Flow cytometry analysis

Flow cytometry analysis was performed as previously described [35]. Briefly, hADMPCs were harvested and re-suspended in staining buffer (PBS containing 1% BSA, 2 mM EDTA, and 0.01% sodium azide) at a density of 1×10^6 cells/mL, incubated for 20 min with a fluorescein isothiocyanate (FITC)-conjugated antibody against CD49b or CD98 (BioLegend, CA, USA) or a phycoerythrin (PE)-conjugated antibody against CD10, CD13, CD29, CD44, CD49a, CD49c, CD49d, CD49e, CD51/61, CD73, CD90, CD105, CD117, SSEA4, HLA-A,B,C (BioLegend), CD133/1 (Miltenyi Biotec, CA, USA), or CD166 (Beckman Coulter, CA, USA). Non-specific staining was assessed using relevant isotype controls. Dead cells were excluded using the Live/Dead Fixable Far Red Dead Cell Stain Kit (Life Technologies). FlowJo software was used for quantitative analysis.

RNA extraction, cDNA generation, and quantitative polymerase chain reaction (Q-PCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) accroding to the manufacturer's instructions. cDNA was generated from 1 µg of total RNA using the Verso cDNA Synthesis Kit (Thermo Scientific, MA, USA) and purified using the MinElute PCR Purification Kit (Qiagen). Q-PCR analysis was conducted using the SsoFast EvaGreen supermix (Bio-Rad, CA, USA) according to the manufacturer's protocols. The relative expression value for each gene was calculated using the $\Delta\Delta$Ct method and the most reliable internal control gene

1 was determined using geNorm Software (http://medgen.ugent.be/~jvdesomp/genorm/). Details of the primers

2 used in these experiments are available upon request.

3

4 **Western blot analysis**

5 Whole cell extracts were prepared by washing cells with ice-cold phosphate-buffered saline (PBS) and lysing

6 them with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific Pierce, IL, USA) according to the

7 manufacturer' s instructions. Nuclear and cytosolic extracts were prepared as follows. Cells were washed with

8 ice-cold PBS and lysed with lysis buffer (50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 137.5 mM NaCl, 10%

9 glycerol, 5 mM EDTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and

10 protease inhibitor cocktail). Then, insoluble nuclei were isolated by centrifugation and lysed with lysis buffer

11 containing 0.5% SDS. Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel

12 electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore),

13 and probed with antibodies against cleaved Notch1 (#2421, Cell Signaling Technology, MA, USA), HIF-1α

14 (#610959, BD Bioscience, CA, USA), hypoxia inducible factor 2α (MAB3472, Millipore), Akt (#9272, Cell Signaling

15 Technology), and phospho Akt (Ser473) (#4060, Cell Signaling Technology). Horseradish peroxidase

16 (HRP)-conjugated anti-mouse or -rabbit IgG antibody (Cell Signaling Technology) was used as a secondary

17 antibody and immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP substrate

18 (Millipore). The band intensity was measured using the ImageJ software.

Fluorescence microscopy

Phase contrast and fluorescence images were obtained using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan) using BZ Analyzer Software (Keyence).

Adipogenic, osteogenic, and chondrogenic differentiation procedures

For adipogenic differentiation, cells were cultured in differentiation medium (Zen-Bio, NC, USA). After 7 days, half of the medium was exchanged for adipocyte medium (Zen-Bio) and this was repeated every 3 days. Three weeks after differentiation, adipogenic differentiation was confirmed by microscopic observation of intracellular lipid droplets with the aid of Oil Red O staining. Osteogenic differentiation was induced by culturing the cells in osteocyte differentiation medium (Zen-Bio). Differentiation was examined by Alizarin Red staining. For chondrogenic differentiation, 2×10^5 hADMSCs were centrifuged at $400 \times g$ for 10 min. The resulting pellets were cultured in chondrogenic medium (Lonza, Basel, Switzerland) for 21 days. The pellets were fixed with 4% paraformaldehyde in PBS, embedded in OCT, frozen, and sectioned at 8 μm. The sections were incubated with PBSMT (PBS containing 0.1% Triton X-100, and 2% skim milk) for 1 h at room temperature, and then incubated with a mouse monoclonal antibody against type II collagen (Abcam, MA, USA) for 1 h. After washing with PBS, cells were incubated with Alexa 546-conjugated anti-mouse IgG to identify chondrocytes (Life Technologies). The

cells were counterstained with 4’-6-diamidino-2-phenylindole (DAPI) (Life Technologies) to identify cellular nuclei.

The sections were also stained with 1% alcian blue (Sigma Aldrich) in 3% acetic acid, pH 2.5 for 30 min.

Determination of HK, PFK, LDH, PDH, and Cox IV activities

Cells (2×10^6) were lysed, and HK, PFK, LDH, or PDH activity was measured using the Hexokinase Colorimetric Activity Kit, Phosphofructokinase (PFK) Activity Colorimetric Assay Kit, Lactate Dehydrogenase (LDH) Activity Assay Kit, or Pyruvate Dehydrogenase Activity Colorimetric Assay Kit (all from BioVision, Milpitas, CA, USA) respectively, according to the manufacturer’s instructions. To measure Cox IV activity, mitochondria were isolated from 2×10^7 cells using a Mitochondria Isolation Kit (Thermo Scientific) and lysed with buffer containing n-Dodecyl β-D-maltoside, followed by measurement with the Mitochondria Activity Assay (Cytochrome C Oxidase Activity Assay) Kit (BioChain Institute, Newark, CA, USA), according to the manufacturer’s instructions.

Results

5% Oxygen hypoxic culture condition increases proliferation capacity and decreases senescence

hADMPCs were cultured under 20% oxygen (normoxia; Nx) or 5% oxygen (hypoxia; Hx) and their proliferation capacities were examined based on the relationship between the number of cultivation days and the population

doubling level (PDL). Nx-cultured hADMPCs ceased proliferation at a PDL of 35–40 (between 46–70 days), whereas continuous cell proliferation beyond 45 PDL was observed when hADMPCs were cultured in the Hx condition (Figure 1A). To investigate whether this increase of PDL in the Hx culture condition resulted from an increase in cell cycle progression and increase in survival rates, 5-ethynyl-2'-deoxyuridine (EdU), an alternative to 5-bromo-2'-deoxyuridine (BrdU), was incorporated into the genomic DNA of the hADMPCs, and the amount of incorporated EdU was quantified by flow cytometry. As shown in Figure 1B, the EdU incorporation rate was significantly higher in Hx-cultured hADMPCs than in Nx-cultured hADMPCs, suggesting that cell growth was increased in the Hx culture condition. In addition, measurement of DNA content in hADMPCs revealed a slight but significant decrease of sub-G1 peaks, which indicates the existence of apoptotic cells with degraded DNA, when the cells were cultured in the Hx condition (Figure 1C). These data suggest that the Hx culture condition increases the proliferation capacity of hADMPCs by promoting their cell growth and survival rates. We also found that Nx-cultured hADMPCs were larger with a more irregular shape (Figure 1D), which suggests that the Hx culture condition prevented hADMPCs from entering senescence [36]. To further investigate this phenomenon, cellular senescence was measured by staining for senescence-associated β-galactosidase (SA-β-Gal), which revealed that SA-β-Gal activity was increased in Nx-cultured hADMPCs at passage 17 (Figure 1E). As it has been hypothesized that senescence results from oxidative stress [20], accumulation of reactive oxygen species (ROS) in hADMPCs was detected using the non-fluorescent probe, CM-H_2DCFDA. Flow cytometry analysis revealed that ROS were generated at higher levels in hADMPCs when cultured in the Nx condition (Figure 1F), suggesting that reduced production of ROS in the Hx condition may prevent the cells from entering replicative senescence.

1

2 **Hypoxic culture maintains some mesenchymal stem cell properties and increases differentiation**

3 We then examined the cell properties of hADMPCs under Nx and Hx conditions. Initially, cell surface antigens
4 expressed on hADMPCs were analyzed by flow cytometry. No significant difference in expression profile between
5 hADMPCs cultured in Nx and Hx was observed; the cells were consistently positive for CD10, CD13, CD29,
6 CD44, CD49a, CD49b, CD49c, CD49d, CD49e, CD51/61, CD54, CD59, CD73, CD90, CD98, CD105, CD166,
7 and HLA-A, B, C, but negative for CD34, CD45, CD117, CD133 (Figure 2 and data not shown). These data were
8 consistent with previous reports describing the expression profiles of cell surface markers of hMSCs [37,38]. To
9 further examine the stem cell properties of hADMPCs, their potential for differentiation into adipocyte, osteocyte,
10 and chondrocyte lineages was analyzed at passage 8. Hx-cultured hADMPCs presented enhanced differentiation
11 into various lineages (Figure 3A and B), indicating that the Hx culture condition improved the stem cell properties
12 of hADMPCs.

13

14 **Hypoxic culture condition activates Notch signaling**

15 To reveal the molecular mechanism by which the Hx culture condition increased the proliferative capacity and
16 maintaining the stem cell properties of hADMPCs, we next examined Notch signaling, which is required for
17 maintaining stem-cell features of various types of stem cells [30,31]. As expected, levels of cleaved NOTCH1, an

activated form of NOTCH1, were significantly increased (> 2-fold) in the Hx culture condition (Figure 4A). Quantitative PCR (Q-PCR) analysis revealed that HES1, a downstream target of Notch signaling, was upregulated in Hx-cultured hADMPCs, which also indicated that Notch signaling was activated in the Hx culture condition (Figure 4B). Administration of the γ-secretase inhibitor DAPT at 1 μM, which was sufficient to inhibit the proteolytic cleavage of NOTCH1 (Figure 4A), decreased the Hx-induced expression of HES1 at both mRNA and protein levels (Figure 4B and C). These data indicate that Hx increased the expression of HES1 through activation of Notch signaling. It has been reported that Notch signaling and hypoxia-inducible factor (HIF) undergo cross talk in hypoxic cells [39-42]. Therefore, HIF-1α and HIF-2α protein levels in hADMPCs were analyzed by western blotting. HIF-1α was stabilized when a chemical hypoxia-mimicking agent, cobalt chloride, was applied in the culture, whereas no obvious increase of HIF-1α was observed in the Hx culture condition (Figure 4D). However, we did not detect any HIF-2α expression even in the presence of cobalt chloride (Figure 4E). Q-PCR analysis revealed that *HIF2A* mRNA was not expressed in these cells (data not shown). From these results, we concluded that neither HIF-1α nor HIF-2α was involved in the Hx-induced increase in the proliferative capacity and stem cell properties of hADMPCs.

To identify the signaling responsible for the observed effect, we next examined the Akt, NF-κB, and p53 signaling pathways. It has been reported that hypoxic conditions induce the activation of Akt and NF-κB signaling [43,44]. In addition, hypoxic conditions have been shown to inhibit p53 activity [45], and crosstalk between these pathways and Notch signaling has also been demonstrated [42,46-48]. As shown in Figure 4F, the

Hx condition increased Akt phosphorylation, which was not decreased by DAPT treatment. These data demonstrate that 5% oxygen activated Akt signaling but not via Notch signaling. Similarly, the hypoxic condition induced nuclear accumulation of p65, which was inhibited by DAPT treatment (Figure 4G). These data suggest that NF-κB signaling is regulated by Notch signaling in hADMPCs. Furthermore, p53 was not activated under the 5% oxygen condition as assessed by detection of phospho-p53 and a p53 reporter assay. However, DAPT treatment significantly increased p53 activity (Figure 4H and I).

Notch signaling is indispensable for acquisition of the advantageous properties of hADMPCs

We next examined the roles of Notch signaling in the proliferative capacity and stem cell properties of hADMPCs in the Hx culture condition. To inhibit Notch signaling, DAPT was added to the medium at a final concentration of 1 μM. DAPT treatment significantly decreased the PDL when hADMPCs were cultured under either 20% or 5% oxygen (Figure 5A). Intriguingly, measurement of the DNA content in hADMPCs revealed that inhibition of Notch signaling by 1 μM DAPT significantly attenuated the decrease in apoptotic cells in the Hx condition (Figure 5B). These data suggest that 5% oxygen increases the proliferation capacity of hADMPCs through Notch signaling by promoting their survival. To examine whether Notch signaling affects the stem cell properties of hADMPCs, differentiation into adipocyte, osteocyte, and chondrocyte lineages was analyzed at passage 8. Hx-cultured hADMPCs underwent greater differentiated into all lineages as described in Figure 3, whereas application of a Notch inhibitor significantly decreased the differentiation capacity to all lineages (Figure 5C-E). In addition,

SA-β-Gal staining revealed that inhibition of Notch signaling by DAPT remarkably promoted senescence in both the Nx and Hx culture conditions, suggesting that the suppression of replicative senescence observed in the Hx condition is mediated by Notch signaling.

Glycolysis is enhanced in the 5% oxygen condition through Notch signaling

Recent studies suggest that the metabolic shift from aerobic mitochondrial respiration to glycolysis extends the life span possibly via reduction of intrinsic ROS production [18,19]. Our results demonstrate that the 5% oxygen condition reduced ROS accumulation in hADMPCs (Figure 1F). In addition, the relationship between Notch signaling and glycolysis has been recently established [49,50]. We therefore considered glycolytic flux by measuring the glucose consumption and lactate production of hADMPCs in the Nx or Hx culture conditions. As shown in Figure 6A, glucose consumption and lactate production were elevated in the Hx culture condition, indicating that a metabolic shift to glycolysis occurred when hADMPCs were cultured in 5% oxygen. In contrast, the Notch inhibitor DAPT markedly reduced glycolytic flux as assessed by glucose consumption and lactate production (Figure 6A). To identify the genes responsible for the glycolytic change, we performed a Q-PCR analysis. As shown in Figure 6B, *SLC2A3, TPI, and PGK1*, encoding glycolytic enzymes, were upregulated in the 5% oxygen condition, whereas these genes were suppressed by DAPT treatment. Interestingly, Hes1 transduction by an adenoviral vector markedly induced the mRNA expression of the same genes (Figure 6E). In addition, SCO2, a positive modulator of aerobic respiration, and TIGAR, a negative regulator of glycolysis, were

transcriptionally downregulated in the 5% oxygen condition, whereas DAPT treatment partially restored the expression level (Figure 6B). Adenoviral expression of Hes1 dramatically reduced *SCO2* and *TIGAR* expression (Figure 6E), which suggests that the Notch-Hes1 signaling modulates the metabolic pathway. We also measured the activities of key enzymes in glycolysis. Hexokinase activity was not changed under hypoxic conditions; however, phosphofructokinase and lactate dehydrogenase were activated in 5% oxygen condition, which was attenuated by Notch inhibition (Figure 6C). In addition, pyruvate dehydrogenase (PDH) and cytochrome c oxidase (Complex IV) activity assays showed that mitochondrial respiration decreased under the hypoxic condition and that DAPT treatment restored it (Figure 6D). Moreover, glycolytic flux in Hes1-expressing hADMPCs was positively correlated with the expression of these glycolytic genes as assessed by glucose consumption and lactate production (Figure 6F). In contrast, HES1 knockdown by adenoviral transduction of *HES1* RNAi resulted in a significant reduction of glycolytic flux (Figure 6G), demonstrating that HES1 is involved in the regulation of glycolysis.

Glycolysis supports the proliferation of hADMPCs

To determine whether aerobic glycolysis is important for the proliferation of hADMPCs, hADMPCs were treated with the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) or the respiration inhibitor sodium azide (NaN_3). We found that hADMPCs were sensitive to treatment with 2-DG even at the low concentration of 0.2 mM (Figure 7A). In contrast, treatment of hADMPCs with NaN_3, rather increased their proliferation at the concentration of 1 mM and

supported their proliferation even at the concentration of 5 mM (Figure 7B). These data suggest that the proliferation of hADMPCs is compromised when aerobic glycolysis is blocked.

Discussion

Recent evidence suggests that hypoxic culture conditions confer a growth advantage, prevent premature senescence, and maintain undifferentiated states in ESCs, iPSCs, and somatic stem cells. However, the molecular mechanism underlying the beneficial effects of culturing these cells at low oxygen conditions remains unclear. Our findings prompted us to hypothesize that Notch signaling in physiological hypoxic conditions (5% O_2) contributes to these effects on hADMPCs by modulating glycolytic flux.

We found that 5% O_2 significantly increased the proliferation capacity, decreased apoptosis, and inhibited senescence of hADMPCs (Figure 1). Moreover, 5% O_2 improved the differentiation of hADMPCs without affecting the expression of their cell surface markers (Figures 2 and 3). Welford et al. reported that HIF-1α delays premature senescence of mouse embryonic fibroblasts under hypoxic conditions (2% O_2) [51]. Tsai et al. reported that hypoxia (1% O_2) inhibits senescence and maintains mesenchymal stem cell properties through accumulation of HIF-1α [26]. Hypoxia was recently reported to enhance the undifferentiated status and stem cell properties in various stem and precursor cell populations via the interaction of HIF with the Notch intracellular domain to activate Notch-responsive promoters [39]. In the current study, the effects observed in 5% O_2 condition were

independent of HIF proteins because accumulation of HIF-1α and HIF-2α was not observed (Figure 4). Instead, our findings suggest that 5% O_2 activated Notch signaling, which contributed advantageous effects of hypoxic culture on hADMPCs. A pharmacological inhibitor of Notch signaling, DAPT, abrogated the hypoxic-induced Notch activation, increased proliferation capacity and lifespan, maintenance of stem cell properties, and prevention of senescence (Figure 4 and 5). Moreover, we also found that 5% O_2 enhanced glucose consumption and lactate production, and these effects were also attenuated by Notch inhibition (Figure 6A) and knockdown of HES1 (Figure 6G). Previously, it has been reported that Notch signaling promotes glycolysis by activating the PI(3)K-Akt pathway [49,50]. However, our results indicate that Akt signaling was not activated by Notch signaling because DAPT did not attenuate hypoxia-induced Akt phosphorylation (Figure 4F). Although Akt is unlikely to be regulated by Notch signaling in hADMPCs, it is clear in our data that Akt signaling was activated by 5% O_2. Therefore, we could not rule out the possibility that the promotion of glycolysis in the 5% O_2 condition was caused by Akt signaling.

Recent evidence suggests that Notch signaling acts as a metabolic switch [49,52]. Zhou et al. demonstrated that hairy, a basic helix-loop-helix transcriptional repressor regulated by Notch signaling, was up-regulated and genes encoding metabolic enzymes, including TCA cycle enzymes and respiratory chain complexes, were down-regulated in hypoxia-tolerant flies. Intriguingly, they also found that hairy-binding elements were present in the regulatory region of the down-regulated metabolic genes. Their work thus provides new evidence that hairy acts as a metabolic switch [52]. Landor et al. demonstrated that both hyper- and hypoactive

Notch signaling induced glycolysis, albeit by different mechanisms. They showed that Notch activation increased glycolysis through activation of PI3K-AKT signaling, whereas decreased Notch activity inhibited mitochondrial function in a p53-dependent manner in MCF7 breast cancer cell lines [49]. Consistent with their reports, our findings that Notch signaling promoted activity of some glycolysis enzymes and inhibited mitochondrial activity (Figure 6) also suggest that Notch signaling functioned as a metabolic switch. While our data showed that Notch inhibition by DAPT resulted in reduced glycolysis (Figure 6A-C), induction of mitochondrial function (Figure 6D) and activation of p53 (Figure 4H and I) are not consistent with the report of Landor el al. This contradiction might be explained by the expression level of endogenous Notch. Landor et al. showed that in breast cancer MDA-M-231 cells, which showed higher endogenous Notch activity, high glucose uptake, and lactate production than MCF7 breast cancer cell lines, Notch inhibition by DAPT significantly reduced glucose consumption and lactate production [49]. As shown in Figure 4A, we observed that hADMPCs in 5% O_2 displayed high Notch activity. Moreover, the lactate-to-glucose ratio was 1.8–1.9 in hADMPCs, suggesting that hADMPCs largely rely on glycolysis for energy production (Figure 6A). In addition, it was reported that hMSCs showed a higher glycolytic rate than primary human fibroblast [53]. It appears that hADMPCs cultured under hypoxic conditions might possess cell properties similar to MDA-M-231 cells or MCF7 cells, in which stable expression of constructs NICD1-GFP produces high Notch activity.

Nuclear translocation of p65 was observed in hypoxic conditions, demonstrating that NF-κB is a direct target of Notch signaling (Figure 4G). Intriguingly, the hypoxic culture conditions in this study upregulated several

genes encoding glycolytic enzymes (*SLC2A3, TPI, and PGK1)* whereas the expression of these genes was suppressed by Notch inhibition. In addition, Hes1 transduction induced mRNA expression of the same genes (Figure 6). It was previously reported that *SLC2A3* expression was regulated by p65/NF-κB signaling, and that Notch/Hes1 is able to induce the activation of the NF-κB pathway in human T-ALL lines and animal disease models [54]. Espinosa et al. demonstrated that Hes1 directly targeted the deubiquitinase CYLD, resulting in deubiquitination and inactivation of TAK1 and IKK, degradation of IκBa, and activation of NF-κB signaling [54]. In our systems, however, we did not observe repression of *CYLD* mRNA in Hes1-overexpressing hADMPCs (data not shown). While *PGK1* mRNA has been reported to be upregulated by NF-κB, it has not clearly been shown to be controlled by NF-κB despite the presence of a NF-κB site in the promoter [55]. Although modulation of *TPI* expression by NF-κB has not been reported, we found several NF-κB binding sites on the human *TPI* promoter (data not shown). As NF-κB is likely to be one of the responsible signals for hypoxic-induced glycolysis [54], further analysis will be required to determine the mechanism by which NF-κB signaling is induced by Notch signaling. Additionally, it will be important to investigate whether NF-κB is really responsible for the observed glycolysis and whether it regulates the expression of *SLC2A3*, *TPI*, and *PGK1* in hADMPCs under 5% oxygen.

In addition, SCO2, a positive modulator of aerobic respiration, and TIGAR, a negative regulator of glycolysis, were transcriptionally downregulated in the 5% oxygen condition, whereas DAPT treatment partially restored expression (Figure 6B). We observed some glycolysis and mitochondrial enzyme activity and found that the activities of COX IV and PFK were consistent with gene expression data (Figure 6C and D). Adenoviral

expression of Hes1 dramatically reduced *SCO2* and *TIGAR* expression (Figure 6E), which suggests that Notch-Hes1 signaling modulates the metabolic pathway. Intriguingly, our results also indicate that Hes1 could suppress the expression of *TIGAR* and *SCO2*, a p53 target gene. It has been reported that Notch signaling suppresses p53 in lymphomagenesis [47]. Moreover, Kim et al. reported that NICD1 inhibits p53 phosphorylation and represses p53 transactivation by interacting with p53 [48]. In addition, DAPT treatment resulted in the enhancement of p53 activity in the hypoxic conditions (Figure 4H and I). Therefore, it is possible that p53 activation was regulated by Notch signaling in hADMPCs, although we did not observe a decrease in p53 activity in hypoxic conditions in this study (Figure 4). Further analysis will be required to determine whether p53 activity is suppressed in hypoxic conditions over a longer period of culture.

Cells undergoing active proliferation utilize large amounts of glucose through glycolysis, producing pyruvate for use in substrates (amino acids and lipids) and the pentose shunt for use in nucleic acid substrates, and also producing NADPH as a reducing agent to counter oxidative stress [18,56]. In the current study, 5% O_2 actually increased proliferation and decreased the accumulation of ROS, which may be involved in the reduction of senescence (Figure 1). Because accumulation of endogenous ROS might be a major reason for replicative senescence [20], enhancing glycolysis in cultured cells may improve the quality of the cells by suppressing premature senescence. Kondoh *et al.* demonstrated that enhanced glycolysis is involved in cellular immortalization through reduction of intrinsic ROS production [14,18,19]. Therefore, it is possible that the extension of lifespan observed in our experimental conditions was caused by the reduction of intracellular ROS

1 levels through enhanced glycolysis by Notch signaling. Our data indicate that aerobic glycolysis is utilized for
2 proliferation of hADMPCs because the glycolytic inhibitor 2-DG attenuates the proliferation rate of hADMPCs
3 (Figure 7A). Intriguingly, the aerobic respiration block by NaN_3 did not decrease the proliferation; rather, it
4 increased proliferation at a low concentration (Figure 7B), which may support our data indicating that the
5 metabolic switch from mitochondrial respiration to glycolysis provides a growth advantage to hADMPCs. However,
6 the question of whether the enhanced glycolysis really contributes to the prolonged lifespan in hADMPCs remains
7 to be determined in this study.

8 In the current study, the molecular mechanism for how Notch signaling is activated in 5% O_2
9 conditions was explored. It has been reported that Notch1 activity is influenced by oxygen concentration
10 [41,42,57]. In melanoma cells, hypoxia (2% O_2) resulted in increased expression of Notch1 by HIF-1α and also by
11 Akt through NF-κB activity [42]. Similarly, in hypoxic breast cancer cells, Notch ligand JAG2 was shown to be
12 transcriptionally activated by hypoxia (1% O_2) in a HIF-1α dependent manner, resulting an elevation of Notch
13 signaling [41]. In contrast, in hESCs continuously cultured in 5% O_2, alteration of the Notch pathway seems to be
14 independent of HIF-1α [57]. In our system, Notch1 activation was not likely dependent on HIF-1α and HIF-2α
15 because these proteins did not accumulate in the Hx condition. In contrast, our results indicate that the 5% O_2
16 condition activated Akt and NF-κB signaling (Figure 4), which suggests that these molecules may activate Notch
17 signaling in hADMPCs. NF-κB was previously shown to increase Notch1 activity indirectly by increasing the
18 expression of Notch ligand Jagged1 in HeLa, lymphoma, and myeloma cells [58]. In addition, Akt regulated

Notch1 by increasing Notch1 transcription through the activity of NF-κB in melanoma cells [42]. Further analysis is required to clarify the mechanism underlying this phenomenon.

In conclusion, the 5% oxygen condition conferred a growth advantage through a metabolic shift to glycolysis, improved the proliferation efficiency, prevented the cellular senescence, and maintained the undifferentiated status of hADMPCs. These observations thus provide new regulatory mechanisms for the maintenance of stemness observed in 5% oxygen conditions. In addition, our study sheds new light on the regulation of replicative senescence, which might have impact for quality control of hADMPC preparations used for therapeutic applications.

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Disclosure Statement

1 The authors declare no conflict of interest. No competing financial interests exist.

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