EGFP-LC3 puncta (Figure 4) and (2) 3-MA significantly diminished the formation of GFP-LC3 puncta and keratinocyte differentiation induced by BNIP3 (Figure 5). These findings suggest that BNIP3 in the epidermis induced both conventional and Atg5/Atg7-independent autophagy. Intriguingly, GFP cleaved from GFP-LC3 also accumulates in the *Atg7*-deficient epidermis (Rossiter *et al.*, 2013), thereby demonstrating the existence of an alternative autophagic pathway (Juenemann and Reits, 2012) in the epidermis. Further investigation will be required to determine whether Beclin 1 and Rab9 are indispensable for the BNIP3-induced autophagy and subsequent differentiation of keratinocytes.

In summary, our data reveal that expression of BNIP3 in granular cells induces autophagy and is involved in the terminal differentiation and maintenance of skin epidermis. Studies on the involvement of autophagy in skin epidermis have attracted considerable attention recently. In addition, increasing evidence suggests the involvement of BNIP3 in the differentiation of several cell types, including oligodendrocytes (Itoh *et al.*, 2003), osteoclasts (Knowles and Athanasou, 2008), and chondrocytes (Zhao *et al.*, 2012); however, the precise role of BNIP3 in this process remains to be investigated. Our study thus provides new insights into the functions of BNIP3 in differentiation and homeostasis.

MATERIALS AND METHODS

Histology and immunofluorescent analysis

Samples and embryos were fixed in 4% paraformaldehyde, embedded in optimal cutting temperature compound, frozen, and sectioned at 10 μm. Sections were then either subjected to hematoxylin and eosin staining or immunohistochemical analysis as previously described (Moriyama *et al.*, 2006). Details are described in Supplementary Materials Online.

Cell culture

HPEKs were purchased from CELLnTEC (Bern, Switzerland) and maintained in CnT-57 (CELLnTEC) culture medium according to the manufacturer's protocol. For induction of differentiation, the medium was changed to CnT-02 (CELLnTEC) at confluent monolayers of HPEKs, followed by adding calcium ions to 1.8 mM. The generation of human skin equivalents was performed using CnT-02-3DP culture medium (CELLnTEC) according to the manufacturer's protocol.

Design of artificial microRNAs and plasmid construction

Oligonucleotides targeting a human BNIP3 sequence compatible for use in cloning into BLOCK-iT Pol II miR RNAi expression vectors (Invitrogen, Carlsbad, CA) were obtained using the online tool BLOCK-iT RNAi Designer. The oligonucleotide sequences used in this study are shown in Supplementary Table S1 online. Cloning procedures were performed following the manufacturer's instructions.

Adenovirus and lentivirus infection

Adenoviruses expressing EGFP, Hes1, BNIP3, and miR *BNIP3* were constructed using the ViraPower adenoviral expression system (Invitrogen) according to the manufacturer's protocol. Lentivirus expressing EGFP-LC3 (from Addgene plasmid 21073, Cambridge, MA) and miR *BNIP3* plasmid was constructed and used to infect keratinocytes as previously described (Moriyama *et al.*, 2012; Moriyama *et al.*, 2013).

RNA extraction, complementary DNA generation, and Q-PCR

Total RNA extraction, complementary DNA generation, and Q-PCR analyses were carried out as previously described (Moriyama *et al.*, 2012). Details of the primers used in these experiments are shown in Supplementary Table S2 online.

Western blot analysis

Western blot analysis was performed as previously described (Moriyama *et al.*, 2012; Moriyama *et al.*, 2013). Details are described in Supplementary Materials Online.

ChIP assay

The ChIP assay was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions. Hemagglutinin-tagged Hes1 was immunoprecipitated with rabbit polyclonal antibody against hemagglutinin tag (ab9110, Abcam, Cambridge, MA). Immunoprecipitated DNA was analyzed by Q-PCR. Relative quantification using a standard curve method was performed, and the occupancy level for a specific fragment was defined as the ratio of immunoprecipitated DNA over input DNA. Details of the primers used in these experiments are shown in Supplementary Table S2 online.

Flow cytometry analysis

For autophagy detection, Cyto-ID Autophagy detection kit (Enzo Life Sciences, Plymouth Meeting, PA) was used according to the manufacturer's instructions. Details are described in Supplementary Materials Online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Role of Notch signaling in the maintenance of human mesenchymal stem cells under hypoxic conditions

Hiroyuki Moriyama[1,*], Mariko Moriyama[1,*], Haruki Isshi[1], Shin Ishihara[1], Hanayuki Okura[2], Akihiro Ichinose[3], Toshiyuki Ozawa[4], Akifumi Matsuyama[2] and Takao Hayakawa[1]

[1] Pharmaceutical Research and Technology Institute, Kinki University, Higashi-Osaka, Osaka, Japan.

[2] Platform of therapeutics for Rare Disease and Health Policy, National Institute of Biomedical Innovation, Kobe, Hyogo, Japan.

[3] Department of Plastic Surgery, Kobe University Hospital, Kobe, Hyogo, Japan.

[4] Department of Dermatology, Osaka City University, Graduate School of Medicine, Osaka, Japan.

*These two authors contributed equally to this work.

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Address correspondence to:
Dr. Hiroyuki Moriyama
Pharmaceutical Research and Technology Institute
Kinki University
3-4-1 Kowakae
Higashi-Osaka
Osaka 577-8502
Japan
Tel&Fax: +81-6-4307-4312
Email: moriyama@phar.kindai.ac.jp

Abstract

Human adipose tissue-derived multilineage progenitor cells (hADMPCs) are attractive for cell therapy and tissue engineering because of their multipotency and ease of isolation without serial ethical issues. However, their limited *in vitro* lifespan in culture systems hinders their therapeutic application. Some somatic stem cells including hADMPCs are known to be localized in hypoxic regions; thus, hypoxia may be beneficial for *ex vivo* culture of these stem cells. These cells exhibit a high level of glycolytic metabolism in presence of high oxygen levels and further increase their glycolysis rate under hypoxia. However, the physiological role of glycolytic activation and its regulatory mechanisms are still incompletely understood. Here we show that Notch signaling is required for glycolysis regulation under hypoxic conditions. Our results demonstrate that 5% O_2 dramatically increased the glycolysis rate, improved the proliferation efficiency, prevented senescence, and maintained the multipotency of hADMPCs. Intriguingly, these effects were not mediated by hypoxia-inducible factor (HIF), but rather by the Notch signaling pathway. 5% O_2 significantly increased the level of activated Notch1 and expression of its downstream gene, *HES1*. Furthermore, 5% O_2 markedly increased glucose consumption and lactate production of hADMPCs, which decreased back to normoxic levels upon treatment with a γ-secretase inhibitor. We also found that HES1 was involved in induction of GLUT3, TPI, and PGK1 in addition to reduction of TIGAR and SCO2 expression. These results clearly suggest that Notch signaling regulates glycolysis under hypoxic conditions and thus likely affects the cell lifespan via glycolysis.

Introduction

Human adipose tissue-derived mesenchymal stem cells (MSCs), also referred to as human adipose tissue–derived multilineage progenitor cells (hADMPCs), are multipotent stem cells that can differentiate into various types of cells, including hepatocytes [1], cardiomyoblasts [2], pancreatic cells [3], and neuronal cells [4-6]. They can be easily and safely obtained from lipoaspirate without posing serious ethical issues and can also be expanded *ex vivo* under appropriate culture conditions. Moreover, MSCs, including hADMPCs, have the ability to migrate to injured areas and secrete a wide variety of cytokines and growth factors necessary for tissue regeneration [7-11]. In addition, because of their hypoimmunogenicity and immunomodulatory effects, hADMPCs are good candidates as gene delivery vehicles for therapeutic purposes [12]. Thus, hADMPCs are attractive seeding cells for cell therapy and tissue engineering. However, similar to other somatic stem cells or primary cells, hADMPCs have limited growth potential and ultimately stop proliferation as a result of cellular senescence [13], which hinders their therapeutic application.

Conversely, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are immortal under standard culture conditions. Recently, several groups have reported that these cells greatly rely on glycolysis for energy production even under high-oxygen conditions [14-16]. This phenomenon is known as the Warburg effect and was originally described for cancer cells by Otto Warburg in the 1920s [17]. Although mitochondrial respiration is more efficient than glycolysis in generating ATP (net yield of 30 ATPs vs. 2 ATPs), glycolysis is able to produce ATP considerably faster than mitochondrial respiration as long as glucose supplies are adequate. Thus,

a metabolic shift from mitochondrial respiration to glycolysis would provide a growth advantage for actively proliferating cells. Moreover, Kondoh *et al.* demonstrated that enhanced glycolysis is also involved in cellular immortalization through reduction of intrinsic ROS production [14,18,19]. Because accumulation of intrinsic ROS levels could be a major reason for replicative senescence [20], enhancing glycolysis in cultured cells might improve the quality of the cells by suppressing premature senescence. One candidate method for induction of glycolysis is application of low-oxygen conditions to activate the transcription factor, hypoxia-inducible factor (HIF). HIF-1 is known to increase the expression of most glycolytic enzymes and the glucose transporters GLUT1 and GLUT3 [20]. Thus, several studies have reported that hypoxia is beneficial for the maintenance of hESCs in a pluripotent state [21,22]. Moreover, low oxygen tension has been reported to enhance the generation of induced pluripotent stem cells both from mouse and human primary fibroblasts [23].

Recently, hypoxic culture conditions have also been reported to confer a growth advantage, prevent premature senescence, and maintain undifferentiated states in somatic stem cells, e.g., hematopoietic stem cells (HSCs) [24], neural stem cells [25], and bone marrow-derived MSCs [26]. These stem cells reside in their local microenvironments called the "stem cell niche", where the oxygen tension is relatively low (in the range of 1%–9%). Thus, hypoxic culture may be beneficial to these stem cells with regard to in vitro proliferation, cell survival, and differentiation. Takubo et al. reported that HSCs activated Pdk through HIF1α in hypoxic culture conditions, resulting in maintenance of glycolytic flow and suppression of the influx of glycolytic metabolites into mitochondria, and this glycolytic metabolic state was shown to be indispensable for the maintenance of HSCs [27]. Several studies have reported that MSCs exhibit a high level of glycolytic metabolism in the presence of high oxygen

levels and further increase their rate of glycolysis upon culture under hypoxia [28,29]. However, a relationship between beneficial effects of hypoxic conditions and metabolic status in addition to involvement of HIFs in the metabolic changes has not been investigated in these reports.

In this study, we aimed to investigate the effect of 5% oxygen on hADMPCs. Our results demonstrate that culture under 5% oxygen increased the glycolysis rate, improved the proliferation efficiency, prevented the cellular senescence, and maintained the undifferentiated status of hADMPCs. Intriguingly, these effects were not mediated by HIF, but rather by Notch signaling, an important signaling pathway required for the development of many cell types and maintenance of stem cells [30,31]. 5% oxygen activated Notch signaling, resulting the upregulation of *SLC2A3*, *TPI*, and *PGK1* in addition to the downregulation of *TIGAR* and *SCO2*, which may contribute to the increase in the glycolysis rate. These observations thus provide new regulatory mechanisms for stemness maintenance obtained under 5% oxygen conditions.

Material and Methods

Adipose tissue samples

Subcutaneous adipose tissue samples (10–50 g each) were resected during plastic surgery from 5 female and 2 male patients (age 20–60 years) as discarded tissue. The study protocol was approved by the Review Board for Human Research of Kobe University Graduate School of Medicine Foundation for Biomedical Research and

Innovation, Osaka City University Graduate School of Medicine, and Kinki University Pharmaceutical Research and Technology Institute (reference number: 12-043). Each subject provided signed informed consent.

Cell culture

hADMPCs were isolated as previously reported [32-35] and maintained in a medium containing 60% DMEM-low glucose, 40% MCDB-201 medium (Sigma Aldrich, St. Louis, MO, USA), 1× insulin-transferrin-selenium (Life Technologies, NY, USA), 1 nM dexamethasone (Sigma Aldrich), 100 mM ascorbic acid 2-phosphate (Wako, Osaka, Japan), 10 ng/mL epidermal growth factor (PeproTech, NJ, USA), and 5% fetal bovine serum. The cells were plated to a density of 5×10^3 cells/cm^2 on fibronectin-coated dishes, and the medium was replaced every 2 days. For hypoxic culture, cells were cultured in a gas mixture composed of 90% N_2, 5% CO_2, and 5% O_2. For maintenance of the hypoxic gas mixture, a ProOx C21 carbon dioxide and oxygen controller and a C-Chamber (Biospherix, NY, USA) were used.

Senescence-associated β-galactosidase staining

Cells were fixed with 2% paraformaldehyde/0.2% glutaradehyde for 5 min at room temperature and then washed 2 times with phosphate-buffered saline (PBS). The cells were then incubated overnight at 37 °C with fresh senescence-associated β-galactosidase (SA-β-Gal) chromogenic substrate solution (1 mg/mL Bluo-gal (Life

Technologies), 40 mM citric acid (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$).

Measurement of reactive oxygen species production

Cells were harvested and incubated with 10 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA). The amount of intracellular ROS production was proportional to the green fluorescence, as analyzed using a Guava EasyCyte 8HT flow cytometer (Millipore, MA, USA) using an argon laser at 488 nm and a 525/30 nm band pass filter, and dead cells were excluded using the Live/Dead Fixable Far Red Dead Cell Stain Kit (Life Technologies).

EdU proliferation assay

For assessment of cell proliferation, hADMPCs were seeded on a fibronectin-coated 6-well plate at a density of 5 × 10^3 cells/cm^2 and cultured for 3 days. Cell proliferation was detected by incorporating of 5-ethynyl-2'-deoxyuridine (EdU) and using the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Life Technologies). Briefly, according to the manufacturer's protocol, cells were incubated with 10 μM EdU for 2 h before fixation, permeabilized, and stained with EdU. EdU-positive cells were then analyzed using the 488 nm laser of a Guava EasyCyte 8HT flow cytometer (Millipore).

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 $1x10^6$ 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 ΔΔCt method and the most reliable internal control gene

was determined using geNorm Software (http://medgen.ugent.be/~jvdesomp/genorm/). Details of the primers used in these experiments are available upon request.

Western blot analysis

Whole cell extracts were prepared by washing cells with ice-cold phosphate-buffered saline (PBS) and lysing them with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific Pierce, IL, USA) according to the manufacturer' s instructions. Nuclear and cytosolic extracts were prepared as follows. Cells were washed with 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% glycerol, 5 mM EDTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and protease inhibitor cocktail). Then, insoluble nuclei were isolated by centrifugation and lysed with lysis buffer containing 0.5% SDS. Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore), and probed with antibodies against cleaved Notch1 (#2421, Cell Signaling Technology, MA, USA), HIF-1α (#610959, BD Bioscience, CA, USA), hypoxia inducible factor 2α (MAB3472, Millipore), Akt (#9272, Cell Signaling Technology), and phospho Akt (Ser473) (#4060, Cell Signaling Technology). Horseradish peroxidase (HRP)-conjugated anti-mouse or -rabbit IgG antibody (Cell Signaling Technology) was used as a secondary antibody and immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP substrate (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 × *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.

Hypoxic culture maintains some mesenchymal stem cell properties and increases differentiation

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

Hypoxic culture condition activates Notch signaling

To reveal the molecular mechanism by which the Hx culture condition increased the proliferative capacity and maintaining the stem cell properties of hADMPCs, we next examined Notch signaling, which is required for 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