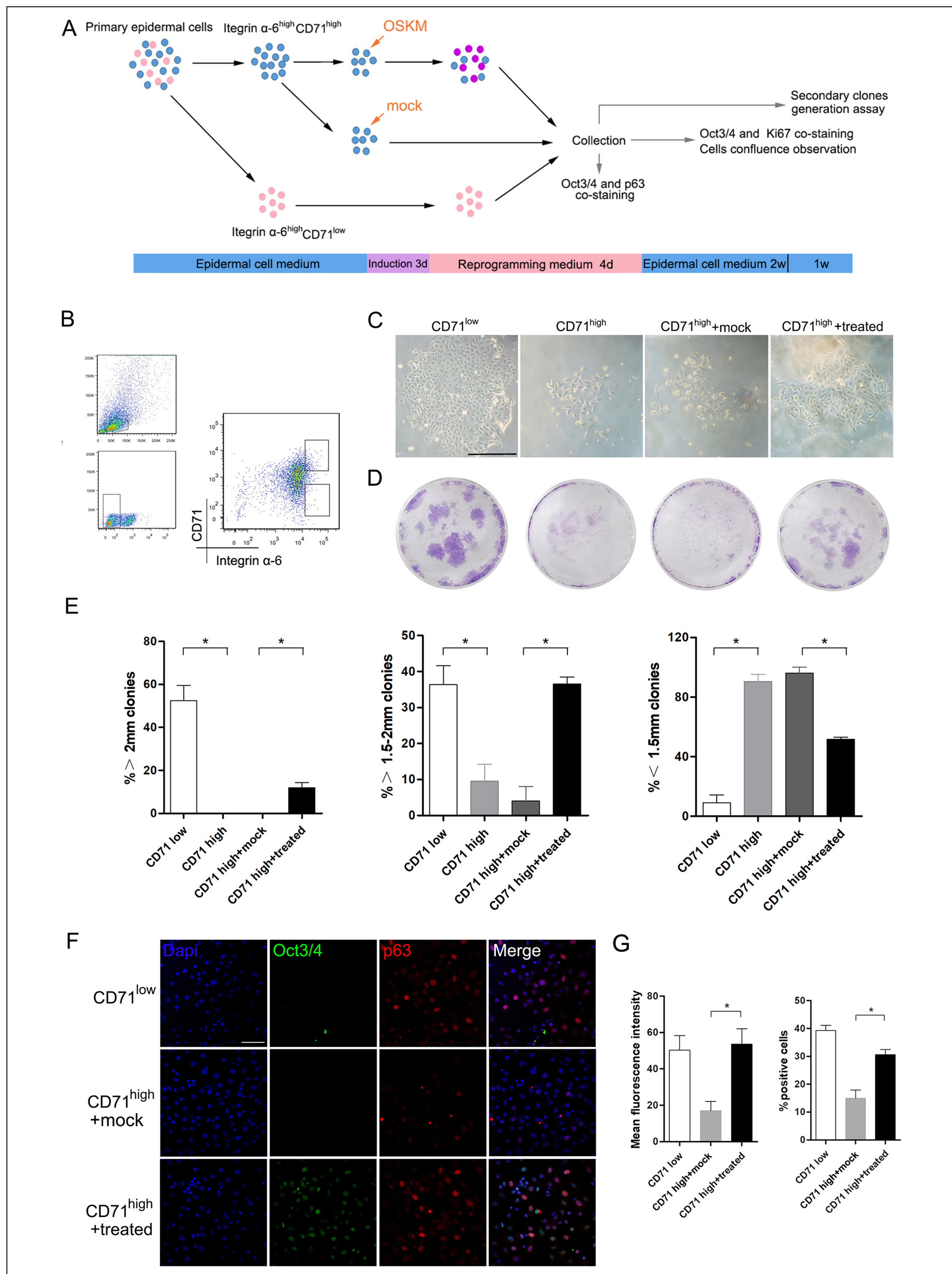

Figure 2. Partial reprogramming restores the self-renewing capacity of senescent epidermal stem cells. **A**, Schematic representation of the partial reprogramming treatment administration protocol. **B**, Primary epidermal cells were sorted into Integrin-α6^{high}CD71^{low} and Integrin-α6^{high}CD71^{high} subsets. Representative dot plots are gated on live cells, vertical lines indicate the cutoff between negative and positive staining based on an isotype-matched negative control. **C**, Higher magnification phase-contrast images of secondary clones generated in each group. Higher magnification phase-contrast images of secondary clones generated by Integrin-α6^{high}CD71^{low} subsets, Integrin-α6^{high}CD71^{high} subsets, and Integrin-α6^{high}CD71^{high} subsets with partial reprogramming treatment and mock infection. Scale bar=100 μm. **D**, Clonogenic assay in a 35-mm dish 3 weeks after plating stained with crystal violet. **E**, Quantification of the plots in (**D**). Percentages of colonies with different sizes (<1.5 mm; 1.5-2 mm and >2 mm) are represented as the means±SD, *p<0.05. **F**, Confocal images of indirect immunofluorescence of Oct3/4 and p63 in Integrin-α6^{high}CD71^{low} cells and Integrin-α6^{high}CD71^{high} cells with partial reprogramming treatment and mock infection. Scale bar=50 μm. **G**, Quantification of p63 expression in (**E**). *p<0.05, data are presented as means±SD.

Partial Reprogramming Enhances the Proliferation Ability of Senescent ESCs

ESCs with high self-renewing capacity and inactive division *in vivo* will obtain high proliferative capacity *in vitro*[19]. Besides the generation of large clones, we investigated whether partial reprogramming could improve the proliferation of senescent ESCs *in vitro*. We collected cells from CD71^{low}, CD71^{high}+treated, and CD71^{high}+mock groups. These cells from the three groups were replaced with the same density and cultured for an additional 2 weeks without OSKM expression before the following assays were performed (Figure 2A). Firstly, exogenous Oct3/4 protein and proliferative protein Ki67 were co-stained in cells from these three groups. Immunofluorescence results revealed that, after stopping OSKM expression for 14 days, cells from CD71^{high}+treated expressed no exogenous Oct3/4 protein and significantly increased Ki67 expression contrasting to CD71^{high}+mock (Figure 3A-C). Moreover, we observed that cells from CD71^{high}+treated group,

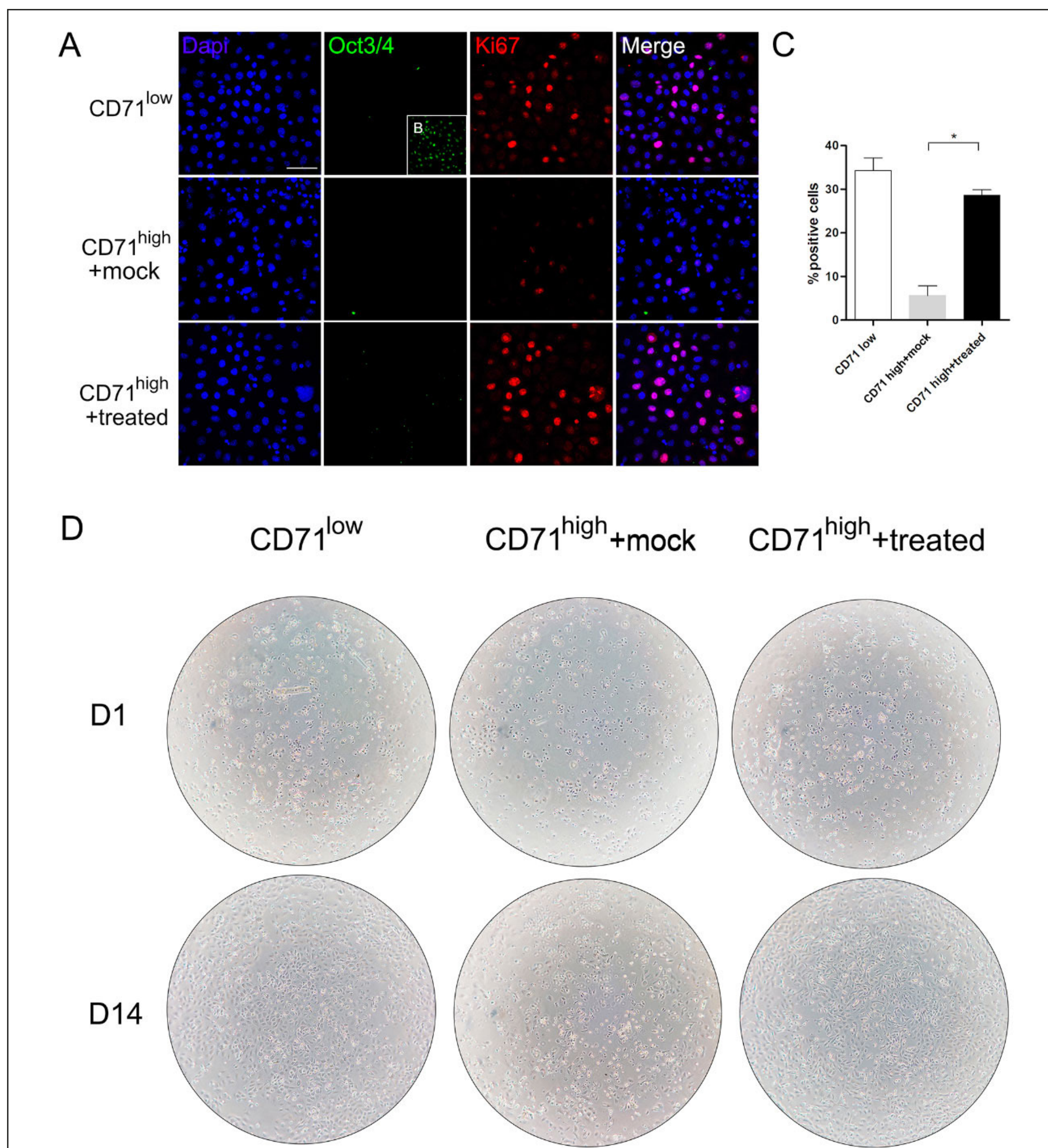

Figure 3. Partial reprogramming increases the proliferation of senescent epidermal stem cells. **A**, Confocal images of indirect immunofluorescence of Oct3/4 and Ki67 in Integrin-α6^{high}CD71^{low} cells and Integrin-α6^{high}CD71^{high} cells with partial reprogramming treatment and mock infection. Scale bar=50 μm. **B**, Positive control of Oct3/4 antibody of Integrin-α6^{high}CD71^{high} cells with OSKM expression. **C**, Quantification of Ki67 expression in (**A**). *p<0.05, data are presented as means±SD. **D**, Low power image in a 35-mm dish of Integrin-α6^{high}CD71^{low} cells and Integrin-α6^{high}CD71^{high} cells with partial reprogramming treatment and mock infection at days 0 and 14.

compared with those from CD71^{high}+mock group, proliferated more quickly and reached nearly 80% confluence 14 days later (Figure 3D). Generally, partial reprogramming increased the proliferative capacity of senescent ESCs independent of OSKM expression.

Partial Reprogramming Allows ESCs to Maintain Their Original Identity

The key to cellular reprogramming used to reverse aging safely is to maintain the original cell identity. To ascertain whether ESCs maintained their original identity after partial reprogramming treatment, we collected cells from CD71^{high} and CD71^{high}+treated. Then we cultured CD71^{high}+treated cells without OSKM expression for 4 days (CD71^{high}±treated). Then we tested the expression of lineage-specific marker genes of mouse skin epithelial cells[23]. Here, we found that senescent ESCs (CD71^{high}) expressed the skin epithelial marker keratin (K) 14 (Figure 4A-B). We collected cells from CD71^{high}+treated cells and cultured them without OSKM expression for 4 days (CD71^{high}±treated). Results of Immunofluorescence and qPCR showed that the protein and mRNA expression of keratin (K) 14 in ESCs were well maintained both with treatment and upon OSKM expression withdrawal (Figure 4A-B). Additionally, ESCs after partial reprogramming treatment obtained higher mRNA expression of *K5*, *K19*, *Integrin-β1*, and *Integrin-α6*, and no different mRNA expression of *K1* (Figure 4B). These results indicated that senescent ESCs maintained epithelial cell identity after partial reprogramming treatment.

Partial Reprogramming Reverses the DNA Methylation Age of Senescent ESCs

Our above results demonstrated that partial reprogramming restored the youthful self-renewal and proliferation in senescent ESCs, and this younger state was maintained for 2 weeks after reprogramming factor withdrawal. The DNA methylation age, which was calculated by age predictor (collectively known as Three-CpG-predictors) on the basis of bisulfite-converted DNA methylation at only three CpGs in age-associated genomic regions (*Prima1, Kcns1,* and *Hsf4*),

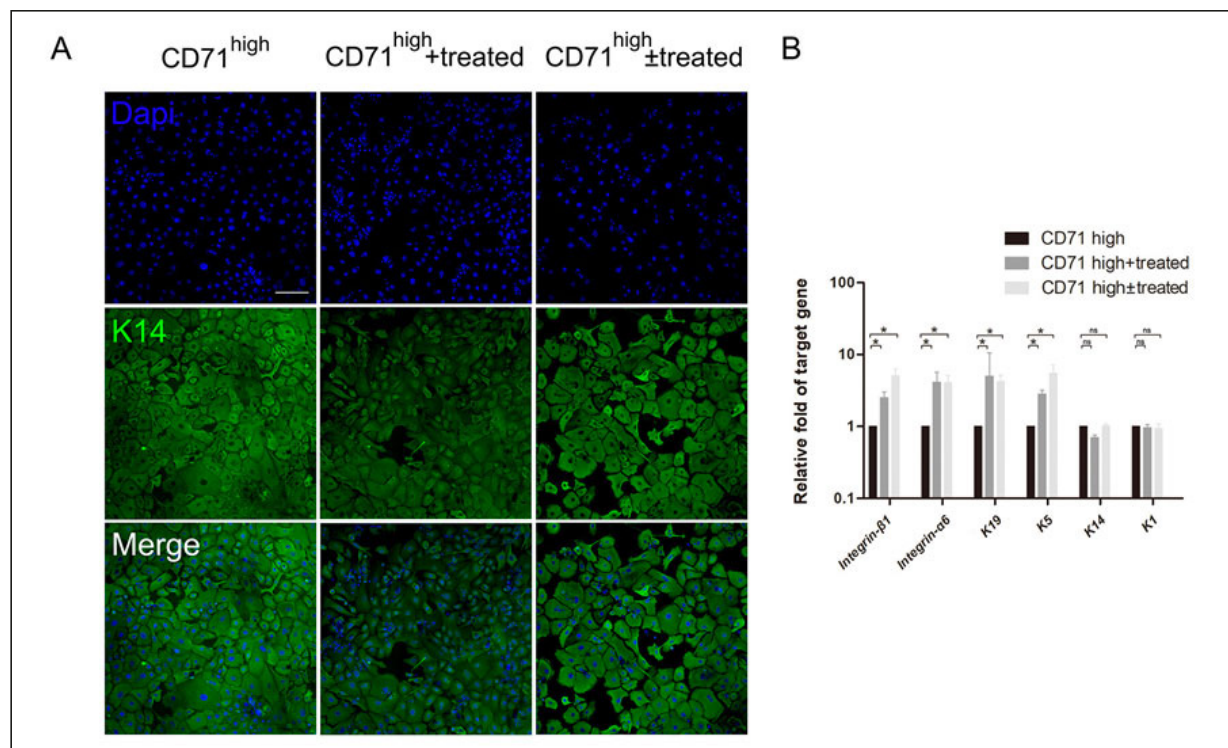

Figure 4. Epidermal stem cell maintains their original identity after partial reprogramming treatment. **A**, Confocal images of indirect immunofluorescence of K14 in Integrin-α6^{high}CD71^{high} cells and Integrin-α6^{high}CD71^{high} cells with partial reprogramming treatment and reprogramming withdrawal. Scale bar=50 µm. **B**, qPCR analysis of *K14, K5, K19, Integrin-β1, Integrin-α6,* and *K1* in Integrin-α6^{high}CD71^{high} cells and Integrin-α6^{high}CD71^{high} cells with partial reprogramming treatment and reprogramming withdrawal as assessed by RT-qPCR (n=3). *p<0.05, ^{ns}p≥0.05, data are presented as means±SD.

could be used to accurately estimate the physiological age of skin in mice[20,24]. Therefore, we collected cells from CD71^{low}, CD71^{high}+mock, and senescent ESCs accepted partial reprogramming treatment (CD71^{high}+4 d). Then we cultured CD71^{high}+4 d cells without OSKM expression for 2 weeks (CD71^{high}+4 d-2 w) (Figure 2A). The DNA methylation ages of ESCs from these four groups were calculated by Three-CpG-predictor. Cells from CD71^{high} experienced an acceleration of DNA methylation with age, whereas partial reprogramming counteracted this effect. This DNA methylation age counteraction, moreover, was still sustained after treating withdrawal for 2 weeks (Figure 5A-B). These results indicated that partial reprogramming reversed the patterns of global DNA methylation in senescent ESCs independent of OSKM expression.

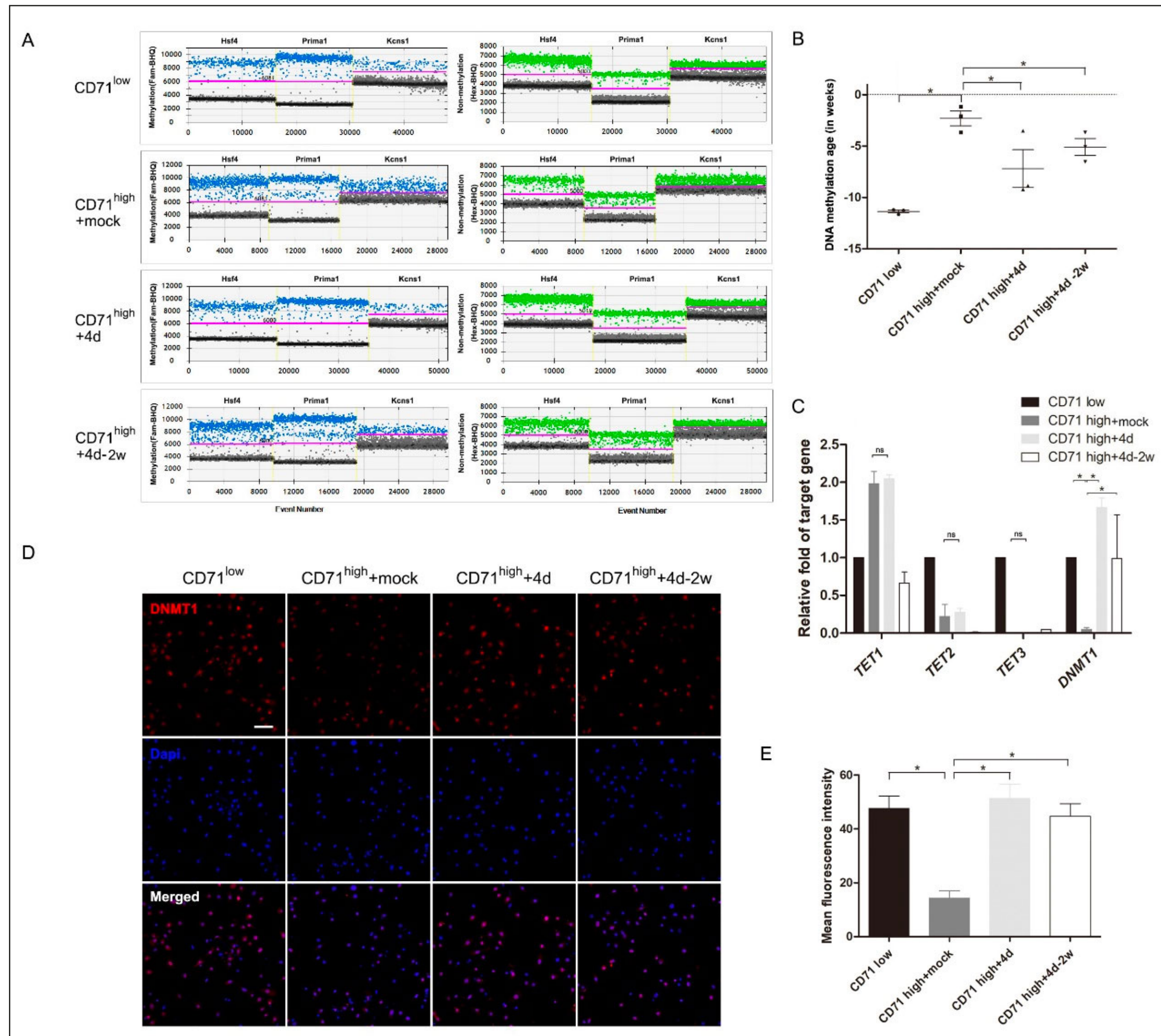

Figure 5. Partial reprogramming reset the patterns of global DNA methylation in ESCs. **A**, Droplet digital quantitative methylation-specific PCR results of representative Integrin-α6^{high}CD71^{low} cells, Integrin-α6^{high}CD71^{high} cells, Integrin-α6^{high}CD71^{high} cells with partial reprogramming treatment, and treated Integrin-α6^{high}CD71^{high} cells after treatment withdrawal for 2 weeks. The methylation signals (blue dots) in *Prima1*, *Kcns1*, and *Hsf4* genomic regions are shown on the right, the non-methylation signals (green dots) in *Prima1*, *Kcns1*, and *Hsf4* genomic regions are shown on the left. The percentage methylation of each gene was determined by Poisson statistics according to the fraction of methylated and non-methylated signals. **B**, The DNA methylation age (in weeks) predicted by the percentage methylation in (**A**) of these four types of cells. **C**, qPCR analysis of *TET1-3* and *DNMT1* in these four types of cells as assessed by RT-qPCR (n=3). **D**, Confocal images of indirect immunofluorescence of DNMT1 in these four types of cells. Scale bar=50 µm. **E**, Quantification of DNMT1 expression in (**D**). *$p<0.05$, $^{ns}p \geq 0.05$, data are presented as means±SD.

Given the effectiveness of partial reprogramming in senescent ESCs and the ability of cell reprogramming to reverse DNA methylation age *in vitro*[25], we speculated that partial reprogramming might promote self-renewal and proliferation by counteracting the effect of aging on the alternation of DNA methylation patterns. *De novo* DNA methylation and active demethylation activities control the global DNA methylation patters[26]. Thus, we detected the mRNA expression levels of DNA hydroxyethyls (*TET1-3*) and DNA methyltransferase (*DNMT1*), which catalyze DNA demethylation and methylation respectively[26], in ESCs from CD71^{low}, CD71^{high}+mock, CD71^{high}+4 d, and CD71^{high}+4 d-2 w. We found that partial reprogramming increased *DNMT1* mRNA expression in senescent ESCs, but had no effect on *TET1*, *TET*2, and *TET3* mRNA expression (Figure 5C). Since young ESCs also had a higher mRNA expression of *DNMT1* compared to senescent ESCs (Figure 5C), we suggested that DNMT1 may play an important role in rejuvenation derived by partial reprogramming. Next, we verified that partial reprogramming significantly increased the DNMT1 protein expression in senescent ESCs (Figure 5D-E). The expression of DNMT1 in ESCs with treatment withdrawal for 2 weeks was much higher than that in senescent ESCs (Figure 5C-E), which implied that partial reprogramming treatment could last for 2 weeks. In conclusion, partial reprogramming may reverse the global DNA methylation patterns of senescent ESCs through increasing DNMT1 expression.

Discussion

Rejuvenating senescent stem cells provides an advanced way to treat AADs[1]. In this study, partial reprogramming technology effectively enhanced the proliferation and self-renewal abilities of senescent ESCs *in vitro* without abolishing their epithelial cellular identity. Moreover, the rejuvenation of adult stem cells could be maintained for at least 2 weeks after reprogramming factor withdrawal, which was more stable than that of differentiated somatic cells. These results suggested that partial reprogramming had a high therapeutic potential to treat AADs by rejuvenating senescent adult stem cells.

Clones grown from ESCs with various self-renewal capacities are of different sizes when cultured *in vitro*[19]. Controversy remains on the surface markers used to size ESCs clones. Kaur et al[18] proposed that Integrin-α6^{high}CD71^{low} could label ESCs with high self-renewal capacity. Consistent with the results of Kaur et al[18], most of the second-generation clones in our research, generated from integrin-α6^{high}CD71^{low} cells, had a dense, healthy appearance with no signs of differentiation, while most of the second-generation clones formed by integrin-α6^{high}CD71^{high} cells were small and differentiated. Therefore, Integrin-α6^{high}CD71^{low} cells had high self-renewal capacity, characterized as young ESCs. By contrast, Integrin-α6^{high}CD71^{high} cells had weak self-renewal capacity, characterized as senescent ESCs, and were used as a research model in this study.

Partial reprogramming alleviates symptoms of AADs by effectively reversing the aging hallmarks of mature somatic cells[12-15]. However, this rejuvenation's function in adult stem cells remains unknown. The findings of our study showed that partial reprogramming significantly improved the self-renewal and proliferation capacity of senescent ESCs without changes in cellular identity *in vitro*. In this study, we treated senescent ESCs by transient reprogramming for 4 days, which significantly improved the quality of the clones generated from these cells. Additionally, our data showed that partial reprogramming treatment increased stemness-related marker p63 expression in senescent ESCs. In summary, partial reprogramming can improve the self-renewal capacity of senescent ESCs. ESCs with high self-renewal capacity are quiescent or low proliferative *in vivo* but activate proliferation when cultured *in vitro*[19]. Culturing *in vitro* for the same duration, we found that senescent ESCs grew at a lower density and expressed proliferation-associated protein Ki67 compared with young ESCs. We exposed senescent ESCs to OSKM expression for 4 days and found that they showed increased proliferative ability, as well as the expression of Ki67 protein. This was similar to the findings of Cheng et al[14] study in which transient expression of OSKM factors significantly enhanced the proliferation of NPCs, which reduced the incidence of IDD in mice. In conclusion, partial reprogramming technology could effectively improve the proliferation of senescent ESCs *in vitro*.

DNA methylation pattern alteration drives the aging process, and methylation changes are accepted as hallmarks of aging[27]. DNA methylation status, therefore, can be used to predict chronological age in a variety of tissues[28]. We calculated the DNA methylation ages of Inte-

grin-α6^{high}CD71^{high} cells and Integrin-α6^{high}CD71^{low} by Three-CpG-predictor. The Integrin-α6^{high}CD71^{high} cells were obtained -2.3±1.2 weeks old and Integrin-α6^{high}CD71^{low} cells were -11.4±0.2 weeks old, which implied that the senescent ESCs (CD71^{high}) obtained an acceleration of DNA methylation with age. This result is similar to Han et al research[24] who confirmed that epigenetic age predictions using these three CpGs could get significant differences between young and old mice in skin tissue. In this study, after being treated with partial reprogramming, senescent ESCs significantly decreased their DNA methylation age and increased DNMT1 expression, which suggested that partial reprogramming treatment may rejuvenate senescent ESCs by DNA methylation pattern resetting regulated by DNMT1. DNA methylation is catalyzed by DNMT and DNMT1 is the major DNMT that maintains the methylation status of the genome[29]. Several pieces of evidence[30,31] point out that DNMT1 is essential for the preservation of the progenitor state of ESCs, and lack of DNTM1 in ESCs would result in severe defects in cell proliferation and self-renewal capacity. Similarly, our results show that partial reprogramming treatment restored the proliferation and self-renewal of senescent ESCs with increasing DNMT1 expression.

In previous research[14,21], senescent differentiated somatic cells, such as skin fibroblasts and NPCs, treated with partial reprogramming reversed their aged phenotype but regained aged hallmarks after 8 days of OSKM expression withdrawal. Interestingly, our study showed that partially reprogramming-treated ESCs still exhibited high proliferative and self-renewal capacities and youthful DNA methylation patterns *in vitro* after stopping the reprogramming process for 2 weeks. These differences between studies may ascribe to the different extents of rejuvenation obtained by cells[12]. The extents of rejuvenation[32] are associated with the reprogramming phases that cells arrive at, and the reprogramming phases are influenced by different epigenetic landscapes between stem cells and differentiated cells[33]. Taking chromatin compaction as an example, compacted heterochromatin blocks, which present a barrier to cellular reprogramming, are prevalent in differentiated cells[27] and are rarely seen in stem cells[5]. Of course, chromosomal changes may not be the only reason for this difference. Any mechanisms regulating cellular reprogramming and stemness maintenance may be involved, such as DNA methylation and so on[7,27]. Further research is required to explore how partial reprogramming rejuvenates aging stem cells.

Conclusions

Taken together, our results indicate that partial reprogramming could reverse the senescent phenotype of adult stem cells, which showed more stability than that of differentiated somatic cells, suggesting a novel way for AADs treatment.

Ethics Approval
The study was approved by the Ethics Committee of Zhongshan Ophthalmic Center, Sun Yat-Sen University (Approval Number: 2022-066).

Informed Consent
Not applicable.

Authors' Contributions
S.Y.R. conceived the project and designed the experiments. G.S.M prepared the manuscript. L.Y.R. performed the qPCR analysis. C.Y.Q, X.S., and W.Q. performed the cell culturing and analyzed the data. C.M.H., L.W.Q., and S.Q. performed the image analysis. L.Y. and L.C.Y. provided lentiviruses. W.Z.C. and W.X.R. provided key revision.

Availability of Data and Materials
The datasets used during the current study are available upon reasonable request.

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Conflict of Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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