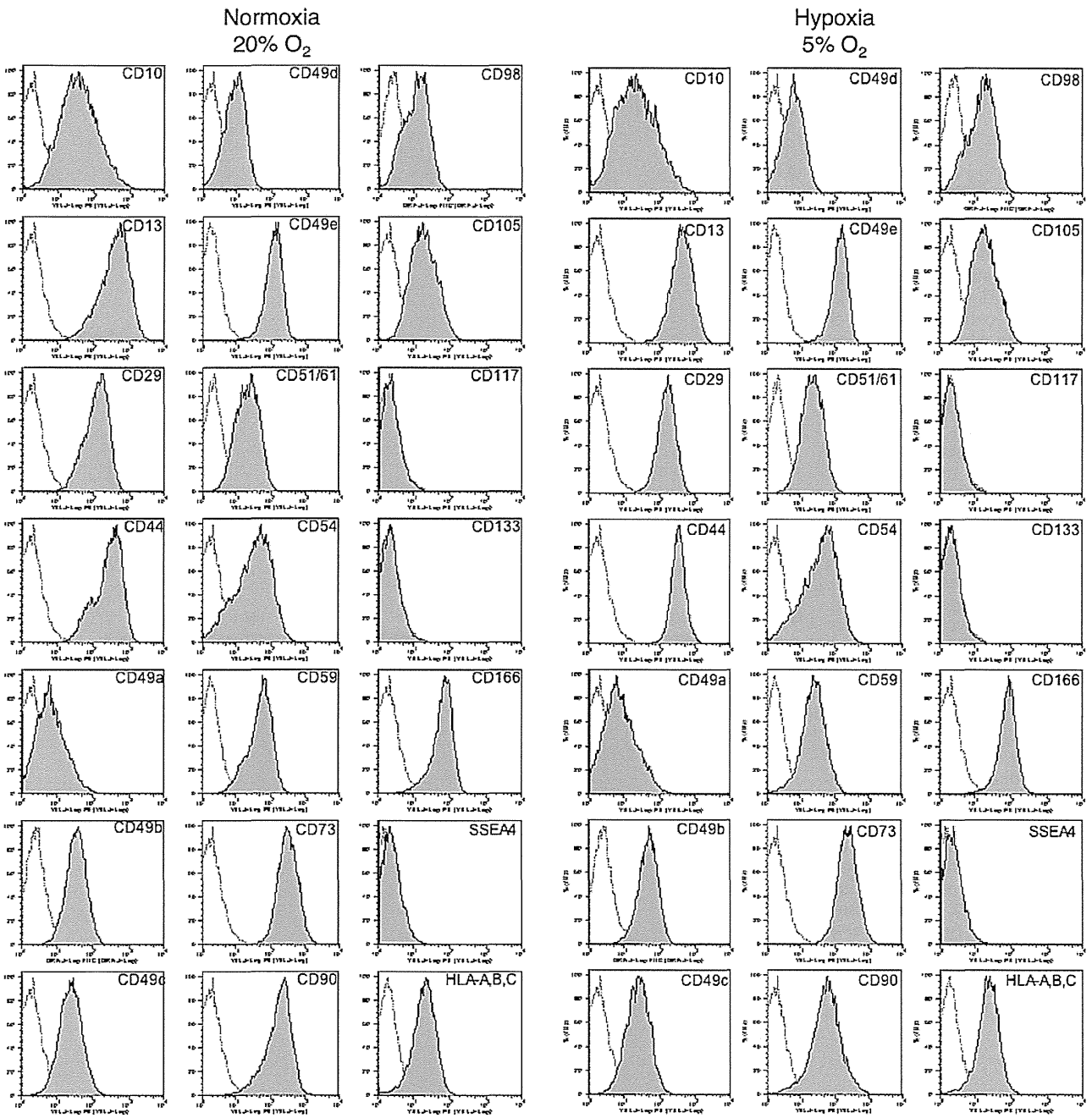

FIG. 2. Hypoxic culture maintains mesenchymal stem cell properties. hADMPCs cultured under normoxia (20% O_2) or hypoxia (5% O_2) were labeled with antibodies against the indicated antigens and analyzed by flow cytometry. Representative histograms are shown. The respective isotype control is shown as a *gray line*.

the Hx culture condition (Fig. 4B). Administration of the γ-secretase inhibitor DAPT at 1 μM, which was sufficient to inhibit the proteolytic cleavage of NOTCH1 (Fig. 4A), decreased the Hx-induced expression of HES1 at both mRNA and protein levels (Fig. 4B, 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 crosstalk in hypoxic cells [38–41]. 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 (Fig. 4D). However, we did not detect any HIF-2α expression even in the presence of cobalt chloride (Fig. 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.

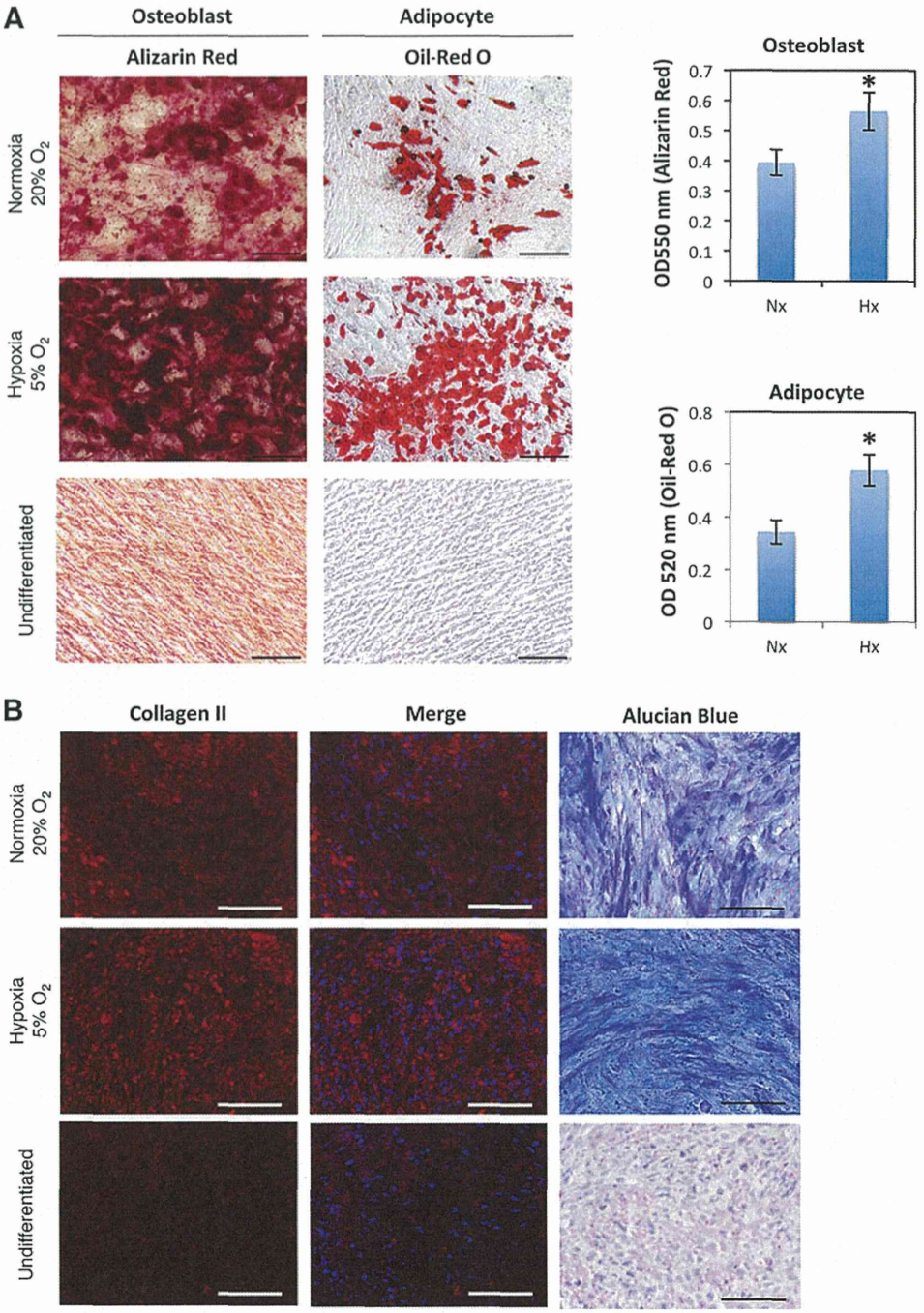

FIG. 3. Hypoxic culture enhances stem cell properties. hADMPCs were expanded under normoxic and hypoxic conditions. **(A)** Normoxic (20% O_2) and hypoxic (5% O_2) cells at passage 8 were induced for 3 weeks to differentiate into osteoblasts and adipocytes and stained with Alizarin Red and Oil-Red O, respectively. The stained dye was extracted, and OD values were measured and plotted as the means of three independent experiments ± SD. $*P < 0.05$. Scale bars, 200 μm. **(B)** Normoxic (20% O_2) and hypoxic (5% O_2) cells at passage 8 were induced for 3 weeks to differentiate to chondrocytes, and immunofluorescent analysis of collagen II (*red*) and Alucian Blue staining were performed. The *blue* signals indicate nuclear staining. Scale bars, 100 μm. Non-induced control cultures in growth medium without adipogenic, osteogenic or chondrogenic differentiation stimuli are shown (Undifferentiated). Color images available online at www.liebertpub.com/scd

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 [42,43]. In addition, hypoxic conditions have been shown to inhibit p53 activity [44], and crosstalk between these pathways and Notch signaling has also been demonstrated [41,45–47]. As shown in Fig. 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 (Fig. 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 (Fig. 4H, 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

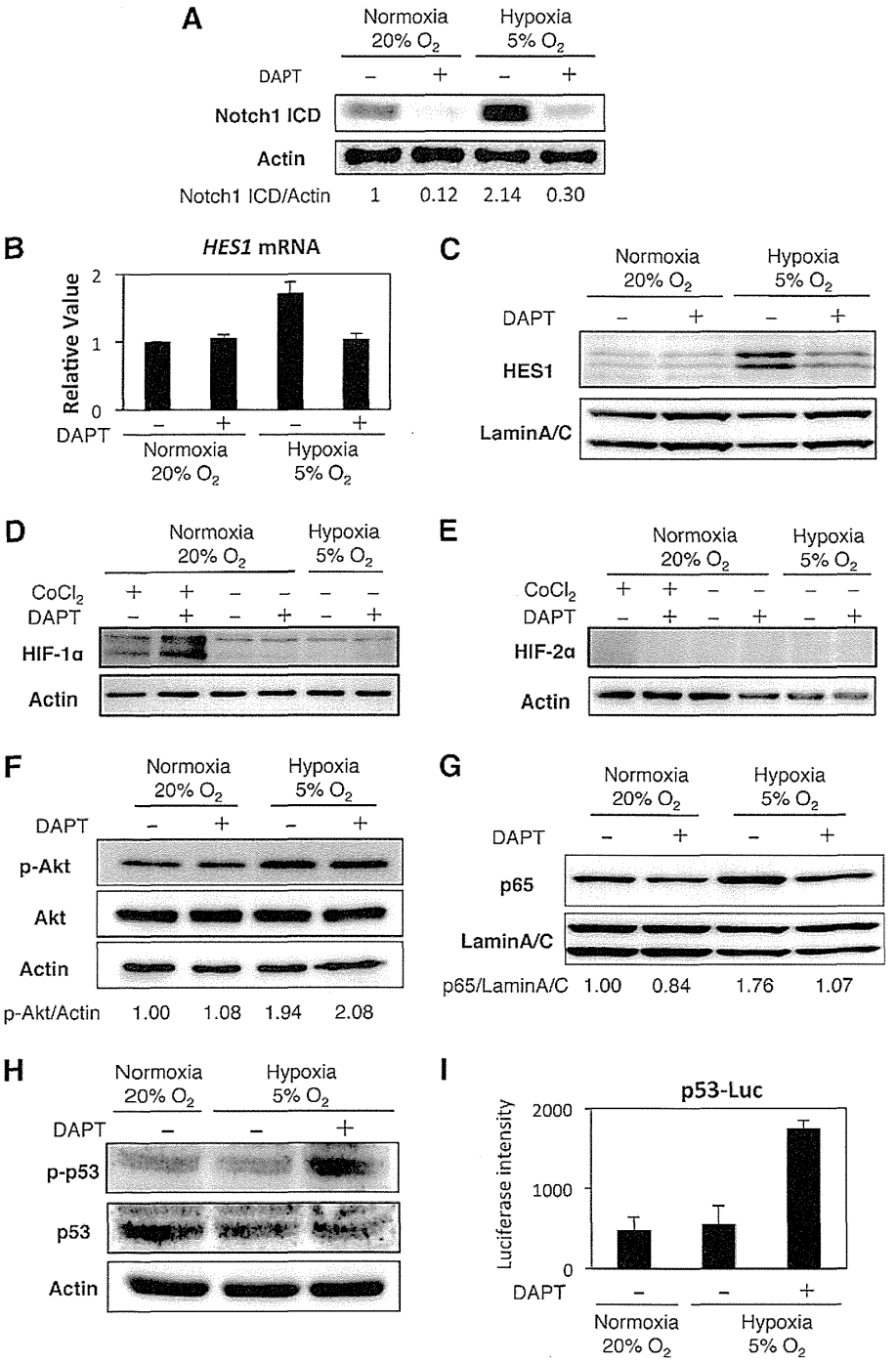

FIG. 4. Hypoxic culture condition activates Notch signaling but not HIF proteins. hADMPCs were expanded under normoxic (20% O_2) and hypoxic (5% O_2) conditions. DAPT (1 μM) was added to inhibit Notch signaling. **(A)** Western blot analysis of intracellular domain of Notch1 (Notch1 ICD) expression. Actin served as the loading control. Numbers below blots indicate relative band intensities as determined by ImageJ software. **(B)** Q-PCR analysis of *HES1*. Each expression value was calculated with the ΔΔCt method using *UBE2D2* as an internal control. **(C)** Western blot analysis of HES1 in nuclear fractions of hADMPCs. Lamin A/C served as the loading control. **(D, E)** Western blot analysis of HIF-1α **(D)** and HIF-2α **(E)**. Cobalt chloride ($CoCl_2$) was added at a concentration of 100 μM to stabilize HIF proteins (positive control). **(F)** Western blot analysis of phosphorylated Akt (p-Akt) and Akt. Actin served as the loading control. Numbers below blots indicate relative band intensities as determined by ImageJ software. **(G)** Western blot analysis of nuclear localization of p65. Lamin A/C served as the loading control. Numbers below blots indicate relative band intensities as determined by ImageJ software. **(H)** Western blot analysis of phosphorylated p53 (p-p53) and p53. Actin served as the loading control. **(I)** Activity of p53 was measured by the p53-luciferase reporter assay. Relative luciferase activity was determined from three independent experiments and normalized to pGL4.74 activity.

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 (Fig. 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 (Fig. 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 differentiation into all lineages as described in Fig. 3, whereas application of a Notch inhibitor significantly decreased the differentiation capacity to all lineages (Fig. 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

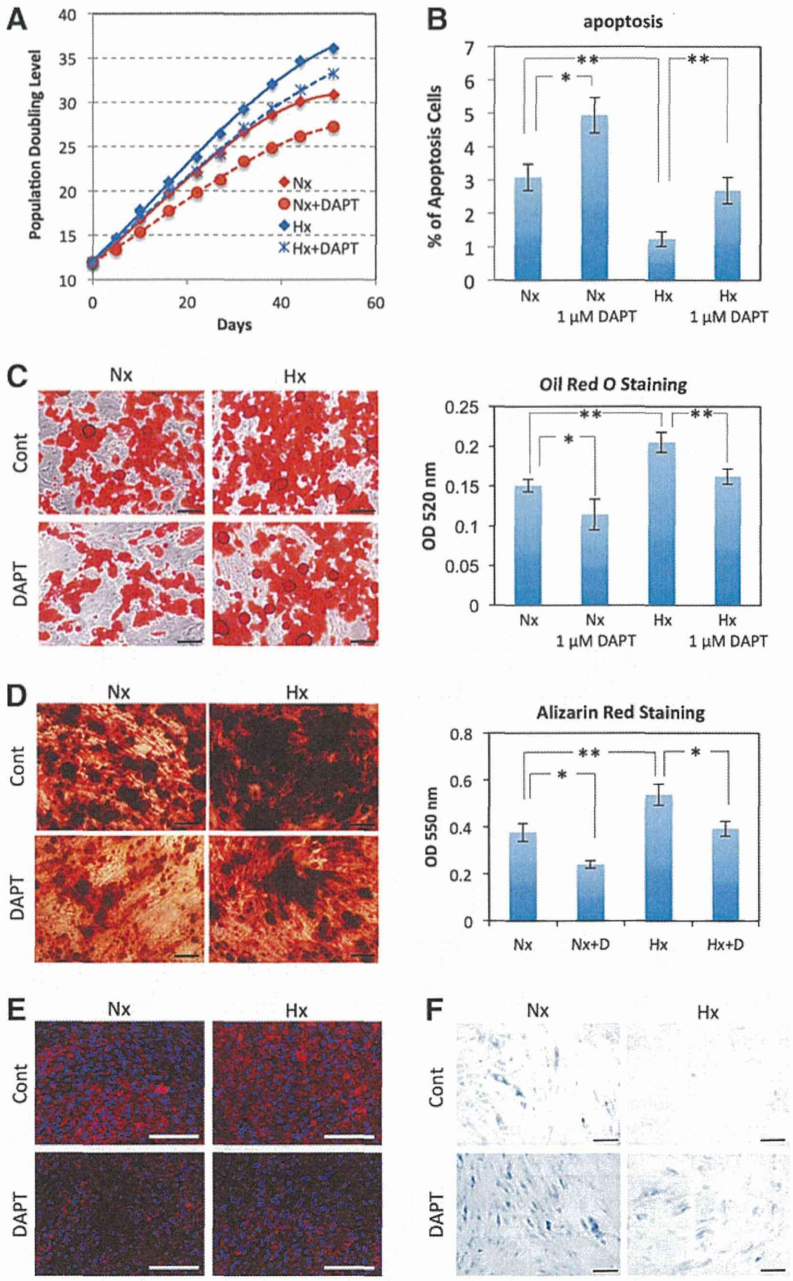

FIG. 5. Notch signaling is indispensable for acquisition of the advantageous properties of hADMPCs. hADMPCs were expanded under normoxic (20% O_2; Nx) and hypoxic (5% O_2; Hx) conditions. DAPT (1 μM) was added to inhibit Notch signaling. **(A)** Growth profiles of hADMPCs under Nx (*red*) and Hx (*blue*) conditions. *Solid lines* represent control cells, and *dotted lines* represent DAPT-treated cells. The number of population doublings was calculated based on the total cell number at each passage. **(B)** Percentages of apoptotic cells with sub-G1 DNA. Results are presented as the mean of three independent experiments ± SD. **(C, D)** hADMPCs at passage 8 were induced for 3 weeks to differentiate into adipocytes **(C)** and osteoblasts **(D)** and stained with Oil Red O and Alizarin Red, respectively. The stained dye was extracted, and OD values were measured and plotted as the means of three independent experiments ± SD. **(E)** hADMPCs at passage 8 were induced for 3 weeks to differentiate into chondrocytes, and an immunofluorescent analysis of collagen II (*red*) was performed. The *blue* signals indicate nuclear staining. **(F)** hADMPCs were stained with SA-β-gal. $*P < 0.05$ and $**P < 0.01$ indicate significant differences (independent *t*-test) between Nx and Hx. Scale bars; 100 μm. Color images available online at www.liebertpub.com/scd

culture conditions, suggesting that the suppression of replicative senescence observed in the Hx condition is mediated by Notch signaling (Fig. 5F).

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 (Fig. 1F). In addition, the relationship between Notch signaling and glycolysis has been recently established [48,49]. 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 Fig. 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 (Fig. 6A). To identify the genes responsible for the glycolytic change, we performed a Q-PCR analysis. As shown in Fig. 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 (Fig. 6E). In addition, SCO2, a positive

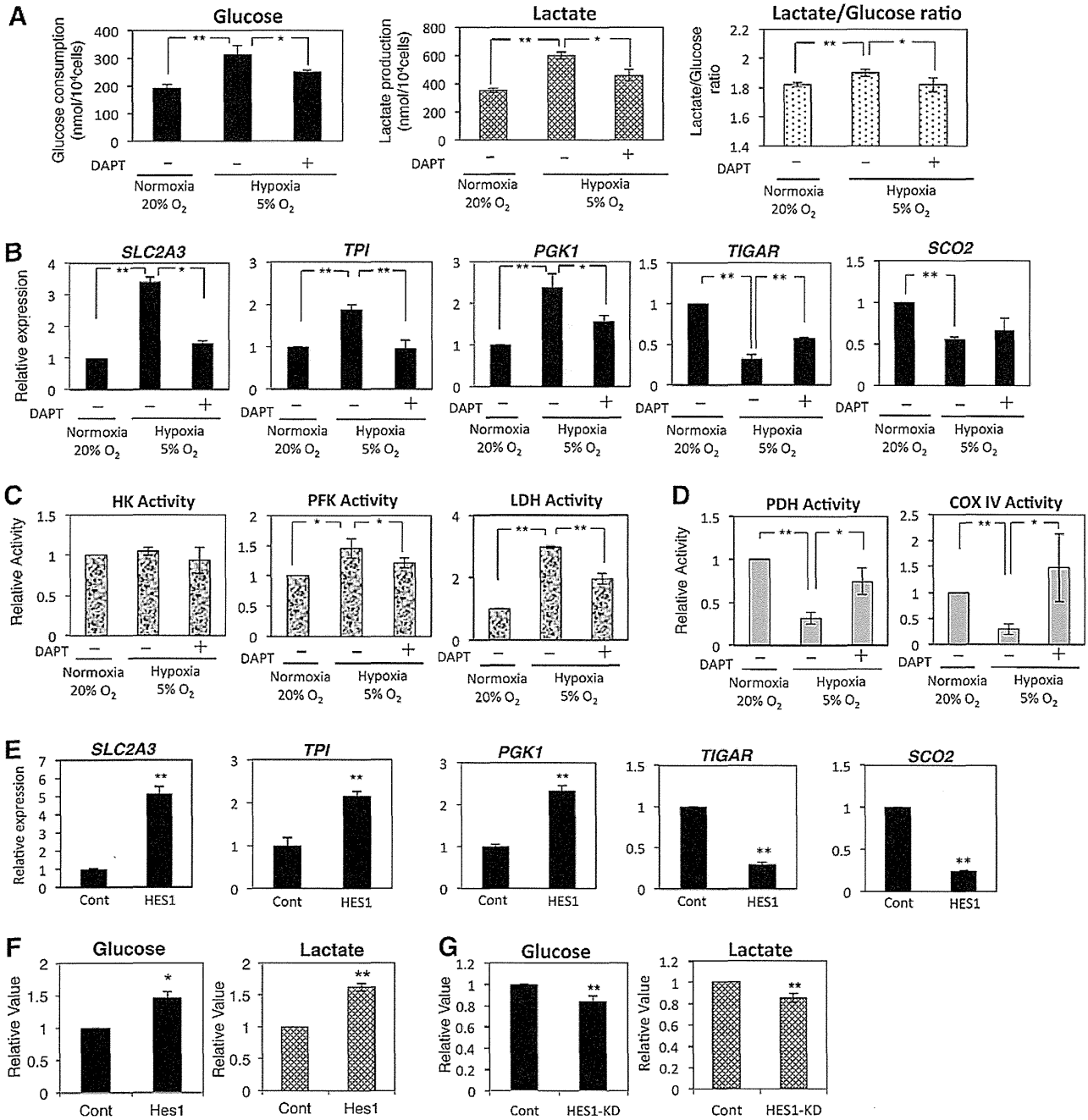

FIG. 6. Glycolysis is enhanced under 5% oxygen through Notch signaling. **(A–D)** hADMPCs were expanded under normoxic (20% O_2) and hypoxic (5% O_2) conditions. DAPT (1 μM) was added to inhibit Notch signaling. **(A)** Glucose consumption and lactate production of hADMPCs were measured and plotted as the means of three independent experiments ± SD. **(B)** Relative mRNA expression of *SLC2A3*, *TPI*, *PGK1*, *TIGAR*, and *SCO2* in hADMPCs. Each expression value was calculated with the ΔΔCt method using *UBE2D2* as an internal control. **(C, D)** Hexokinase (HK), phosphofructokinase (PFK), lactate dehydrogenase (LDH) **(C)**, pyruvate dehydrogenase (PDH), and Complex IV (Cox IV) **(D)** activities were measured and the value of relative activity was plotted as the means of three independent experiments ± SD. **(E, F)** hADMPCs were transduced with either mock (Cont) or HES1 and then cultured for 3 days. **(E)** Relative mRNA expression of *SLC2A3*, *TPI*, *PGK1*, *TIGAR*, and *SCO2* in hADMPCs. Each expression value was calculated with the ΔΔCt method using *UBE2D2* as an internal control. **(F)** Glucose consumption and lactate production of hADMPCs were measured and plotted as the means of three independent experiments ± SD. **(G)** hADMPCs were transduced with either scrambled control RNAi (Cont) or RNAi against HES1 (HES1-KD), and then cultured for 3 days. Glucose consumption and lactate production of hADMPCs were measured and plotted as the means of three independent experiments ± SD. $^{**}P < 0.01$. $^{*}0.01 < P < 0.05$.

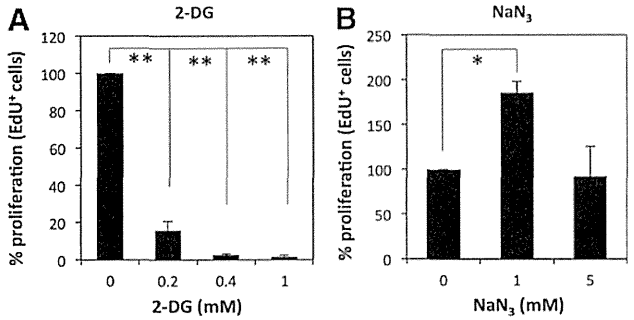

FIG. 7. Glycolysis supports proliferation of hADMPCs. hADMPCs were treated with 0, 0.2, 0.4, and 1 mM 2-deoxy-D-glucose (2-DG) **(A)** or 0, 1, and 5 mM sodium azide (NaN_3) **(B)** for 24 h. Cells were then allowed to incorporate EdU for 2 h, and the EdU-positive cells were analyzed by flow cytometry. The percentages for the 0 mM control were plotted as the means of three independent experiments ± SD. $*P<0.05$; $**P<0.01$.

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 (Fig. 6B). Adenoviral expression of Hes1 dramatically reduced *SCO2* and *TIGAR* expression (Fig. 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, PFK and LDH were activated in 5% oxygen condition, which was attenuated by Notch inhibition (Fig. 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 (Fig. 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 (Fig. 6F). In contrast, HES1 knockdown by adenoviral transduction of *HES1* RNAi resulted in a significant reduction of glycolytic flux (Fig. 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 a low concentration of 0.2 mM (Fig. 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 (Fig. 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 (Fig. 1). Moreover, 5% O_2 improved the differentiation of hADMPCs without affecting the expression of their cell surface markers (Figs. 2 and 3). Welford et al. reported that HIF-1α delays premature senescence of mouse embryonic fibroblasts under hypoxic conditions (2% O_2) [50]. Tsai et al. reported that hypoxia (1% O_2) inhibits senescence and maintains MSC 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 [38]. 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 (Fig. 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 (Figs. 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 (Fig. 6A) and knockdown of HES1 (Fig. 6G). Previously, it has been reported that Notch signaling promotes glycolysis by activating the PI(3)K-Akt pathway [48,49]. However, our results indicate that Akt signaling was not activated by Notch signaling, because DAPT did not attenuate hypoxia-induced Akt phosphorylation (Fig. 4F). Although Akt is unlikely to be regulated by Notch signaling in hADMPCs, it is obvious 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 [48,51]. Zhou et al. demonstrated that hairy, a basic helix-loop-helix transcriptional repressor regulated by Notch signaling, was upregulated and genes encoding metabolic enzymes, including TCA cycle enzymes and respiratory chain complexes, were downregulated in hypoxia-tolerant flies. Intriguingly, they also found that hairy-binding elements were present in the regulatory region of the downregulated metabolic genes. Their work, thus, provides new evidence that hairy acts as a metabolic switch [51]. 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 [48]. Consistent with their reports, our findings that Notch signaling promoted activity of some glycolysis enzymes and inhibited mitochondrial activity

(Fig. 6) also suggest that Notch signaling functioned as a metabolic switch. While our data showed that Notch inhibition by DAPT resulted in reduced glycolysis (Fig. 6A–C), induction of mitochondrial function (Fig. 6D) and activation of p53 (Fig. 4H, 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 [48]. As shown in Fig. 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 (Fig. 6A). In addition, it was reported that hMSCs showed a higher glycolytic rate than primary human fibroblast [52]. 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 (Fig. 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 (Fig. 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 [53]. 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 [53]. 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 an NF-κB site in the promoter [54]. 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). Since NF-κB is likely to be one of the responsible signals for hypoxic-induced glycolysis [53], further analysis will be required to determine the mechanism by which NF-κB signaling is induced by Notch signaling. In addition, 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 (Fig. 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 (Fig. 6C, D). Adenoviral expression of Hes1 dramatically reduced *SCO2* and *TIGAR* expression (Fig. 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 [46]. Moreover, Kim et al. reported that NICD1 inhibits p53 phosphorylation and represses p53 transactivation by interacting with p53 [47]. In addition, DAPT treatment resulted in the enhancement of p53 activity in the hypoxic conditions (Fig. 4H, 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 (Fig. 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 [18,55]. 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 (Fig. 1). Since 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 levels through enhanced glycolysis by Notch signaling. Our data indicate that aerobic glycolysis is utilized for proliferation of hADMPCs, because the glycolytic inhibitor 2-DG attenuates the proliferation rate of hADMPCs (Fig. 7A). Intriguingly, the aerobic respiration block by NaN_3 did not decrease the proliferation; rather, it increased proliferation at a low concentration (Fig. 7B), which may support our data indicating that the metabolic switch from mitochondrial respiration to glycolysis provides a growth advantage to hADMPCs. However, the question of whether the enhanced glycolysis really contributes to the prolonged lifespan in hADMPCs remains to be determined in this study.

In the current study, the molecular mechanism for how Notch signaling is activated in 5% O_2 conditions was explored. It has been reported that Notch1 activity is influenced by oxygen concentration [40,41,56]. In melanoma cells, hypoxia (2% O_2) resulted in increased expression of Notch1 by HIF-1α and also by Akt through NF-κB activity [41]. Similarly, in hypoxic breast cancer cells, Notch ligand JAG2 was shown to be transcriptionally activated by hypoxia (1% O_2) in an HIF-1α-dependent manner, resulting in an elevation of Notch signaling [40]. In contrast, in hESCs continuously cultured in 5% O_2, alteration of the Notch pathway seems to be independent of HIF-1α [56]. In our system, Notch1 activation was not likely dependent on HIF-1α and HIF-2α, because these proteins did not accumulate in the Hx condition. In contrast, our results indicate that the 5% O_2 condition activated Akt and NF-κB signaling (Fig. 4), which suggests that these molecules may activate Notch signaling in hADMPCs. NF-κB was previously shown to

increase Notch1 activity indirectly by increasing the expression of Notch ligand Jagged1 in HeLa, lymphoma, and myeloma cells [57]. In addition, Akt regulated Notch1 by increasing Notch1 transcription through the activity of NF-κB in melanoma cells [41]. 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 an impact for quality control of hADMPC preparations used for therapeutic applications.

Acknowledgments

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Author Disclosure Statement

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

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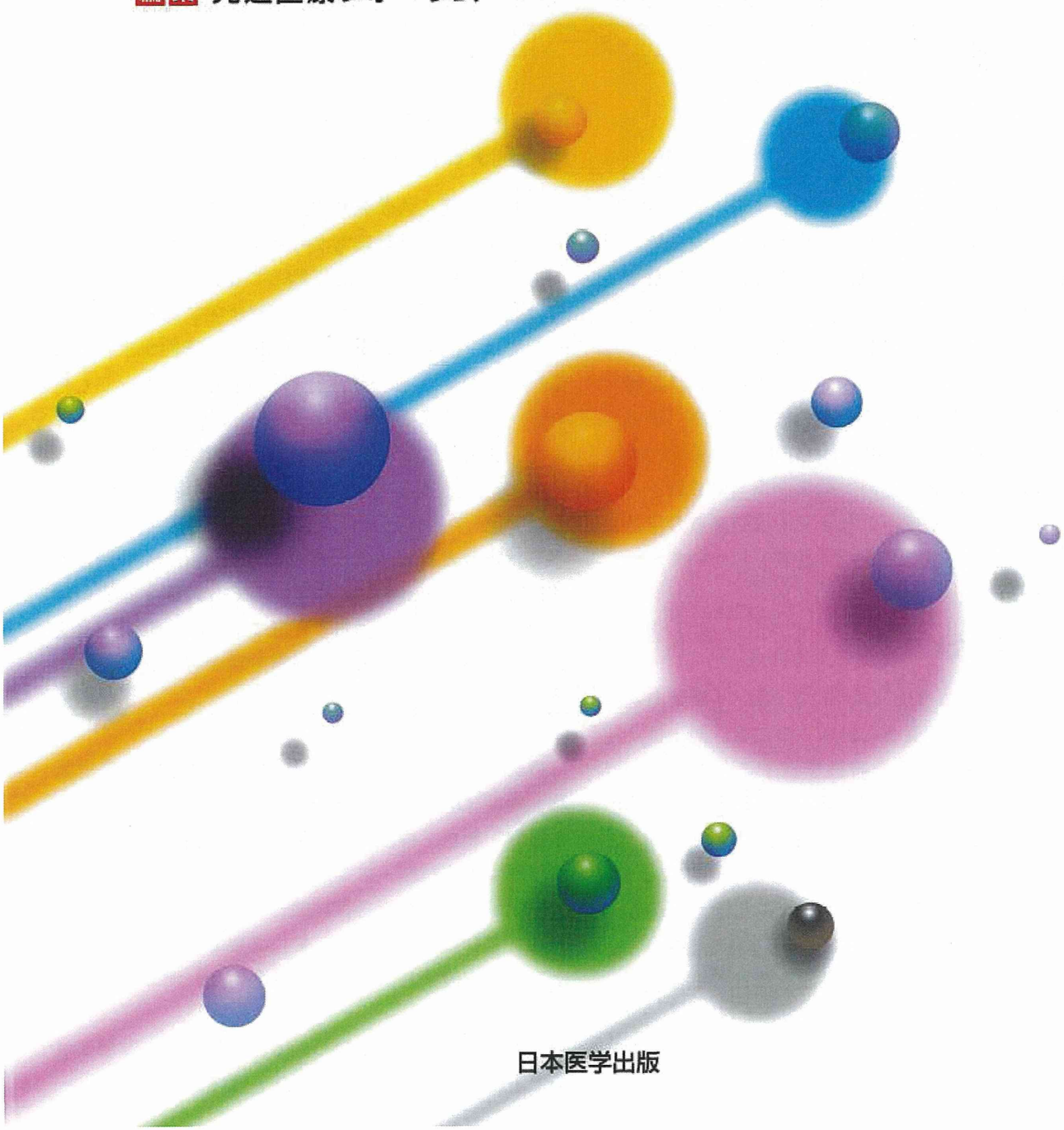
先進医療 NAVIGATOR II

再生医療・がん領域の実用化へのTOPICS

編集 先進医療フォーラム



日本医学出版

2 細胞医療での申請にあたっての注意点
—品質の観点から—

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はじめに

再生医療製品においても品質，有効性，安全性の3つが確保されて初めて医療として成立する。有効性と安全性は臨床試験でしか評価ができないが（安全性の一部は非臨床試験で評価する），品質は臨床試験（ヒト幹細胞臨床研究あるいは治験）前に評価が可能であるからこそ，品質の確保は重要である。試験物，あるいは製品の品質管理を行わなくてはならないのは，研究開発段階での品質の役割としての被験者保護（患者保護）の観点（倫理的妥当性）からの品質確保のためであり，臨床試験の質を高め，結果を正当に評価するためでもある。本稿では，これまでの細胞医療の申請においておざなりにされてきた「品質」について解説することとした。

品質の確保のために

「品質」の定義は，ICH-Q6A によれば，「原薬あるいは製剤の意図した用途への適切さのこと。同一性，含量，物質の純度のような特性を指すこともある。」とされる。承認段階での品質が保証されている状態を，平易に述べれば，「いつ・誰が・どこで・作っても，同じ品質のものをつくれる仕組みができていること」といえる。「同じ品質のもの」であることは，製造方法と最終製品の規格で管理・保証する。「いつ・誰が・どこで」に関しては，何らかの制限を設けて管理する必要があり，主に GMP で確認することとなる。

ヒト幹細胞臨床研究あるいは治験段階から臨床展開までの品質保証

再生医療製品の研究開発にあたって，ヒト幹細胞臨床研究あるいは治験段階から臨床展開までの品質保証の方策について述べる。当然のこととして，開発段階では製品の品質管理も試験・研究状態にある。品質に関しては，開発期間中通して品質の一貫性が求められる。ここでいう「一貫性」とは，「違いがあっても良いが，どこが違うのかわかっている状態」と捉えればよい。開発後期である第Ⅲ相あるいは検証型治験においては市販品との「同等性」が要求される。「同等性」とは，「科学的に有意差が認められず，同等と判断しうる状態」と捉える。一貫性は biocomparability，同等性は biocompatibility と理解しても大きな間違いはないだろう。これら品質を保証する手段として，最終製品の品質管理と原材料の品質管理が行われる。

最終製品の品質管理

最終製品について，細胞数並びに生存率・確認試験・純度試験・細胞由来の目的外生理活性物質に関する試験・製造工程由来不純物試験・無菌試験・マイコプラズマ否定試験・エンドトキシン試験・効能試験・力価試験・力学的適合性試験といった一般的な品質管理項目及び試験を参考として，必要で適切な規格及び試験方法を設定し，その根拠を明らかにすることが望ましい。

細胞数並びに生存率については，細胞の生存率が低いことによる有効性の減弱を阻止するという観点と，

死滅細胞は血栓形成促進傾向にあること等による安全性の観点から議論される。細胞数として通知上は記載されているが，投与時に細胞懸濁液として投与する場合，その濃度についての評価が必須である。なんとなれば，細胞濃度が濃すぎると塞栓症の危険性が上昇すると想定され，安全性と有効性を支える品質の担保として重要な評価項目となるからである。これまでの経験では，生存率は70%以上として定義し，実測値は90%以上，という申請が多い。

確認試験とは，目的とする細胞・組織の形態学的特徴，生化学的指標，免疫学的指標，特徴的産生物質その他適切な遺伝型あるいは表現型のうち，重要細胞特性指標を選択して，目的とする細胞であることを確認することである。「目的とする」細胞・組織であると強調されている通り，確認試験で不純物としての夾雑細胞については言及されていない。この点が，純度試験との違いである。ただし，目的である細胞として単に間葉系幹細胞としての規格設定では十分とはいえない。なんとなれば，品質項目は，安全性と有効性を担保するために確認する項目であるからである。間葉系幹細胞であれば，紡錘状（spindle shape）の付着細胞であり，免疫学的にはCD105/CD166等が陽性で，CD45等が陰性として定義されよう。また，MHC class Ⅱの発現を認めないという品質指標も想定される。確認試験には，「細胞そのものを同定するための指標（CD105陽性等）」と，「安全性の観点から期待される指標（MHC class Ⅱ陰性等）」，case-by-caseであり常に頭を悩ませる「有効性を期待させる指標」が検討されるべきである。

細胞の純度試験では，目的細胞以外の未分化細胞，異常増殖細胞，形質転換細胞の有無や混入細胞の有無等の細胞の純度について，目的とする細胞・組織の由来，培養条件等の製造工程，中間製品の品質管理等を勘案し，試験項目，試験方法及び判定基準を示すこととなっている。特に多能性幹細胞由来細胞製剤にあっては，分化抵抗性多能性幹細胞の残存が議論されることとなり，その残存比率の評価法と規格値の設定が必須である。低分子化合物での純度試験と異なり，すべての目的以外の細胞を同定することは困難であり，目的細胞以外の細胞による毒性の発揮などを非臨床安全性試験で検証したうえで，marginをかけたうえでの規格値設定とならざるを得ない。非臨床試験でのワーストケースを活用し，投与用量に可能な範囲で非線形性をもたせた過剰用量にて得られた安全性試験で確認することが現実的である。

細胞由来の目的外生理活性物質に関する試験は，確認試験での目的生理活性物質評価と相対するものである。もし，細胞由来の各種目的外生理活性物質のうち，製品中での存在量如何で患者に安全性上の重大な影響を及ぼす可能性が明らかに想定される場合には，適切な許容量限度試験を設定することとなっている。「明らかに想定される」という通知上の記載に行間を読んでいただきたい。細胞特性によってはこれら試験が求められることなるが，非臨床試験で安全性にて懸念すべき事項が見い出されなければ検討する必要はないのではないかと考えている。

製造工程由来不純物試験については，通知に記載の通り，原材料に存在するかまたは製造過程で非細胞成分，培地成分，資材，試薬等に由来し，製品中に混入物，残留物，または新たな生成物，分解物等として存在する可能性があるもので，かつ，品質及び安全性の面からみて「望ましくない物質等」については，当該物質の除去に関するプロセス評価や当該物質に対する工程内管理試験の結果を考慮してその存在を否定するか，または適切な試験を設定して存在許容量を規定することとなっている。医薬品等を細胞製造工程で使用し，その残存が想定される場合であっても，使用した資材のすべてが最終製品に残存している（全く洗浄除去されていない）と仮定しても，1回臨床投与量よりも少ない場合には，議論したうえで品質規格として設定しないという考えもあろう。抗生物質の残存に関しては，多くの場合この手法が活用できる。

無菌試験・マイコプラズマ否定試験およびエンドトキシン試験にあっては，局法に基づいて行うのが望ましい。ただし，局法と同等であると確認された試験法あるいは自ら局法と同等性を確認した試験法であれば，局法でなくとも品質管理に用いることが可能である。

効能試験・力価試験・力学的適合性試験については，細胞製剤の特性を考慮したうえでいずれかを選択すればよい。例えば，胚性幹細胞から肝細胞を再生して投与し，低アルブミン血症の改善を期待する場合，アルブミンを産生分泌する程度を品質管理項目とすればよい。細胞・組織から分泌される特定の生理活性物質の

分泌が当該ヒト体性幹細胞加工医薬品等の効能または効果の本質である場合(例えばインスリン分泌細胞等)には，その目的としている必要な効果を発揮することを示すために，当該生理活性物質に関する検査項目及び規格を設定すべきである。再生軟骨細胞組織のように力学的強度をその製品特性として期待される場合には，適用部位を考慮した力学的適合性及び耐久性を確認するための規格を設定することとなる。

原材料の品質管理

原材料及び製造関連物質については，原材料となるヒト細胞・組織と，目的とする細胞・組織以外の原材料及び製造関連物質とに分けられる。原料が受け入れから出荷まで一貫して存在するものであって，材料がそこに振りかけられ洗浄されるというイメージが理解しやすい。

原材料となるヒト細胞・組織については，起源及び由来，選択理由，原材料となる細胞・組織の特性と適格性に関しては，出発原材料となる細胞について，申請者らの研究成果を踏まえ議論すればよい。出発細胞・組織そのものに重点が置かれた記載となるべきであり，原材料として用いられる細胞・組織について，当該細胞・組織を原材料として選択した理由を説明することとは，例えば分化誘導における優位性など *ex vivo* での細胞特性や，細胞製剤投与後の活性などに重点が置かれる。iPS 細胞のように Master Cell Bank を用いる場合には，当該 iPS 細胞ラインの説明が適切である。

目的とする細胞・組織以外の原材料及び製造関連物質とは，培地であったりサイトカインであったり，場合によってはフィーダー細胞もこれに該当する。目的とする細胞・組織以外の原材料及び製造関連物質を明らかにし，その適格性を示すとともに，必要に応じて規格を設定し，適切な品質管理を行うことが必要であると記載されている。

製造工程の項には，受け入れ検査，細菌，真菌及びウイルス等の不活化・除去，組織の細切，細胞の分離，特定細胞の単離等，最終製品の構成要素となる細胞の作成，細胞株の樹立と使用，細胞のバンク化，製造工程中の取り違え及びクロスコンタミネーション防止対策の各章項目がある。これらは，GMP として管理される品質に深く関係し，すべて記載され管理されるべき項目である。

再生医療製品における GMP

GMP とは，Good Manufacturing Practice であり製造管理および品質管理に関する基準である。

GMP の 3 原則は，

1. 間違い防止…人為的な誤りを最小限にする
2. 汚染防止…汚染・品質低下を防止する
3. 品質保証システム…高い品質を保証するシステムを設計する

であり，実行して記録に残すことが重要である。

GMP ではハードウエアとソフトウエアの両立が必要で，ハードとしての施設・設備・機器と，ソフトとしての文書・製造・試験方法・清掃・組織・教育訓練を両輪として初めて成り立つ品質保証システムであるといえる。

GMP ではルールを決めることが肝心である。ルールを決めることとは，それを基準書，標準書，手順書などといった文書体系に落とし込む作業であり，ルールを決め（文書化），ルール通りに実行し（記録化），チェックし（評価・検討），改善する（ルール見直し）というサイクルで品質として作りこんでいく作業に他ならない。サイクルを回して品質を作りこむこととは，換言すれば GMP 管理程度には試験の段階に応じて強弱があって良いということを示す。製造の GMP は開発段階や工程の重要度に応じて使い分けることとすれば，Ⅰ相試験では「SOP（Standard Operation Procedure：標準作業手順書）があり，記録を保存する」で十分だが，第Ⅱ相試験では「SOP があり，工程，作業，設備が評価され，記録を確認し，保存する」ことが求められる。第Ⅲ相になれば，製造販売承認後と同等性が求められるため，「SOP があり，工程，作業のバリデーションが実施・記録され，品質保証部門がその記録を承認し，保存する」こととなろう。SOP は，あらゆる作業に策定が求められる文書である。SOP に求められる要素は，それがどのような作業であれ，わかりやすく，必要なことはすべて書かれていること。作業ごとにかつすべての作業に存在しすぐ見ることができ，そして最新であることである。これらを念頭に，SOP 文書体系の構築をされたい。

おわりに

これまでの細胞医療の申請においておざなりにされてきた「品質」について解説した。GMP管理程度には試験の段階に応じて強弱があって良いということは，これから細胞医療の申請を行う研究者にとっては，心強く，これまでに申請の経験のある研究者にとっても励みになる。細胞医療の裾野が広がり，細胞医療水準もまた向上させること，切にお願いしたい。

XZX

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本書のポイント

●なぜ今再生医療新法ができたのか？
＞再生医療関連法の成立過程や各法規の経緯および既存法規との違いを解説！
●再生医療にはどのような規制があるのか知りたい。
＞再生医療新法や医薬品医療機器等法や重要な部分であるGCTP省令・構造施設の規制について解説
●現状での再生医療の開発動向や開発の際の注意点が知りたい。
＞自家培養軟骨の開発事例を紹介し、培養用加工施設の例や交叉汚染防止の注意点・取り組みにも言及
☆その他にも以下ポイントを解説！

◎再生医療の国際的な開発ガイドラインの現状と各国際標準化における状況(ISO/TC150、ISO/TC198)
◎欧米における細胞加工製品・再生医療製品の安全性評価制度(HCT/P、ATMP)の枠組み
◎再生医療製品の品質管理基準(GCTP省令)や製造工程開発・品質規格の考え方や保存・搬送、購買管理の考え方
◎薬事申請・製品化前までに行うべき前提条件と注意点
◎再生医療に必要な技術開発のポイントと日常管理のための培養ツールの開発事例

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Topical Rebamipide Treatment for Superior Limbic Keratoconjunctivitis in Patients With Thyroid Eye Disease

YASUHIRO TAKAHASHI, AKIHIRO ICHINOSE, AND HIROHIKO KAKIZAKI

• PURPOSE: To evaluate efficacy of topical rebamipide for superior limbic keratoconjunctivitis (SLK) in patients with thyroid eye disease.

• DESIGN: Retrospective, observational case series.

• METHODS: Thirty-three eyes from 20 thyroid eye disease patients with SLK who received topical rebamipide (Mucosta ophthalmic suspension unit dose 2%; Otsuka Pharmaceutical Co, Ltd; chemical name, (2RS)-2-(4-chlorobenzoylamino)-3-(2-oxo-1, 2-dihydroquinolin-4-yl) propanoic acid) were included. The following items were evaluated before and 4 weeks after the start of treatments: presence or absence of SLK, rose bengal staining score, area and density classification of fluorescein staining, Schirmer test I results (without topical anesthesia), tear film break-up time, Hertel exophthalmometry values, and margin reflex distances 1 and 2.

• RESULTS: Twenty-eight eyes showed complete disappearance of SLK after treatment (84.8%; $P < .001$). The other 5 eyes (15.2%) demonstrated significant improvement, but had residual punctate rose bengal staining and fluorescein staining near the superior corneal limbus. All 5 eyes exhibited at least 1 of the following findings: proptosis of more than 17.7 mm and upper or lower eyelid retractions or both. Incidence of upper eyelid retraction was significantly higher in eyes with SLK than in those without SLK at the 4-week follow-up ($P = .021$). The severity of rose bengal staining and fluorescein staining improved significantly after treatment ($P < .001$). Although the Schirmer test results remained constant before and after the treatment ($P = .212$), tear film break-up time increased significantly in the posttherapeutic state ($P = .009$). No serious adverse events were reported.

• CONCLUSIONS: Topical rebamipide improved SLK in patients with thyroid eye disease, suggesting a first-line treatment in such patients. (Am J Ophthalmol 2014;157:807–812. © 2014 by Elsevier Inc. All rights reserved.)

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REBAMIPIDE IS A QUINOLINONE DERIVATIVE THAT increases production of mucin-like substances in the cornea and conjunctiva.[1–5] It also exerts anti-inflammatory effects, promoting wound healing.[4,6] Recent studies showed that rebamipide ameliorated corneal and conjunctival epithelial damage and increased tear film stability in patients with dry eye.[1,2] However, it is still unclear whether rebamipide improves superior limbic keratoconjunctivitis (SLK).

SLK typically is caused by local mucin deficiency in the upper conjunctiva, resulting in abnormal friction and inflammation between the upper eyelid and the superior corneal limbus.[7,8] This is associated predominantly with thyroid eye disease (TED),[9–12] in which proptosis and enlarged eyelid fissures aggravate tear deficiency, abnormal friction, and ocular surface inflammation.[10,13,14] In general, conservative procedures, such as topical treatment (artificial tears, vitamin A, silver nitrate, α-blocker, tacrolimus, cyclosporine, autologous serum, lodoxamide tromethamine, ketotifen fumarate, and cromolyn sodium), botulin toxin A and triamcinolone acetonide, bandage contact lenses, and punctal plugs, as well as a surgical conjunctival resection partially or completely improve SLK.[7,8,15–29] However, SLK in TED tends to be more refractory, requiring systemic steroids, radiation therapy, blepharoplasty, strabismus surgery, and orbital decompression for treatment.[10]

Because local mucin deficiency and underlying inflammation cause SLK, we speculated that topical rebamipide may improve SLK. SLK in TED usually requires more intense treatment than common SLK. If topical rebamipide improves SLK in TED, instillation as a first-line treatment in the clinic could be applied to such patients. We therefore examined the efficacy of topical rebamipide for SLK in TED patients.

METHODS

INSTITUTIONAL REVIEW BOARD APPROVAL WAS OBTAINED from Aichi Medical University (no. 13-015). We performed a retrospective chart review of all TED patients with SLK who were treated with topical rebamipide from May 2012 through June 2013. The diagnosis of TED was the presence of at least 1 of the characteristic eyelid signs (eyelid fullness, eyelid retraction, and eyelid lag) as well as presence of thyroid autoimmunity.[30] Patients with at

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